序

名誉教授　柴原　浩章

この度、兵庫医科大学医学部産科婦人科学教室の『臨床研究・症例報告集Ⅵ』を発行する運びとなりました。私がかつて在職していた当時の教室員達が、2017 年から 2020 年の間に寸暇を惜しみながら、汗してまとめてくれた貴重な研究成果の記録集であり、思い出も詰まった内容となっています。

既に教室も開講以来 50 年を超え、初代・礒島晋三教授、二代目・香山浩二教授、三代目・小森慎二教授、四代目の私までが繋いできた襷は、2024 年 4 月には五代目の馬淵誠士教授に無事受け継がれました。これまでに教室に在籍されました多くの先生方のご期待に沿い、今後も産科婦人科学の各分野からの新しい数多くの知見を、馬淵教授のリーダーシップのもと世界に発信し続けていただきたいと思います。

本書の編集作業にご尽力いただいた教室の皆様に感謝致しますとともに、お手に取られました先生方の明日からの診療や研究に少しでもお役立ていただけることを期待しています。それでは引き続きまして、同門会の先生方からのご支援とご指導を宜しくお願い申し上げます。

兵庫医科大学産科婦人科学教室　臨床研究・症例報告集Ⅵ
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—Review—

The inability of human sperm with chromosomal abnormalities to penetrate the oocyte in assisted reproductive technology (ART): risk factors and the role of seminal plasma

Seiji Watanabe[1]*, Atsushi Fukui[2], Rika Nakamura[3], Rie Fukuhara[3] and Ayano Funamizu[3]

[1]*Department of Anatomical Science, Hirosaki University Graduate School of Medicine, Aomori 036-8562, Japan*
[2]*Department of Obstetrics and Gynecology, Hyogo College of Medicine, Hyogo 663-8501, Japan*
[3]*Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine, Aomori 036-8562, Japan*

Abstract: The combination of ICSI and a sperm chromosome assay provides a useful opportunity to directly investigate relationships between chromosomal aberrations and head morphology, motility or *in vitro* aging. An increase of aneuploidy was identified in sperm with large heads. Structural chromosome aberrations were frequently found in elongated heads. These types of sperm should therefore be avoided for ICSI and can easily be identified under a microscope. In sperm with normal heads, the chromosomal aberration rate was initially low but increased gradually after removal of the seminal plasma (3.3% to 15.9%); in immotile sperm, the increase of DNA damage was more pronounced (4.5% to 48.1%). Therefore, there are motile and immotile sperm populations in human ejaculates that are potentially vulnerable to *in vitro* culture conditions. Since these sperm can maintain DNA integrity in the seminal plasma for at least 3 h, and even after cryopreservation, the seminal plasma appears to be an efficient suppressor of DNA damage. We report here our attempts at using ICSI using sperm stored in the seminal plasma (SP-ICSI) to treat infertile patients with normozoospermia and provide data to confirm the safety of this technique. For poor quality semen, SP-ICSI may be more effective in achieving a successful outcome.

Key words: Human sperm, Chromosome aberrations, Amorphous, Immotile, *In vitro* aging

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Received: May 29, 2017
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*To whom correspondence should be addressed.
e-mail: sage@hirosaki-u.ac.jp

Introduction

In 1983, Mikamo and Kamiguchi [1] combined the gradual fixation-air drying method, which is a very effective technique for the preparation of chromosomal specimens from oocytes, and interspecific *in vitro* fertilization (IVF) between human sperm and zona-free hamster oocytes [2], which permitted the analysis of the chromosomes in individual human sperm. Since then, these authors have published a series of papers relating to the characteristics of chromosomal abnormalities in human sperm. This work demonstrated that the incidence of structural chromosome aberration is very high in human sperm, with a mean incidence of 14%, and shows significant individual variation [3]. This was considered by Edmonds *et al.* [4] to underlie the low outcome rate in human reproduction. However, the precise mechanisms underlying the high incidence of DNA damage in human sperm has yet to be elucidated. This study was conducted after obtaining the ethical approval of our institute.

The development of intracytoplasmic sperm injection (ICSI) has created a fundamental new problem in how we should select a sperm for ICSI, particularly in terms of selecting sperm with good genetic integrity. However, human sperm chromosome assays are not able to assess genetic integrity for ICSI because it is impossible to determine the precise features of how sperm penetrate an oocyte. To resolve this issue, this paper describes our attempts to analyze chromosomes of individual sperm by ICSI into mouse oocytes with a piezo-micromanipulator,

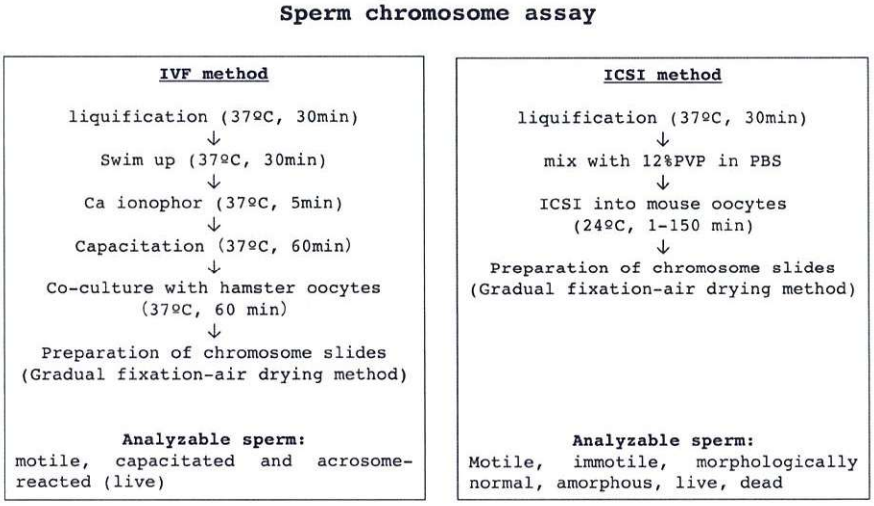


Fig. 1. Two methods to analyze sperm chromosomes. There are two methods that can be used for the analysis of human sperm chromosomes with IVF or ICSI. The IVF method requires sperm to be capacitated and then penetrated into zone-free hamster oocytes prior to chromosome analysis; two and a half hours are necessary for the sperm preparation involved. With the ICSI method, sperm can be injected into mouse oocytes soon after liquefaction.

a technique originally developed by Kimura *et al.* [5]. This paper also describes our results pertaining to human sperm chromosomes. Fig. 1 shows the differences between the sperm chromosome assay when used with IVF or ICSI; ICSI allows us to study the relationship between chromosomes and sperm morphology, motility and viability.

Sperm Head Abnormality

By injecting sperm with a particular feature into mouse oocytes, it is possible to investigate the associated genetic risk. We compared the risk of structural and numerical chromosome aberrations between motile sperm with aberrant and normal heads from normozoospermia donors [6] (Fig. 2 and 3).

Small heads

The incidence of numerical chromosome aberrations did not differ between sperm with small heads (<4 μm in length and <3 μm in width) and those with normal heads which were present in semen from a normozoospermic donor; this suggests that small heads do not result from loss of chromosomes (hypoploidy). This result is inconsistent with globozoospermia patients who show a significant increase of sperm aneuploidy when analyzed by multi-color FISH analysis [7, 8]. Since these studies evaluated a sperm population without taking into account motility, morphology and viability, there is a possibility that the genetic risk associated with round sperm is overestimated on account of contamination by dead and amorphous sperm. On the other hand, the incidence of structural chromosome aberrations was slightly lower in sperm with small heads than with normal heads. Sperm with a small nucleus, containing tightly condensed DNA, may be therefore be resistant to *in vitro* environments responsible for DNA damage.

Large heads

No increase in the incidence of structural chromosome aberrations, which are caused by single- and double-stranded DNA damage, was found in sperm with large heads (>5 μm in length and >3 μm in width). On the other hand, sperm with large heads were associated with a risk of diploidy. We have experienced a case of a patient in which all sperm in the ejaculate had a diploid large head. In such patients, chromosome analysis will be required prior to ICSI. However, the risk was very low in the normozoospermic donors we examined, and accordingly, the total incidence of chromosome aberrations did not differ between sperm with large and normal heads. Therefore, sperm with large heads appear to represent an alternative to sperm with normal heads for ICSI, depending upon the particular circumstances of the case involved. It is not clear how frequent diploid large-headed sperm are spontaneously produced in the testis of hu-

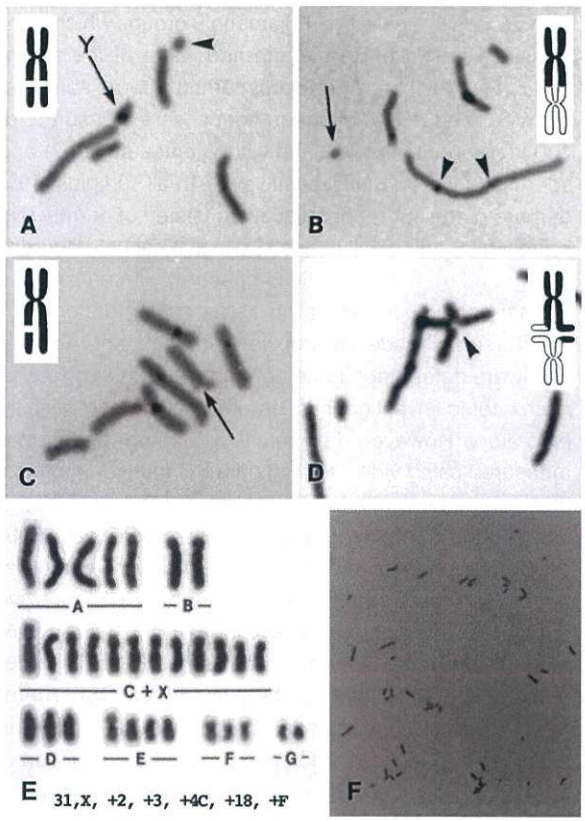


Fig. 2. Types of chromosome abnormality in human sperm. Giemsa-stained human sperm chromosomes formed in murine oocytes around 20 hours after fertilization. A-D shows 4 types of structural chromosome aberrations resulting from DNA breaks. A, Chromosome-type breakage. The arrowhead shows a chromosome fragment caused by a double-strand DNA break. B, Chromosome-type exchange. A dicentric chromosome, which has two centromeres (arrowheads), results from the reconstruction of two chromosomes with a double-strand DNA break. C, Chromatid-type break. A chromatid break (arrow) is derived from a single-strand DNA break that is not repaired. D, Chromatid-type exchange. The arrowhead shows a chromosome reconstructed with two chromosomes having single-strand DNA break. E, Aneuploidy. Thirty-one chromosomes were found in a sperm with a large head. F, Diploidy. Forty-six chromosomes were found in a sperm with a large head. The images in B, E and F were reproduced from our previous paper [6].

mans. Fortunately, this type of aberrant sperm is not predominant in normozoospermic semen and can be easily discriminated from the normal-headed sperm under a comparably low magnification (×200).

Elongated heads

Despite the fact that elongated- and large-headed sperm are the same length, structural chromosomal aberrations were found in 33.3% of the elongated sperm heads (length >5 μm), and their DNA was shredded. This inconsistency between the elongated and large heads highlights the possibility that sperm DNA is packed in normal heads in a manner that retains the nuclear length/width ratio within the normal range (approximately 5:3) and thus avoids collateral damage. Although it is not clear why the DNA of elongated sperm is severely damaged, it has been reported that endogenous DNA nick formation gradually increases during spermiogenesis but is undetectable in mature sperm [9]. Therefore, in the elongated sperm head, DNA nicks may remain, even after the completion of spermiogenesis. Head length is a crucial factor in avoiding fertilization of an oocyte with genetically abnormal sperm. Later in this paper, we provide further evidence to support this fact in infertile men by TUNEL assay [10, 11]. Given the above, elongated sperm are not recommended, even for ICSI treatment. Genetic alteration resulting from a DNA break (s) appears to be maintained and is heritable; this differs from cases of aneuploidy, in which aberrant embryos are mostly aborted during early pregnancy.

Pointed heads

Pointed heads is a morphological abnormality in the acrosomal region, which can be barely distinguished at a magnification of ×400 in normozoospermic sperm. While there was not any significant structural or numerical chromosome aberrations in sperm with this abnormality, we observed a significant reduction (10%) of the oocyte activation rate in mouse oocytes following ICSI. We did not expect that minor acrosomal abnormalities would influence oocyte activation and thus prevent the sperm nucleus from decondensing, because factors responsible for the induction of oocyte activation are localized in the equatorial region of human sperm. Although there is a possibility that human sperm activate mouse and human oocytes in different manners, it is very important to carefully evaluate the morphology of sperm destined for ICSI at a magnification of ×400.

Sperm Head Size

In our previous data (Fig. 3), we noticed that the incidence of structural chromosomal aberrations was slightly lower in sperm with small heads (6.3%) compared with those with normal heads (8.6%), suggesting the possibility that the DNA of sperm with tightly condensed heads is

not comparably damaged.

Therefore, to minimize the risk of choosing sperm with DNA fragmentation for ICSI, the most preferable sperm head size was determined by comparing the frequency of TUNEL-positive cells [11, 12] among 9 groups which were classified based on the length and width of the sperm head (Fig. 4). In 7 normozoospermic sperm samples, the width and length of sperm heads were measured for 100 individual sperm stained with Giemsa stain (G) and sperm that were TUNEL-positive (T). In all samples, 50% or more of the sperm population consisted of normal (N), narrow (Na) and small heads (S). In five cases (donors 1, 2, 3, 5 and 6), the populations of sperm with narrow and normal heads were similar. In three cases (donors 5, 6 and 7), small-headed sperm were predominant. All samples were determined to be normozoospermic by routine microscopic investigations carried out in our hospital laboratory. However, data relating to sperm head sizes measured using video scaling on a PC monitor suggests that many sperm with a slightly narrow head were classified into the normal sperm population. Sperm with DNA fragmentation were mostly those with normal or larger heads (groups E, L, Sh and W) and TUNEL-positive sperm with narrow (Na) or small heads (S) were rarely found. Amorphous sperm can be identified easily under a microscope and thus can be avoided for ICSI. Therefore, we should pay attention to the fact that the risk of selecting a DNA-damaged sperm from the population of sperm with a normal head is higher than from popula-

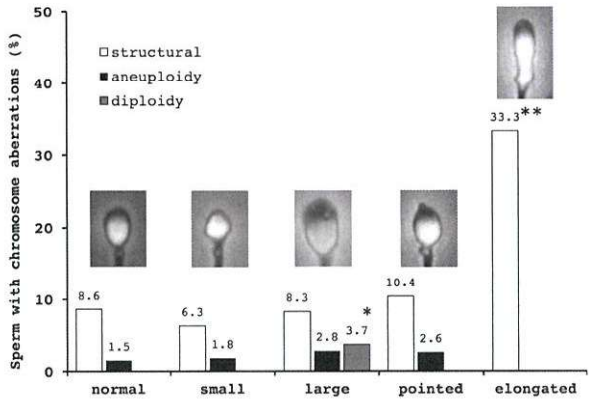


Fig. 3. Relationship between sperm head morphology and chromosome aberrations. The incidence of structural chromosome abnormality, aneuploidy and diploidy was compared among sperm with normal, small, large, pointed and elongated heads obtained from normozoospermic males. *Significant at the $P<0.05$ level. Images of the sperm heads are reproduced from our previous paper [6].

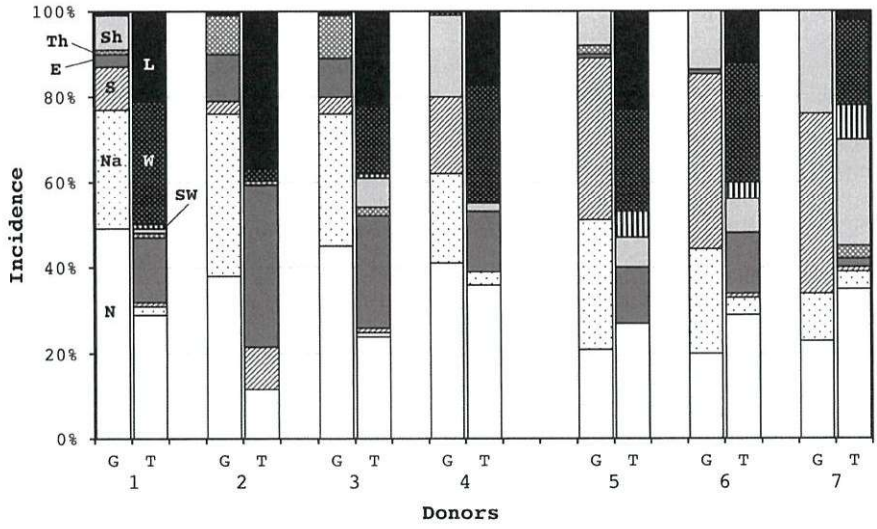


Fig. 4. The relationship between sperm head sizes and DNA fragmentation. In semen samples from 7 normozoospermic IVF patients, we compared the head sizes of 100 sperm stained with Giemsa's solution (G) and 100 DNA-damaged sperm detected by TUNEL assay (T). N, normal (based on WHO criteria [13]); Na, narrow (<2.5 μm in width); S, small (<4 μm in length and <2.5 μm in width); E, elongated (>5 μm in length); Sh, short (<4 μm in length); W, wide (>3.5 μm in width); L, large (>5 μm in length and >3.5 μm in width); Th, Thin (>5 μm in length and <2.5 μm in width); SW, short and wide (<4 μm in length and >3.5 μm in width).

tions with narrow or small heads. This difference in risk is approximately fivefold [11]. The application of narrow- or small-headed sperm in ICSI may reduce the risk of pre- and postimplantation embryo loss caused by sperm chromosomal damage. DNA integrity may be maintained in sperm nuclei packaged more tightly with protamine.

In Vitro Aging

Munne and Estop [14] previously reported that oocyte development is reduced after the penetration of sperm stored *in vitro* for more than 6 h, suggesting that sperm DNA are gradually damaged over time *in vitro*. Therefore, sperm aging appears to be recognized as a factor that prevents the successful treatment of infertile males by IVF. However, *in vitro* culture is a necessary requirement for sperm to induce "capacitation," a vital physiological change required to penetrate oocytes; accordingly, sperm aging appears to be accepted as an inevitable risk. For the same reason, the sperm chromosome assay for IVF cannot assess sperm DNA damage caused in the few hours that it undergoes sperm capacitation. This limitation was removed after Kimura *et al.* [5] applied a piezo-micromanipulator to carry out ICSI into mouse oocytes. Since then, we have successfully utilized this device for the human sperm chromosome assay with ICSI into mouse oocytes [15], and the resultant data has allowed us to investigate the effect of short-term (a few hours) *in vitro* aging on sperm DNA. The results of this analysis are shown in Fig. 5. The incidence of structural chromosome aberrations did not differ between motile and morphologically-normal sperm that penetrated murine oocytes by either IVF and ICSI during 2.5 h after ejaculation (10.6% *versus* 8.6%). However, when the aberration rates were recalculated for every 30 mins of prior incubation for ICSI, we identified a remarkable increase of the aberration rate at 30 and 120 mins (Fig. 5B). This data shows that there are three types of motile normal sperm with genetic fragility in ejaculated semen. The first is the sperm in which DNA was damaged in the testis or the epididymis. These are detectable using the sperm chromosome assay 30 min after sperm preparation and accounted for 3.3% of normal motile sperm. The second is the sperm in which DNA was newly fragmented at 30 min after sperm preparation; this population increases the population of sperm with DNA fragmentation to approximately 10%. This phenomenon is caused by the removal of seminal plasma or exposure to culture medium. Evidence for this is also described in the section relating to seminal plasma herein. The third group is sperm in which DNA was fragmented at more than

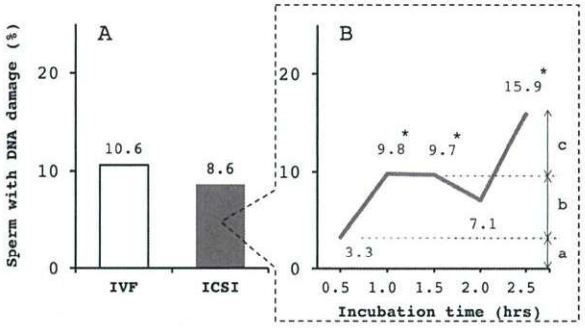


Fig. 5. Time-dependent increase of structural chromosome aberration in motile and morphologically normal human sperm. A, The total incidence of DNA-damaged sperm in IVF and ICSI methods in a normozoospermic donor. B, Alterations of the aberrant sperm incidence. a, sperm DNA initially damaged; b, sperm DNA damage resulting from potential membrane damage; c, sperm DNA damage newly produced *in vitro*. *Significant at the $P < 0.05$ level.

2 h after sperm preparation, creating a second peak of the DNA fragmentation rate (15.9%). This phenomenon could be akin to the process of "*in vitro* aging" previously reported for murine sperm, which resulted from the effect of active oxygen species produced by sperm movement [16]. The individual variation in DNA damage which was demonstrated by the sperm chromosome assay with IVF may arise from the difference in sperm sensitivity to the removal of seminal plasma and short-term *in vitro* aging. This result provides us with valuable information indicating that sperm preparation protocols generally used in the IVF laboratories are not necessarily suitable for sperm applied for ICSI.

Immotile Sperm

In cases of severe asthenozoospermia, embryologists may consider the use of immotile sperm with normal heads for ICSI. In general, the hypoosmotic swelling test (HOST) is the recommended method with which to distinguish live cells, which involves the swelling of sperm in a hypoosmotic solution.

A combination of ICSI and the sperm chromosome assay enables us to analyze the genetic integrity of immotile sperm. Previously, an author of this study revealed a mechanism for producing DNA damage in immotile sperm [17]. Surprisingly, of the immotile sperm directly selected from normozoospermic donor ejaculates, only 4.5% were genetically abnormal, although 48% were dead cells whose nuclei were stained red with 0.1%

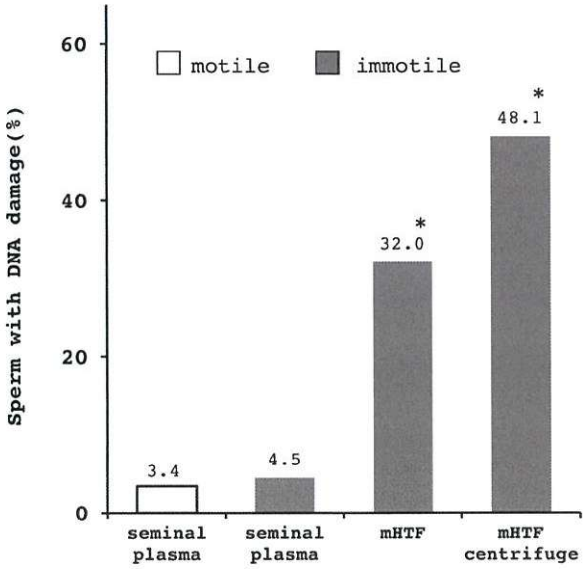


Fig. 6. An increase of DNA damage in immotile normal-shaped sperm. Initially, almost all immotile sperm were genetically normal (95.5%). In some of these, however, DNA was quickly damaged by the culture medium and centrifugation. *Significant at the $P<0.05$ level.

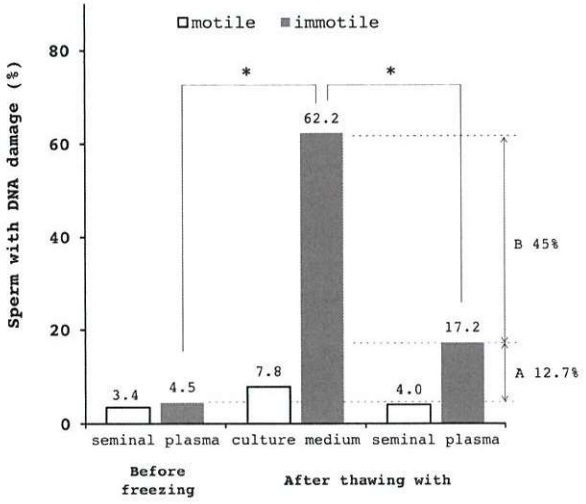


Fig. 7. Inhibitory effect of seminal plasma upon DNA damage in immotile normal-shaped sperm. The seminal plasma completely, or efficiently, inhibited DNA damage in motile or immotile sperm, respectively. A, sperm with DNA directly damaged by ice crystals; B, sperm with DNA damaged by the thawing process due to potential membrane damage. *Significant at the $P<0.05$ level.

eosin-Y solution due to the poor integrity of the plasma membrane. In contrast, when immotile sperm were transferred and stored in several types of culture media for approximately 2 h, the incidence of structural chromosome aberration increased up to 32%. Moreover, centrifugation with a culture medium, a technique generally performed to remove seminal plasma in ART, resulted in DNA damage in 48.1% of immotile sperm (Fig. 6). This result shows that both dead and live immotile sperm mostly maintain their DNA integrity in seminal plasma, although the DNA of dead sperm is vulnerable to the culture media used to remove the seminal plasma. The osmotic pressure of seminal plasma is higher (over 300 mOsm) than those (270–280 mOsm) of commercial media generally used for ART. It is likely that sperm with poor membrane integrity cannot absorb the influx of water and ions required to equilibrate the difference in osmotic pressure, thus resulting in DNA damage. This is supported by the proven fact that the influx of sodium ions caused DNA lesions in mouse sperm when their plasma membranes were artificially damaged by sonication [18]. Accordingly, in cases of severe asthenozoospermia, the live/dead sperm ratio provides an indicator for predicting the results of subsequent ART treatment. If the live sperm ratio is high, HOST is efficient in selecting genetically normal sperm. In contrast, when almost all sperm are dead, the use of HOST does not make sense because the hypotonic solution quickly destroys their DNA.

Efficiency of the Seminal Plasma

As mentioned above, our data showed that culture media induces DNA damage in both motile and immotile sperm. This means that the seminal plasma is an excellent preservative material that can prevent sperm DNA damage. In the seminal plasma, the incidence of structural chromosome aberrations in the motile and immotile sperm remained less than 5% for at least 3 h after ejaculation (Fig. 6). Therefore, an attempt was made to confirm whether DNA damage caused by freezing and thawing (F/T) operations is suppressed within the seminal plasma [17]. Cryopreserved semen samples were thawed and then centrifuged with a culture medium or seminal plasma. The results of our chromosomal analysis are shown in Fig. 7. The incidence of DNA damage in the F/T immotile sperm increased to 62.2% after thawing in culture medium and was higher than with fresh immotile sperm (Fig. 5, 48.1%). Therefore, 14.2% (62.2–48.1%) of F/T immotile sperm appears to be injured accidentally by the formation of ice crystals during the freezing process. In contrast, the seminal plasma significantly suppressed DNA damage and rescued 45% (62.2–17.2%) of the F/T immotile sperm, although DNA received irreversible inju-

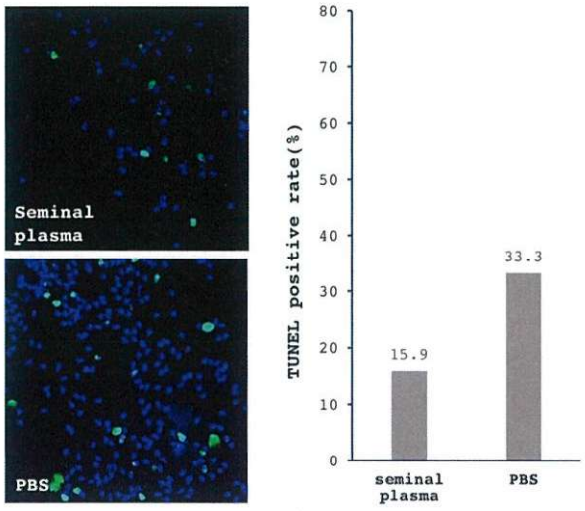


Fig. 8. The incidence of TUNEL-positive sperm in an IVF patient sperm sample before and after the removal of the seminal plasma.

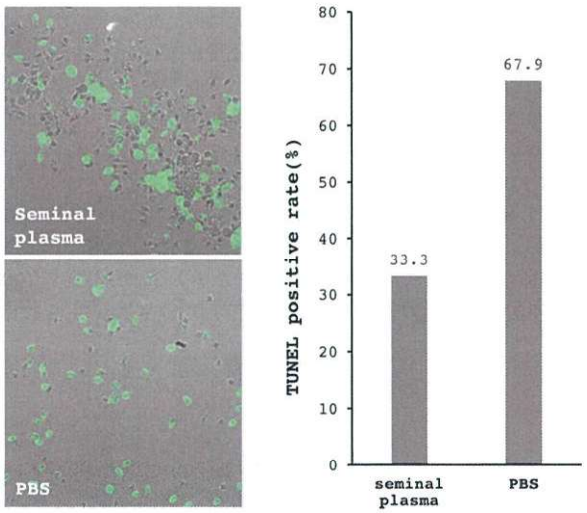


Fig. 9. The incidence of TUNEL-positive sperm in an ICSI patient who experienced repeated failures of ICSI treatment. It was difficult to find genetically normal sperm after the removal of seminal plasma.

Table 1. The success rate of SP-ICSI and ICSI

	SP-ICSI	ICSI	
Oocytes penetrated	59	61	
Oocyte fertilized	36/59 (61.0%)	38/61 (62.3%)	NS
Excellent quality embryos at day 3	25/28 (89.3%)	25/27 (92.6%)	NS
Embryos transferred or cryopreserved	15/28 (53.6%)	15/27 (55.6%)	NS
Fresh ET cycles	7	7	
Pregnancy rate	3 (42.9%)	2 (28.6%)	NS

ry due to ice crystals in 12.7% of the F/T immotile sperm (17.2–4.5%). The rates of DNA-damaged immotile sperm caused by ice crystals were very similar when compared between the medium and seminal plasma groups. The above indicates that immotile sperm samples after the F/T operation consist of cells whose DNA and plasma membrane were intact and cells with intact DNA and a fragile plasma membrane. Thus, the seminal plasma protects the fragile plasma membrane, which probably allows water and ions to influx and injure sperm DNA.

The effect of the seminal plasma and culture media upon sperm DNA damage shown in this review appears to affect other sperm DNA assays, such as TUNEL, HALO and Acridine orange tests. A typical sperm sample presented with an increase in the number of TUNEL-positive cells before and after the suspension of sperm in PBS, as shown in Fig. 8. When a denaturing process with acetic acid is additionally combined, the rate of TUNEL-positive cells increases further. Therefore, it is important to consider the difference between initial and potential DNA damage when applying cytogenetic sperm analysis.

Intracytoplasmic Injection of Sperm Selected from the Seminal Plasma

Based on the effect of seminal plasma upon sperm DNA, ICSI of sperm selected from the seminal plasma (abbreviated as SP-ICSI) was attempted in the hospital of our institute. The results are shown in Table 1. In the SP-ICSI group, sperm selected from normozoospermic patient ejaculates were transferred into a 7% PVP droplet to remove the seminal plasma by gentle pipetting for a few seconds and then injected into oocytes. In the normozoospermic patient ejaculates used, we found no difference in either oocyte development or implantation when compared between conventional ICSI and SP-ICSI groups, suggesting that SP-ICSI is as safe as conventional ICSI and that sperm washing and swim-up proce-

dures are not necessary. For infertile patients with poor semen quality, SP-ICSI will be more effective, although more data is required to confirm this. We have experienced the case of a severe asthenozoospermic patient who produced only short-tailed sperm and the case of a normozoospermic patient who has repeatedly received ART treatments; both were able to obtain normal cleavage embryos using SP-ICSI. We also found via the live/dead sperm test and TUNEL assay that most sperm from these patients were damaged after sperm washing (Fig. 9).

Sperm Vacuoles

Since the ICSI of sperm which are morphologically selected under very high magnification (IMSI, ×6,600 magnification) was introduced [19–21], increasing attention has been focused upon the relationship between large vacuoles on the sperm heads and ICSI outcome. During IMSI, sperm are selected based on several factors determined by the motile sperm organelle morphology examination (MSOME), and accordingly, it is not clear what factor is mostly responsible for ICSI outcome. However, these IMSI studies were misleading if large sperm head vacuoles caused a significant decrease of ICSI outcome, although amorphous sperm with large vacuoles were actually used in the study. As shown above, it has already become clear that amorphous sperm heads strongly correlate to DNA damage [6, 22]. Therefore, we conducted a sperm chromosome assay and TUNEL assay [10] to examine the potential influence of large vacuoles. The above indicates that a large vacuole found in motile and oval-shaped sperm did not relate to an increase in the rate of DNA fragmentation in the normozoospermic IVF or ICSI patients examined. We also noticed that such large vacuoles are visible at x400 magnification, which is the standard lens combination equipped on the inverted microscopes generally used for ICSI. In addition, a few small vacuoles were found in more than 90% of normal sperm, whereas the aberration rates shown by TUNEL and chromosome assays were very low (3.5% and 4.1%, respectively). Therefore, we cannot see the advantage of spending a long time looking for sperm head vacuoles under very high magnifications in semen samples from normozoospermic patients. Our data indicated the possibility that SP-ICSI is highly effective in improving ICSI outcome in severely infertile male patients.

Conclusion

Using the sperm chromosome assay with ICSI revealed detailed characteristics of DNA damage in human sperm. Our results seem to lead to the conclusion that sperm of any type of semen quality should be stored in seminal plasma to maintain DNA integrity prior to ICSI. On the other hand, there is also an advantage of using SP-ICSI to reduce processing times and the use of consumables for sperm preparation. As a next step, it is necessary to demonstrate the advantages of using narrow sperm over normal sperm in clinical applications.

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会員質問コーナー Q&A

㉘8 不育症の診断と治療

回答／福井淳史

Q：妊娠初期の流産を繰り返してしまいました．次の妊娠が不安です．どうしたらよいでしょうか？

（兵庫県　E.F.）

A：いわゆる「不育症」は単一の診断名ではなく，複数の病態を含みます．厚生労働科学研究班（齋藤班）では，「妊娠はするけれど2回以上の流産・死産もしくは生後1週間以内に死亡する早期新生児死亡によって児が得られない場合」つまり，22週以前の流産を繰り返す反復流産（流産を2回以上繰り返した場合），習慣流産（流産を3回以上繰り返した場合）に加え，死産・早期新生児死亡を繰り返す場合を含めて「不育症」と定義されます[1]．不育症の定義に合致する事例の約半数は偶発的流産であり，特別な治療を行わなくても次回妊娠予後は良好ですが，残りの半数では，凝固異常や夫婦の染色体異常，子宮形態異常内分泌異常，代謝異常，免疫学的異常などの共通のリスク因子が認められることがあります．今回のケースは不育症にあてはまると考えられ，系統的なアプローチを行い，適切に精査・加療を行っていくことが肝要です．

しかし，不育症は，検査方針やリスク因子ごとの治療方針が定まっていないことや，流産・死産してしまったというストレスがさらに流産・死産の要因になること，何もリスク因子がなく，たまたま胎児染色体異常を繰り返しただけの全く健康なカップルが半数くらい存在することなどから，産婦人科医にとって難解な疾患となっていました．これに対し，平成20～22年度に先に述べた厚生労働省科学研究費補助金「不育症治療に関する再評価と新たなる治療法の開発に関する研究」（研究代表者：齋藤　滋，富山大学教授）において，不育症に関するさまざまなリスク因子評価がなされ，平成23年3月に「不育症治療に関する再評価と新たなる治療法の開発に関する研究班を基にした不育症管理に関する提言[1]」が発表され，以降，不育症に関する一定した検査・治療方針が広く日本に拡がってきています[2]．本研究班は現在もAMED成育疾患克服等総合研究事業「不育症の原因解明，予防治療に関する研究」として継続され，不育症のリスク因子の解明，新治療法開発に関する研究が続けられています．

流産の原因は，胎児染色体異常に起因することが多いですが，不育症の場合には母体の子宮形態の異常，甲状腺や卵巣の内分泌異常，糖尿病などの代謝異常，抗リン脂質抗体症候群や凝固因子異常などもリスク因子となります．さらに，非自己である胎児が免疫学的に拒絶され，流産となる場合もあり得ます．中隔子宮など子宮形態異常の場合，主として子宮鏡を用いた子宮形成術の有効性が示されており，内分泌異常や代謝異常の場合には内科的な原疾患治療が有用です．また血栓性素因を有する場合にはヘパリンやアスピリンを用いた抗凝固療法を行います．さらにわれわれは，NK細胞の機能異常（NK細胞活性の増加やNK細胞機能分担の異常）といった母体が免疫学的に胎児を攻撃しやすい状態になっていると考えられる不育症に対して，それらを是正するため免疫グロブリン療法やイントラリピッド療法などの治療を行い，良好な成績を得ています[3]．

しかし，不育症の約60％はリスク因子不明の原因不明不育症と診断されます．一般に不育症に関するリスク因子を検索したものの原因を指摘できない場合，患者側は「何度も流産を繰り返し，次の妊娠が怖い」「妊娠しても怖くて仕方がない」というようなことを訴え，何らかの治療を求めてくることが多いです．さらに，医療者側にとっても何

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もしないで経過をみるということはあまり好まれず，患者からの要望もあれば“とりあえず”アスピリンなどを処方することもあるのではないかと推察されます．しかし，原因不明不育症に対するアスピリン療法やヘパリン療法の有効性は否定的であり[4,5]，最も有効であろうとされているのはtender loving careです．すなわち，原因不明不育症と診断された場合には，検査結果に問題がないことを十分に説明し，患者および家族の理解を得たうえで無治療で十分に経過をみながら次回妊娠に臨んでも，妊娠が継続する可能性も十分考えられるのではないでしょうか．

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不妊につながる感染症

平野 由紀　松岡 理恵　坂根 理矢　原田 佳世子　田中 宏幸　柴原 浩章

- クラミジア感染症は卵管性不妊症に関与するSTIであり，現時点で有効なワクチンは存在しない．
- クラミジア感染や淋菌などのSTIに感染していると，HIV感染を受けやすくなる．
- クラミジア感染による卵管性不妊症の予防には，クラミジア感染などのSTIに関する知識向上のための啓蒙活動が肝要である．

はじめに：不妊症の原因と性感染症

不妊症の原因は，報告により多少の差はあるが，最も頻度が高いものが卵管性不妊症であり，女性不妊症全体の30～40％を占める（**図1**）．不妊症の発症に関連する性感染症には*Chlamydia trachomatis*（以下，クラミジア），淋菌，human papilloma virus（HPV）感染などがあるが，その60％以上でクラミジア感染の関与が報告されている[1]．したがって，本稿では主にクラミジア感染症との関連を中心に述べる．

クラミジアは近年世界的に最も頻度が高いsexually transmitted infections（STI）の病原体で[2]，卵管性不妊症，異所性妊娠や骨盤腹膜炎の発生と密接に関係[3, 4]し，その蔓延はわが国でも社会的な問題となっている[5]．また発展途上国では出産時の眼感染による失明の原因ともなる深刻な感染症である[6]．わが国における最近の性感染症の動向を**図2**[7]に示すが，クラミジア感染症は，同時感染することの多い淋菌感染症の罹患率がほぼ横ばいであることと比べ，1990年代以降急増傾向にあったが，2002年をピークに減少傾向となり，2009年からは横ばいである．定点調査には一定の限界はあるが，検査方法に大きな変更はないので減少しているといえる．これは性教育などの啓蒙活動による感染症対策が功を奏した成果と推定できるが，2008年以降は膠着した状態であることから，感染症対策は今後も推進する必要がある．

ひらの ゆき，まつおか りえ，さかね りや，はらだ かよこ，たなか ひろゆき，しばはら ひろあき：兵庫医科大学産科婦人科学講座（〒663-8501　兵庫県西宮市武庫川町1-1）

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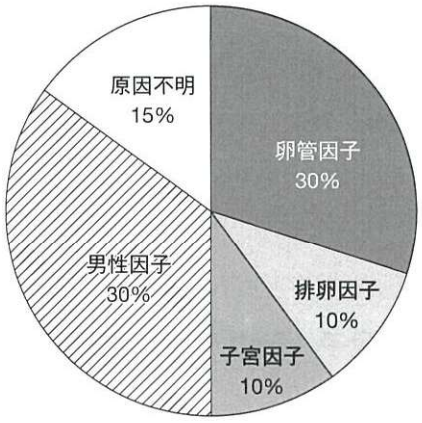


図1　不妊原因の頻度

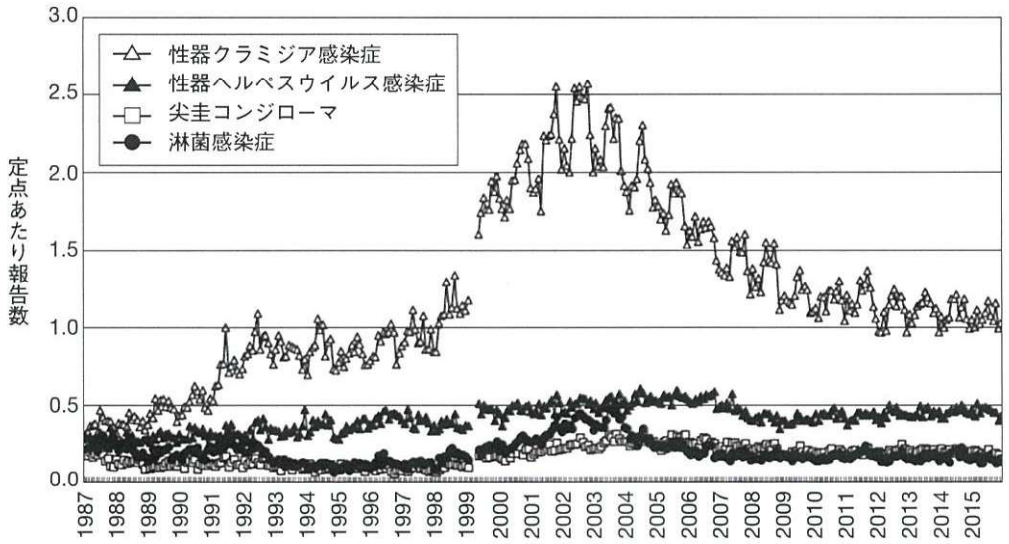


図2　感染症発生動向調査による性感染症の年次推移（女性）

（文献7より）

クラミジア感染は無症候性に進行することが多く，未治療でセックスパートナーに感染させるおそれがあり，感染を広く蔓延させる原因となる．

クラミジア感染は女性では主に子宮頸管炎と骨盤内炎症性疾患（pelvic inflammatory disease：PID）を発症する．その炎症症状は軽度な場合が多く，症状の自覚がないため受診機会を欠いて長期持続感染となる場合があり，卵管性不妊症の原因となる卵管閉塞や卵管周囲癒着などの誘因となる．

一方，男性のクラミジア感染では漿液性～粘液性の尿道分泌物の増加，尿道瘙痒感，排

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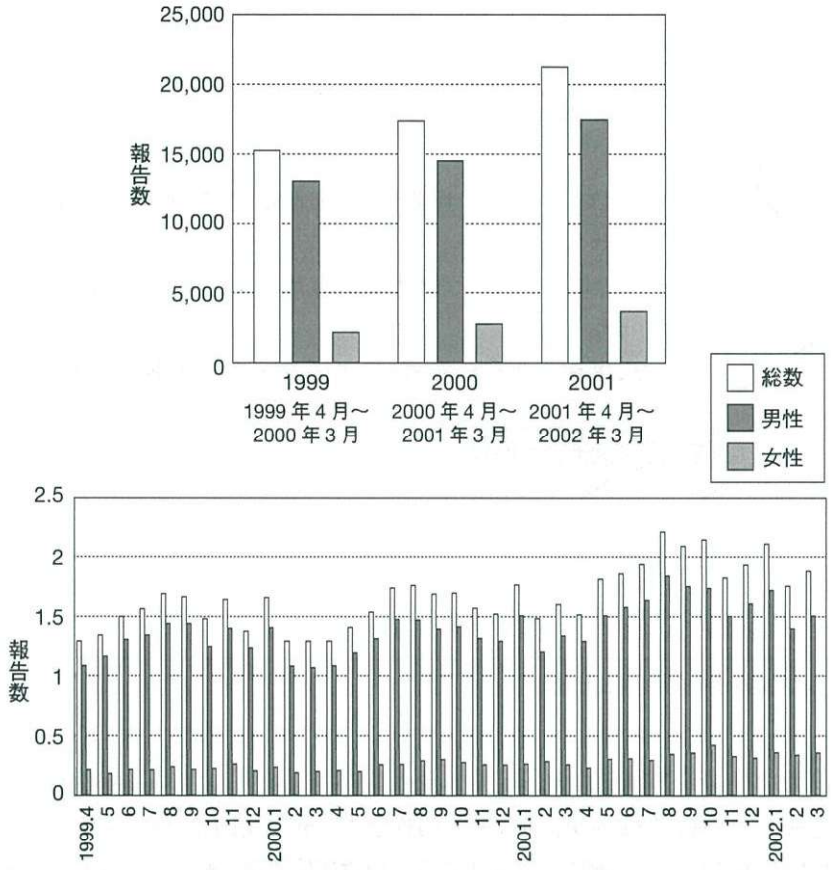


図3　淋菌感染症発生動向

1999年4月1日より施行された感染症法では定点数や定点医療機関に変更があったため（感染症週報2000年第46週号4頁を参照），それ以前のデータとの比較は困難である．

尿痛などを示すが，淋菌性尿道炎と比して症状は軽く半数は無症状である．精巣上体炎まで波及した際には陰嚢の腫脹・疼痛・発熱を呈し，閉塞性無精子症の原因ともなるが，不妊とかかわる造精機能への影響については論争がある[8~11]．

クラミジアと同時感染の多い淋菌感染症は世界中に存在し，わが国では20歳台の年齢層に最も多い．なお，報告数のなかで女性の数が男性より極端に少数である理由は，女性は自覚症状に乏しく受診の機会が少ないことも要因の1つと考えられる（**図3**）[12]．最近の疫学的研究によれば，淋菌感染によりHIVの感染が容易になると報告されており，その意味でも淋菌は重要な疾患である．

卵管機能からみた不妊症の発生機序

卵管は単に卵子や精子が通過するだけの管腔臓器ではなく，卵管采による卵子のpick

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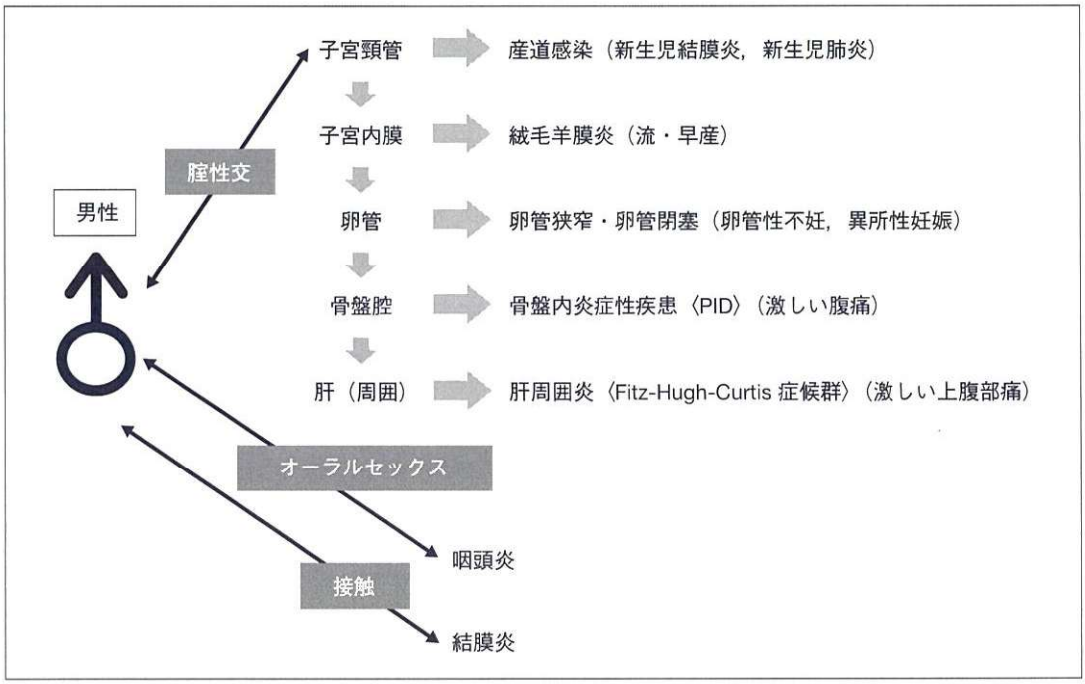


図4　クラミジア感染症の進展様式と症状

up，精子の輸送，受精の場の提供，受精卵の発育促進・輸送といった妊娠成立過程において非常に重要な機能を果たす．また卵管は子宮からの上行性感染を受けやすく，機能障害に陥りやすい．女性側の不妊原因のうち卵管機能障害は約 30～40%と最も多く，かつ難治性であるのはこのような卵管の特性による．

クラミジアの感染経路

性行為（オーラルセックスの場合は咽頭感染）により子宮頸部の上皮細胞に感染したクラミジアが急性期の子宮頸管炎を引き起こす．自覚症状がある場合には潜伏期間は 1～3 週間程度とされるが，女性の場合は約 80%が無症状である．ただし無治療の女性の約 40%において，子宮付属器炎や骨盤腹膜炎に移行する．無治療のまま慢性期に移行した約 10%において，卵管への炎症波及による狭窄，閉塞や癒着による卵管性不妊症をきたす[13]．なお一部の女性では肝周囲まで炎症が波及し，これを Fitz-Hugh-Curtis 症候群と呼ぶ．腹腔内感染が持続すると激烈な腹痛を伴った急性腹症を発症し救急搬送されることもある．子宮付属器炎は無症候であっても，後遺症として卵管内腔の上皮細胞の障害による受精卵の通過障害，慢性持続感染による卵管筋層の膠原線維の増殖による卵管内腔の狭小化などによる卵管性不妊症，あるいは卵管周囲癒着による卵管可動性の障害による異所性妊娠の発生原因となる．

また妊婦のクラミジア感染症は妊婦の約 5%に存在し，絨毛羊膜炎を誘発しプロスタグ

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ランジンの産生により子宮収縮が促進され流・早産の原因となりうる．さらに分娩時のクラミジア感染症は産道感染による新生児結膜炎，咽頭炎，肺炎の原因になる（**図4**）．

クラミジア感染による卵管性不妊症発症の病態

卵管上皮に感染したクラミジアは細胞質内に封入体を形成し，線毛細胞を破壊する結果，線毛運動による配偶子や胚の輸送能が障害される．さらに卵管炎が持続すると炎症は上皮から深部へと波及し，間質が線維化する．この線維化により卵管壁が硬化し，蠕動運動による卵管の輸送能も障害され，卵管内腔は狭窄あるいは閉塞する．炎症が卵管外にまで波及すると卵管周囲に膜状癒着を生じ，卵管采による卵子の pick up 障害の原因となったり，一部では卵管留水腫を形成することがある．これらの卵管傷害はクラミジア感染の治療後も不可逆性に存続し，難治性の卵管性不妊症の原因となる．

卵管傷害以外にもクラミジア感染と不妊症との関係は深く[14]，着床前期における着床障害，着床後期における自然流産[15]，体外受精による胚発生障害など[16, 17]とも関連する可能性が報告されている．これらのようにクラミジアが持続感染へと移行する場合に，クラミジアに特異的な chlamydial heat shock protein 60（cHSP60）[18]に対する宿主の免疫応答活性化が深く関与することが知られている．cHSP60 は遅延型過敏反応を生じ，感染部位に自己免疫反応が起きる．これらが局所の慢性炎症，卵管傷害の原因となると考えられている[18, 19]．

前述のようにクラミジアによる広範な炎症には，cHSP60 に対する宿主の免疫応答の活性化が深く関与する．クラミジア持続感染ではクラミジア菌体は増殖しないが，免疫原性の高い cHSP60 は産生され続け，cHSP60 に対する宿主であるヒトの免疫応答活性化により，再感染の際の遅延型過敏反応を惹起する．この遅延型過敏反応がクラミジア持続感染の際の広範な炎症に関与している．またクラミジア感染後の卵管性不妊症や異所性妊娠例には cHSP60 抗体が高頻度に検出され，cHSP60 抗体の卵管障害発症へのかかわりが示唆されている[20]．

診断

クラミジア抗原検査[21]

子宮頸管の分泌物か，擦過検体からクラミジア検出を行う．分離同定法，核酸検出法，核酸同定法，EIA 法（enzyme immunoassay：酵素免疫抗体法）があるが，高感度であることから核酸増幅法が推奨される．核酸増幅法には従来の polymerase chain reaction（PCR）法に加えて，transcription-mediated amplification（TMA）法，standard displacement amplification（SDA）法がある．PCR 法は頸管粘液や血液などによる増幅阻害物質のための偽陰性に注意が必要である．TMA 法，SDA 法は感度・特異度ともに優れ，クラミジアと淋菌の同時検出が可能である．

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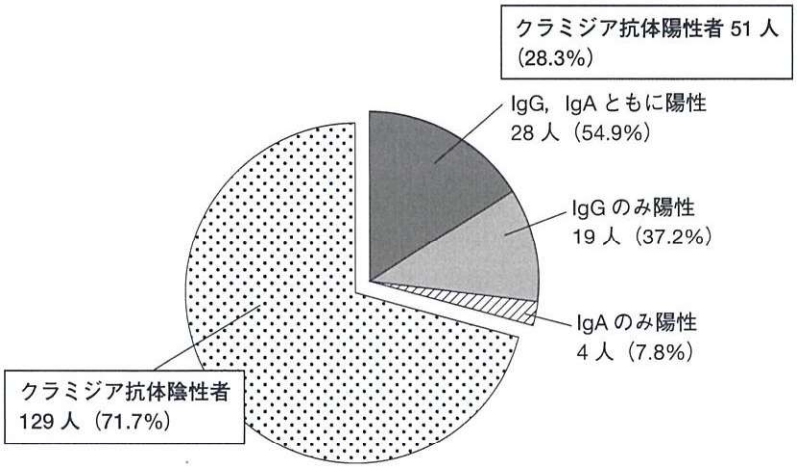


図 5　不妊外来初診女性 180 人におけるクラミジア抗体の検出頻度
（自治医科大学；2000 年 1 ～ 12 月）

オーラルセックスによるクラミジア咽頭炎例に対しては，咽頭擦過物またはうがい液を用いて TMA 法，SDA 法で検出でき保険適応がある．子宮頸管からクラミジアが検出される場合は，無症状であっても，10～20%は咽頭からもクラミジアが検出される[22]．慢性の扁桃腺炎や咽頭炎のうち，セフェム系抗菌薬による治療に反応しない症例の約 1/3 にクラミジア感染が存在するが，生殖器への感染に比べ，治療に難渋することが報告されている[23, 24]．

ところで女性のクラミジア感染症は，その感染範囲が腹腔内全体に及ぶこともあることから，腹腔内感染があっても子宮頸管からは検出できない可能性があることを念頭に置くべきである．したがって症状や診察所見でクラミジア感染を疑う場合は，血清クラミジア抗体検査を行う．

血清クラミジア抗体検査

前述のように，卵管あるいは腹腔内に進行したクラミジア感染が存在しても，子宮頸管からクラミジア抗原を検出できない偽陰性症例の存在が指摘されてきた．下腹痛や性交痛を訴える症例に対しては，血清クラミジア抗体を検査する意義は深い．一方，クラミジアに関して無症状であっても，不妊カップルへの初期スクリーニングとしてのクラミジア検査は，感染既往の有無を評価できる．なお，以前筆者らが不妊外来に通院中の女性の血清クラミジア抗体を測定した結果によると，約 30%に IgG あるいは IgA 抗体のいずれかが陽性であった（**図 5**）[25]．子宮卵管造影法（hysterosalpingography：HSG）によりクラミジア感染の影響を調べた結果，卵管通過障害や卵管周囲癒着などの不妊症発生の原因となることが判明した（**図 6**）[26]．また血清クラミジア IgG 抗体価は卵管通過障害の予測に有用であることも判明した[27]．

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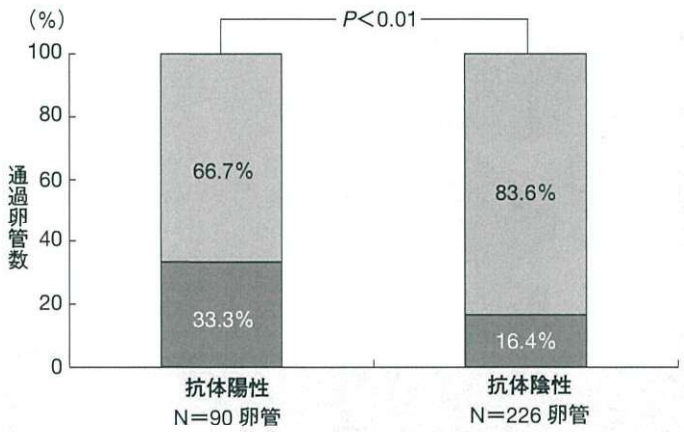


図6　クラミジア抗体と HSG による卵管通過所見との関係
（自治医科大学；2000 年 1 ～ 12 月）
（文献 26 より）

■クラミジア感染による卵管性不妊症の 1 次スクリーニング

血清クラミジア抗体検査は前述のように卵管性不妊症のスクリーニングに有用である．また最近の報告ではクラミジア抗体陽性例では HSG や腹腔鏡検査で卵管に異常を認めなくても，その後の自然妊娠率が低下するとの報告がある[28]．

卵管性不妊症の 1 次スクリーニングとして，一般に HSG を施行する．HSG には診断的意義だけでなく，検査後の妊娠率が上昇するという治療効果も報告されている[29]．HSG は子宮，卵管の形態，および卵管通過性を診断すると同時に，拡散像により骨盤内の癒着の有無を推定できるが，その診断能には限界がある．後述する経腟的腹腔鏡検査（transvaginal hydrolaparoscopy：THL）と HSG 所見を比較すると，卵管通過性の一致率は 82.4%で，卵管周囲癒着の一致率は 67.6%に過ぎなかった[30]．

HSG と THL の所見が不一致であった 1 例を**図 7** に示す．本症例においては，クラミジア抗体が陽性で抗菌薬内服による治療後の HSG により右卵管近位端閉塞，左卵管遠位端狭窄を疑い THL を施行したが，両側卵管通過性は良好で，左卵管・卵管采周囲癒着の診断に至った[31]．

HSG 以外には，超音波造影剤による超音波卵管造影検査も卵管性不妊症評価には有用である[32]．同時にソノヒステログラフィ（sonohysterography：SHG）も行うことにより，子宮腔内のポリープや筋腫なども評価できる．これら両者をあわせて SHSG（sonohysterosalpigography）と呼ぶ．HSG と SHSG の両者の診断能に差はなく[33]，利点として患者および術者への X 線被曝を回避できる．ただし拡散像の検討が不可能であるため，クラミジア抗体陽性者，子宮内膜症患者，あるいは骨盤内手術既往のある女性など骨盤内癒着のリスクのある患者に対しては，次に述べる 2 次スクリーニング検査も考慮する．

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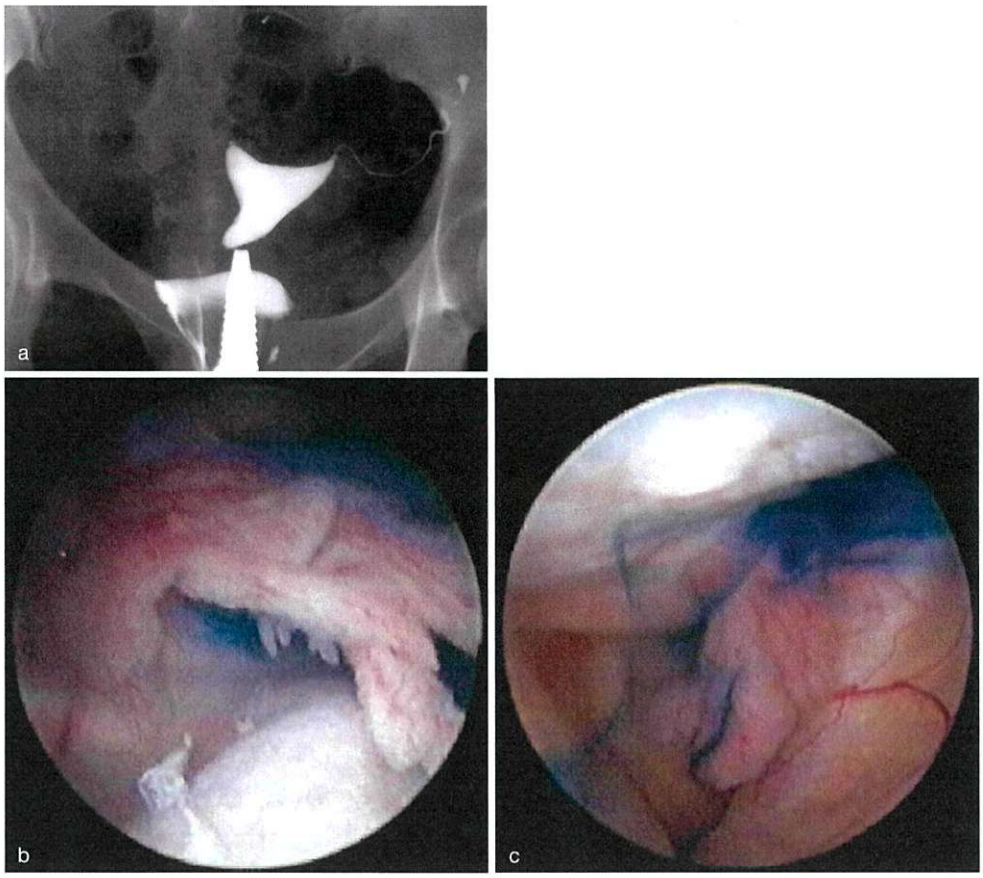


図7　HSG 所見と THL 所見の比較

a：右卵管閉塞，左卵管の狭窄を疑った HSG 所見
b：THL により右卵管采より色素の流出を確認
c：THL により左卵管采より色素の流出を確認．左卵管・卵管采周囲癒着あり

■クラミジア感染による卵管性不妊症の２次スクリーニング

１次スクリーニングにより卵管性不妊を疑う患者に対しては，２次スクリーニングとして腹腔鏡検査を行う．

腹腔鏡検査では HSG で診断が不可能な卵管周囲の癒着を診断できる．したがって卵管性不妊症を疑う症例には，積極的に施行すべき検査といえる．なお卵管性不妊の診断だけが目的であれば，後述する THL が従来の経腹的な腹腔鏡検査より低侵襲な方法で，かつ十分な診断能を有する[31]．腹腔鏡には従来の経腹法および経腟法があり，以下にそれぞれの特徴を述べる．

1）経腹的腹腔鏡

卵管の疎通性は子宮内に子宮マニュピレータなどを挿入し，インジゴカルミンを注入し検査する．腹腔内観察に引き続き，卵管采の通過性改善，癒着剝離術，子宮内膜症病巣焼灼術，ovarian drilling（LOD）や卵巣生検などの手術が可能であるという利点を有す

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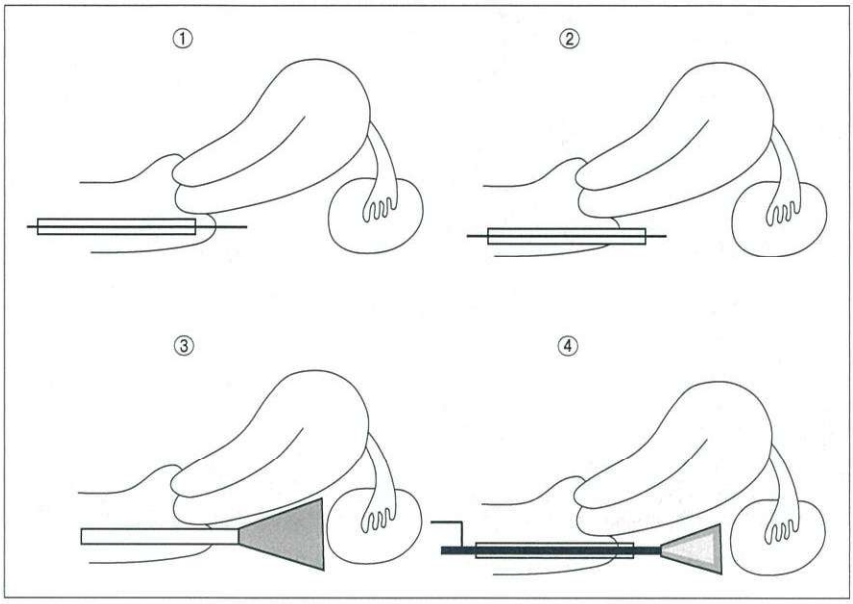


図 8　THL による観察の手順
①後腟円蓋に Veress 針を穿刺
② Cannula 挿入
③生理食塩水注入
④スコープを挿入し，子宮後面より子宮・付属器・骨盤腹膜を観察

る[34]．

2）THL

THL は後腟円蓋よりアプローチし，骨盤腔内を生理食塩水で満たし，腸管が上腹部に浮遊した状態で子宮後面・付属器・骨盤腹膜を観察する（**図 8**）．なおダグラス窩閉鎖，強度の子宮後屈，骨盤内腫瘍，急性骨盤腹膜炎には禁忌である．

THL の適応は，HSG による卵管異常所見，血中クラミジア抗体陽性，原因不明不妊症，その他，である[23, 35]

図 9 に原因不明不妊女性における正常な卵管所見（**図 9a～d**），およびクラミジア感染による卵管性不妊に特徴的な THL 所見（**図 9e～h**）を示す[36]．クラミジア感染のない正常卵管采構造（**図 9a**，**b**）に比し，クラミジア感染既往のある不妊女性では卵管采構造の破壊，周囲との癒着を認める（**図 9e**，**f**）．また卵管内膜に関しても正常所見（**図 9c**，**d**）と比べ，クラミジア感染女性では浮腫，虚血，腔内癒着などの所見を認める（**図 9g**，**h**）．なお色素通過試験でも閉塞を示した．

THL は付属器周囲の癒着，pick up 障害の有無も容易に確認でき，卵管・腹膜因子の診断を可及的に低侵襲性に行うことができる．また THL は初期の子宮内膜症の診断に対しても，経腹的腹腔鏡に劣らない診断能を有する[37]．進行した内膜症病変や凍結骨盤症例に THL は禁忌となるが，われわれも初期症例の診断，および子宮内膜症性の不妊を疑う場合の確定診断に有用であることを報告してきた[38]．なお手術的 THL として，PCOS

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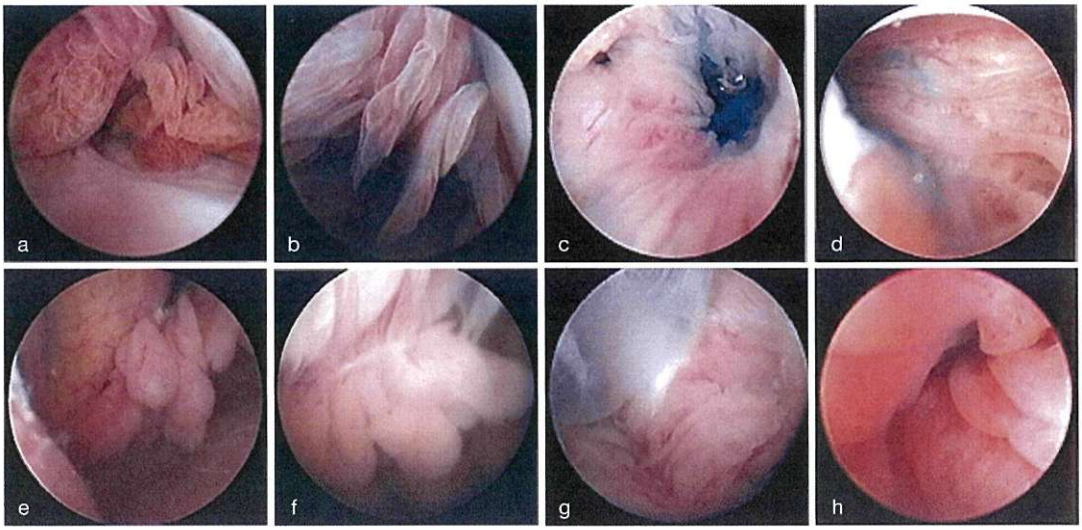


図 9　THL 正常所見（a ～ d）とクラミジア感染既往患者所見（e ～ h）

（文献 36 より）

の卵巣に対する ovarian drilling も可能である（THLOD）[39~41].

卵管性不妊症の治療法

クラミジア感染に対する薬物療法

一般的にクラミジア感染の治療にはマクロライド系，テトラサイクリン系，ニューキノロン系を選択する．クラミジア菌体には細胞壁がないので，細胞壁合成阻害薬であるペニシリン系やセフェム系などのβラクタム系薬剤は無効である．

以下に日本性感染症学会の『性感染症診断・治療ガイドライン 2016』で推奨する治療法を示す．経口薬は子宮頸管炎や子宮内膜炎などの軽症例に用いる．妊婦に対しては経口薬の①~③を用いる．

治療の際にはパートナーの治療も同時に必要である．また HSG や子宮鏡検査，あるいは腹腔鏡検査などを行う際に，腹腔内にクラミジアが存在したまま施行すると，クラミジア感染を腹腔内に蔓延させ，卵管性不妊あるいは PID を医原性に惹起する可能性があることから，これらの不妊検査や治療を行う前にクラミジア感染の有無を検査し，陽性者には下記に示す治療をまず行うべきである．

■経口投与

①アジスロマイシン（マクロライド系，ジスロマック®）1,000 mg 1 日 1 回　1 日間　妊婦・非妊婦：推奨レベル A

②アジスロマイシン（マクロライド系，ジスロマック®SR）2 g 1 日 1 回　1 日間　妊婦・非妊婦：推奨レベル B

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③クラリスロマイシン（マクロライド系，クラリス®，クラリシッド®）200 mg 1日2回 7日間　非妊婦：推奨レベルA，妊婦：推奨レベルB
④ミノサイクリン（ミノマイシン®）100 mg 1日2回　7日間　非妊婦：推奨レベルD（保険適用外）
⑤ドキシサイクリン（ビブラマイシン®）100 mg 1日2回　7日間　非妊婦：推奨レベルD（保険適用外）
⑥レボフロキサシン（ニューキノロン系，クラビット®）500 mg 1日1回　7日間　非妊婦：推奨レベルA
⑦トスフロキサシン（オゼックス®，トスサキシン®）150 mg 1日1回　7日間　非妊婦：推奨レベルD
⑧シタフロキサシン（グレースビット®）100 mg 1日2回　7日間　非妊婦：推奨レベルB

■点滴投与

急性腹症を呈するPIDや劇症型の上腹部痛を訴える肝周囲炎の場合．

ミノサイクリン（ミノマイシン®）100 mg 1日2回　3〜5日間，その後内服に変えてもよい．

不妊症の治療方法

卵管性不妊症例には外科的方法，あるいは体外受精が適応となる．かつては開腹あるいは腹腔鏡下に行うマイクロサージャリーも行われたが，体外受精が臨床応用されて以来，次第に体外受精を選択する傾向にある．一方，卵管鏡の登場により，卵管鏡下に再疎通術を行う卵管鏡下卵管形成法（falloposcopic tuboplasty：FT）も行われている．FTの適応となる卵管病変部は卵管間質部，卵管狭部の近位部が対象になるが，クラミジア感染の影響を受けやすい卵管膨大部や卵管采などの遠位部の病変はFTの適応とはならない．遠位部も治療をする場合は，腹腔鏡手術とFTを併用することになる．しかしながらクラミジア感染が原因である場合，卵管の開口術を行っても卵管内膜自体が障害を受けているため，再閉鎖や異所性妊娠（卵管妊娠）の発生も危惧される．

■体外受精・胚移植（IVF-ET）

卵管の機能障害の程度を正確に判断し，的確に治療する技術はいまだ開発されておらず，卵管の機能を*in vitro*で代行するIVF-ETが，卵管性不妊の治療に頻用されている．

一方，卵管留水腫の存在がIVFによる妊娠率に影響するかについては一定の見解がない[42]．Ajonumaら[43]は卵管留水腫の存在がIVFによる妊娠率に影響するとの立場から，卵管留水腫の貯留液を解析した結果，卵管上皮細胞に存在するcystic fibrosis transmembrane conductance regulator（CFTR）が感染に伴い活性化され，sodium epithelial channels（EnaC）の機能を障害する結果，卵管内分泌液の産生亢進と吸収阻害に働く．そこで卵管采が閉塞する場合，卵管留水腫の貯留液が子宮腔内へ逆流する結果，胚と子宮内膜の間に機械的障壁となりうるとしている点で興味深い．

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おわりに

不妊症につながる感染症をクラミジア感染症を中心に，卵管性不妊症の現況と，最近の診療法について述べた．現時点でクラミジア感染による卵管傷害は不可逆性とされ，IVF-ET は治療の切り札と位置づけられている．

クラミジア感染や淋菌などの STI に罹患していると，局所の傷害のために HIV を伝播しやすくなるとともに，感染を受けやすい状況になる．

WHO は特に発展途上国で骨盤内炎症性疾患とその後遺症予防のためのワクチン開発が重要との見解を示している[44]が，これまでのところクラミジアに対する有効なワクチンは存在しない．

クラミジア感染による卵管性不妊症の予防には，まずクラミジア感染の蔓延を回避することが必要である．したがって将来の卵管性不妊症や，HIV 感染を含む他の STI を減少させるために，国民の STI に関する知識向上のための啓蒙活動が必要である．

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不育症診療
—新たな治療戦略とその限界—

福井淳史* 柴原浩章*

不育症は，検査方針やリスク因子ごとの治療方針が定まっていないことや，流産・死産してしまったというストレスがさらに流産・死産の要因になること，リスク因子がなく，たまたま胎児染色体異常をくり返しただけのまったく健康なカップルが半数くらい存在することなどから，産婦人科医にとって難解で取り組みにくい疾患である。しかし，系統的な検査により，不育症を診断することにより治療方針も明確となり，正しい治療を行うことができ，その結果を十分に説明して理解を得ることで，患者にも安心して治療を受けてもらうことができる。

はじめに

不育症は，妊娠はするものの2回以上の流産・死産もしくは生後1週間以内に死亡する早期新生児死亡によって児が得られない場合，つまり，22週以前の流産を繰り返す反復流産(連続2回以上の流産の繰り返しをいう)，習慣流産(連続3回以上の流産の繰り返しをいう)に加え，死産・早期新生児死亡を繰り返す場合と定義される。不育症の定義に合致する事例の約半数は偶発的流産であり，特別な治療を行わなくても次回妊娠予後は良好であるが，残りの半数では凝固異常や夫婦の染色体異常，子宮形態異常などの共通のリスク因子が認められることがあり，それらに対する精査・加療が必要となる。

いわゆる「不育症」は，検査方針やリスク因子ごとの治療方針が完全には定まっておらず，流産・死産してしまったというストレスがさらに流産・死産の要因になることや，リスク因子がなくたまたま胎児染色体異常をくり返しただけのまったく健康なカップルが半数くらい存在することから，難解な疾患であるとされてきた。2011年3月，厚生労働省不育症研究班(齋藤班)より「不育症治療に関する再評価と新たなる治療法の開発に関する研究班を基にした不育症管理に関する提言」[1]が発表され，以降不育症に関する一定した検査・治療方針が広く日本に広がってきているものと推測される。しかし，不育症に関する研究は日進月歩であり，先に示したわが国から出された提言をはじめ，世界各地から種々のガイドライン[2)~5)]が刊行されている。

本稿ではそれらのなかから，現在行いうる不育症の診断と治療について概説する。

1．不育症診療のコツ

1 適切な診断

先に述べたように不育症患者の診療では，治療方針がいまだ一定していないこともあり，常に最新の知識を身につける必要がある。しか

* A. Fukui, H. Shibahara 兵庫医科大学産科婦人科

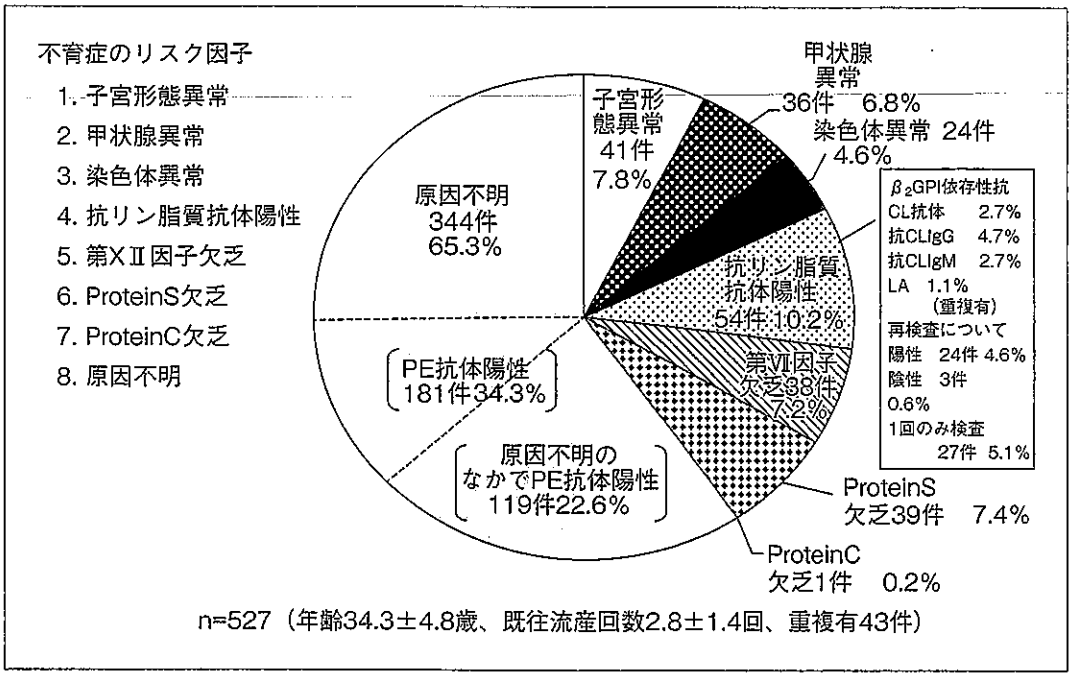


図1 不育症のリスク別頻度

し，現状では産婦人科医にとって不育症の診断・治療に関する知識を得ることは容易ではなく，なかなか不育症の検査・治療を行うことができないとする報告[6]もあり，わが国においても，より容易に不育症に関する最新の知識が得られるような環境となることが望まれる。わが国では，先述の厚労省不育症研究班での研究期間終了後も，不育症のリスク因子の解析が日本医療研究開発機構(Japan Agency for Medical Research and Development；AMED)不育症研究班に引き継がれており，さらなる詳細の解析・報告が期待されている。これらによると，既知の不育症リスク因子として判明するのは40%ほどであり，残りの60%はリスク因子不明(原因不明)と診断される(図1)。既知の不育症リスク因子には，子宮形態異常，甲状腺機能低下症・高プロラクチン血症などの内分泌異常，プロテインS低下やXII因子低下などの血栓性素因，抗リン脂質抗体症候群，転座などの夫婦染色体異常が挙げられ，これらに関しては，ある一定の治療方針が示されている[1]。

また2017年，欧州ヒト生殖医学会(European Society of Human Reproduction and Embryology；ESHRE)から発表された不育症に関するガイドラインによれば，検査項目として推奨されているのは，ループスアンチコアグラント，抗カルジオリピン抗体(IgG，IgM)，抗β2GP1抗体および甲状腺検査(TSHと抗TPO抗体)，子宮形態異常の検査〔3D超音波あるいはソノヒステログラフィー(sonohysterography；SHG)を推奨〕のみである。しかし，結論の出ていない，あるいは不育症と弱い関連がある検査項目として卵巣予備能検査，末梢血あるいは子宮内膜ナチュラルキラー(natural killer；NK)細胞検査などがあり，今後詳細な検討が必要であると思われる。

2 不育症のリスク因子とその対応

1. 詳細な病歴の聴取

不育症診療において，詳細な病歴の聴取は非常に重要である。患者年齢が高くなれば流産率が増加することはよく知られていることであるが，さらに流産回数が増加すればその後の流産も増加する[5]。家族歴の聴取により転座や遺伝的要因による流産が推定される場合もある。また，それまでの妊娠の詳細(流産時期，胎嚢の大きさ，胎児心拍の有無，胎児の大きさなど)を聴取することにより，それぞれの妊娠の流産時期を知ることができ，流産時期が一定しているか

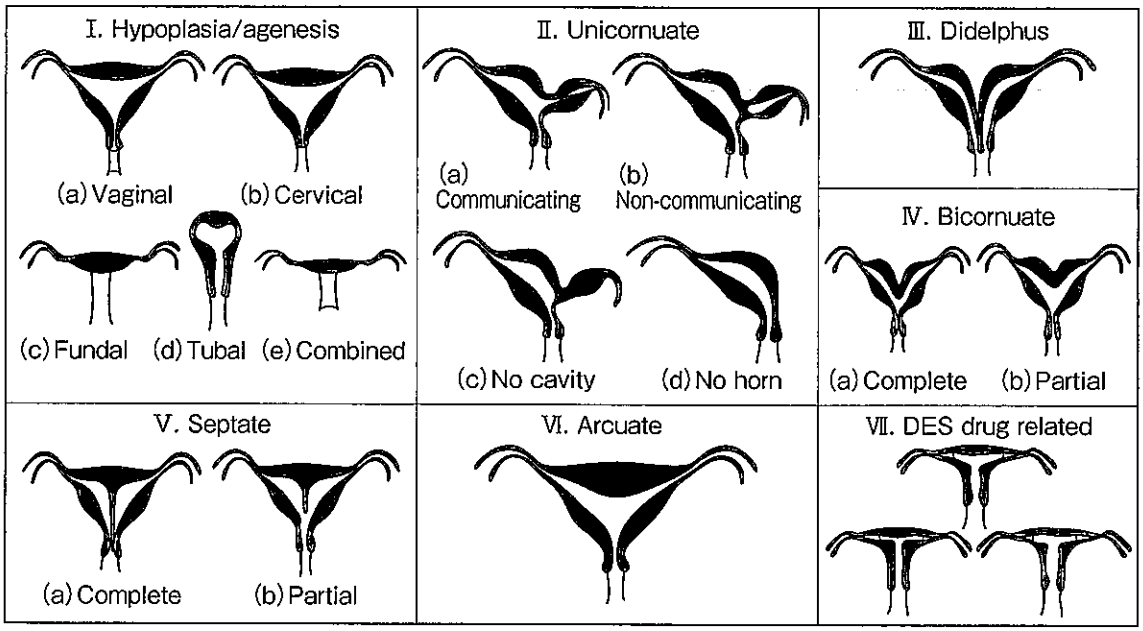


図2 米国生殖医学会による子宮形態異常の分類 (文献8より)

否かなどから不育症の原因推定につながるような重要な情報を得ることができる。

2. ライフスタイル

ストレスが流産の原因になるか否かについては議論の余地があるが，関連性があるとの報告もあることから，過度のストレスは避けるべきである。また喫煙，過度のアルコール・カフェイン摂取と流産との関連が示されており，妊娠を希望する場合，注意を促す必要がある。さらに過度の肥満ややせは妊娠に対して不利に作用するため，適切なダイエットや食事摂取を促すことも大切である。

3. 子宮形態異常

わが国[7]，米国[3]，欧州[2]いずれのガイドラインにおいても子宮形態異常は不育症のリスク因子になりうるとされている。子宮形態異常の分類法としては，米国生殖医学会(American Society for Reproductive Medicine；ASRM)(図2)[8]およびESHRE(図3)[9]から出されている分類法が用いられることが多い。また最近，ASRMから子宮中隔に関する分類法が報告された[10]。子宮形態異常の評価法としてこれまでは子宮卵管造影(hysterosalpingography；HSG)が広く行われてきた。しかしHSGには被曝の問題があり，卵管の通過性を確認する必要がないのであれば，被曝量をできる限り減らすためにも3D超音波あるいはSHG(子宮腔内に生理食塩水を注入しながら超音波を行う方法)により子宮形態を把握することが望ましい。またMRIによる子宮形態評価を行う場合もあるかと推測されるが，MRIはfirst lineの検査法としては推奨されておらず，3D超音波が行えない場合に行われるべきであるとされ，実際3D超音波を施行することにより子宮形態の把握は十分可能である[2]。さらに子宮鏡検査を併用することにより，実際の形態の詳細を把握することができる。なお先天性子宮形態異常を有する場合，腎欠損など腎尿路系の異常を有する場合があるので適宜超音波断層法，MRI，CT，点滴注入腎盂造影法(drip infusion pyelography；DIP)など腎尿路系の確認を行うとよい。

子宮形態異常，特に中隔子宮は流早産との関連が示されている。中隔切除は子宮鏡下に行うことで低侵襲に手術を完遂することが可能であり，有効性に関する報告は多い[10]。後天性子宮形態異常には，子宮筋腫，子宮内膜ポリープ子宮腺筋症，子宮腔癒着症(アッシャーマン症候群)などがあるが，これらが不育症のリスク因子になるかどうかについては結論が得られていない。しかし，後天的な子宮形態異常として粘

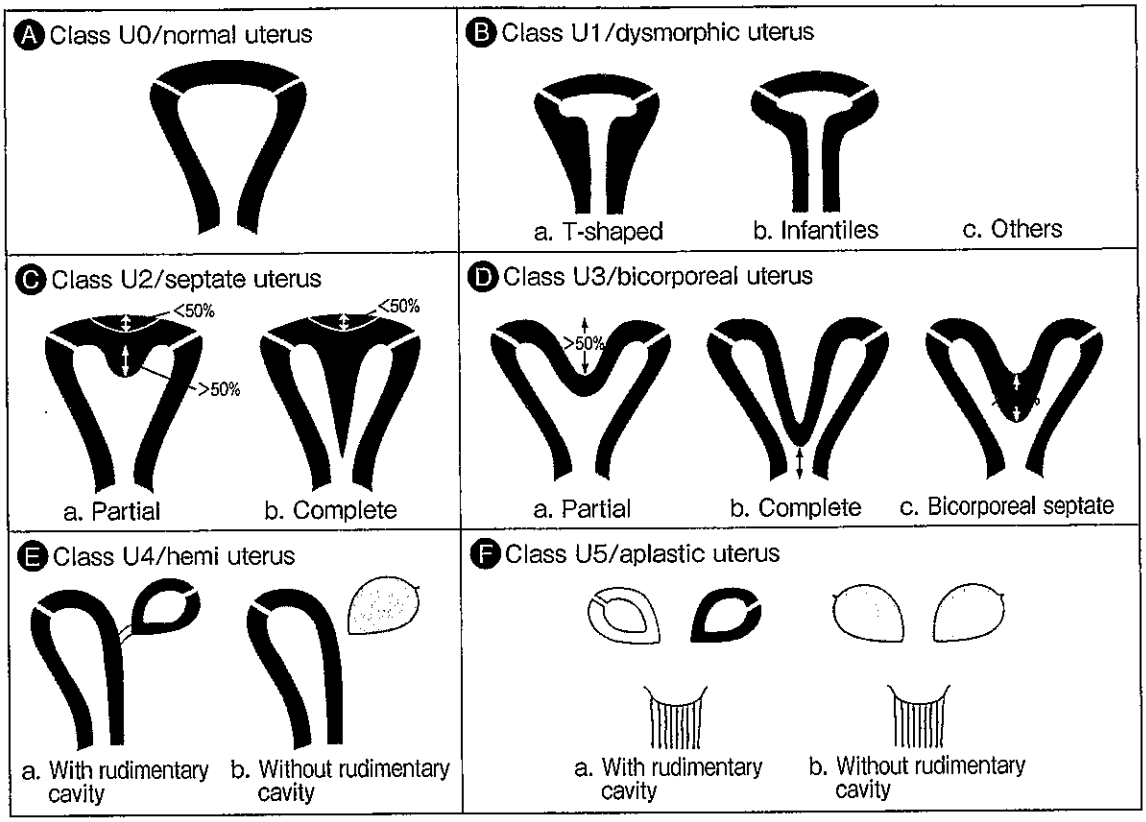


図6 欧州ヒト生殖医学会による子宮形態異常の分類 (文献9より)

Class U2の中隔子宮は子宮の漿膜側の凹みが子宮筋層厚の50%を超えないものであり，かつ内腔への突出が子宮筋層厚の50%を超えるものである。

Class U3の双角子宮は子宮の漿膜側の凹みが子宮筋層厚の50%を超えるものである。さらに双角中隔子宮(U3c)は中隔の厚さが子宮筋層厚の150%を超えるものである。

膜下子宮筋腫は流産率を増加させ生産率を低下させるため，摘除が望ましい[11]。

4. 染色体検査

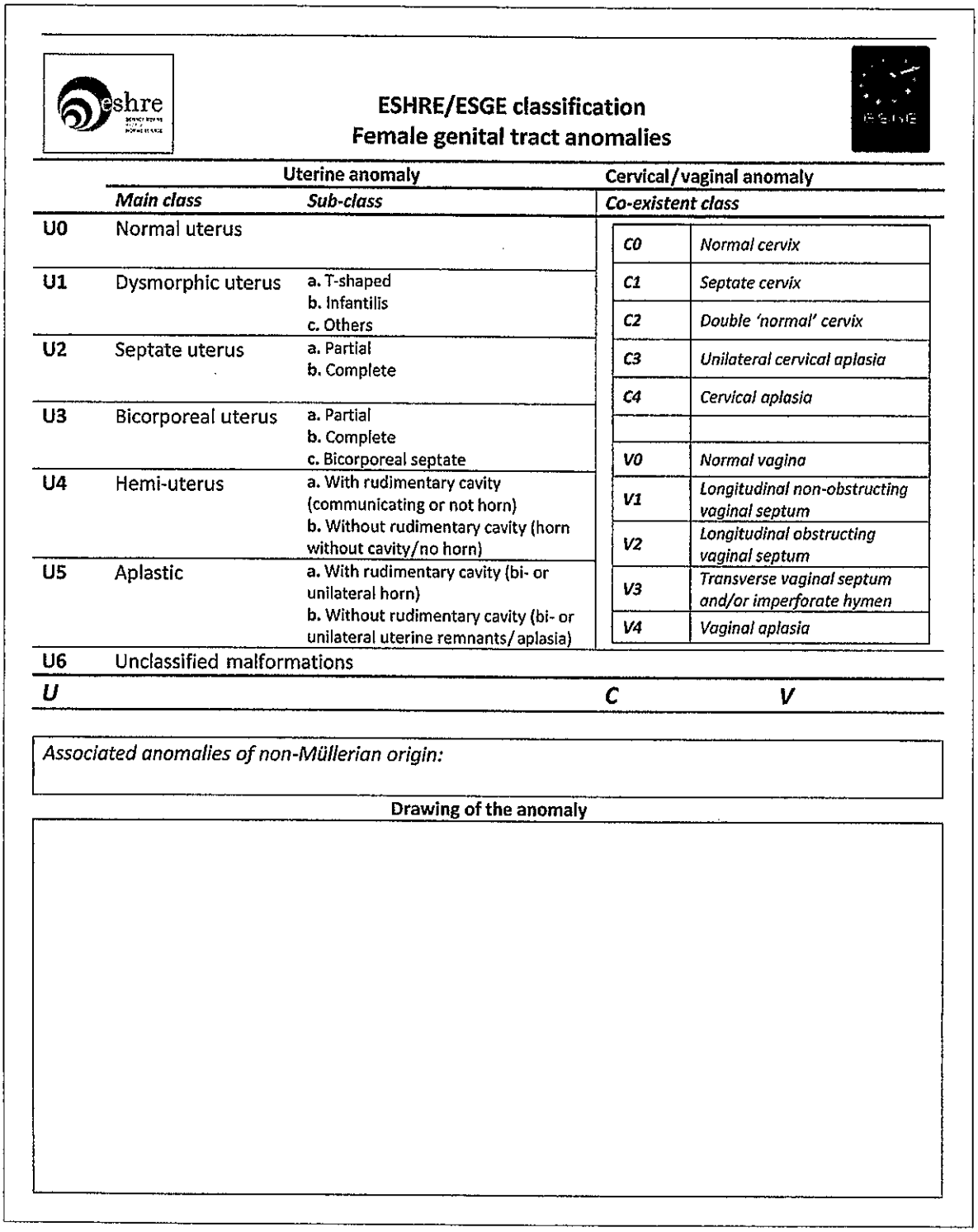
妊娠初期流産の約60%は偶発的な胎児染色体異常に起因するものである。カップルのうちどちらかが相互転座やロバートソン転座，逆位などの均衡型染色体異常を有する頻度は約2～5%である[5]。カップルの染色体核型分析を行うことによりリスク評価が可能であるが，転座保因者に対する治療が存在しないため，十分な遺伝学的カウンセリング体制の下に検査を行う必要がある。またカップルのどちらに転座があるかを明らかにするかどうかについて，カップルの意志を十分に確認しなければならない。カップルの染色体検査をすべての不育症カップルに行うか否かについては議論のあるところであり，先天異常児の出産歴，出生児や流産絨毛の不均衡型転座歴など十分なリスク因子を評価した上で施行されるべきである[2]。

5. 先天性血栓性素因

プロテインS欠乏症，プロテインC欠乏症，アンチトロンビン欠乏症などの血栓性素因は，血栓症を引き起こすことは示されている。同時に生殖ロスの原因となる可能性から治療介入されることもあるかと思われるが，不育症のリスク因子となるかの結論は得られていない。いずれのガイドラインにおいても先天性血栓性素因の検査をすることを推奨していない。

6. 抗リン脂質抗体症候群

抗リン脂質抗体症候群(antiphospholipid syndrome；APS)は不育症患者の約10%に認められる。APSは動脈・静脈血栓症ならびに不育症，妊娠高血圧症候群などの産科合併症を主要な臨床所見とし，抗リン脂質抗体が検出されることにより診断される(表1)。抗リン脂質抗体としてはAPSの診断基準に示されているルー



ESHRE/ESGE classification
Female genital tract anomalies

	Uterine anomaly		Cervical/vaginal anomaly
	Main class	*Sub-class*	*Co-existent class*
U0	Normal uterus		
U1	Dysmorphic uterus	a. T-shaped b. Infantilis c. Others	
U2	Septate uterus	a. Partial b. Complete	
U3	Bicorporeal uterus	a. Partial b. Complete c. Bicorporeal septate	
U4	Hemi-uterus	a. With rudimentary cavity (communicating or not horn) b. Without rudimentary cavity (horn without cavity/no horn)	
U5	Aplastic	a. With rudimentary cavity (bi- or unilateral horn) b. Without rudimentary cavity (bi- or unilateral uterine remnants/aplasia)	
U6	Unclassified malformations		

C0	*Normal cervix*
C1	*Septate cervix*
C2	*Double 'normal' cervix*
C3	*Unilateral cervical aplasia*
C4	*Cervical aplasia*
V0	*Normal vagina*
V1	*Longitudinal non-obstructing vaginal septum*
V2	*Longitudinal obstructing vaginal septum*
V3	*Transverse vaginal septum and/or imperforate hymen*
V4	*Vaginal aplasia*

U ***C*** ***V***

Associated anomalies of non-Müllerian origin:

Drawing of the anomaly

図3 欧州ヒト生殖医学会による子宮形態異常の分類(つづき)

プスアンチコアグラント，抗カルジオリピン抗体(IgG，IgM)，抗β2GP1抗体に加え，APSの診断基準には含まれてはいないが，フォスファチジルセリン依存性抗プロトロビン抗体(aPS/PT抗体)，抗フォスファチジルエタノールアミン抗体(IgG，IgM)が研究用として測定可能である。なお抗リン脂質抗体症候群の診断は，初回検査および12週間以上あけた再検査でも陽性であることが必須である。

抗リン脂質抗体症候群を有する不育症女性に対しては抗凝固療法，すなわち低用量アスピリン(バイアスピリン1錠/日など)と未分画ヘパ

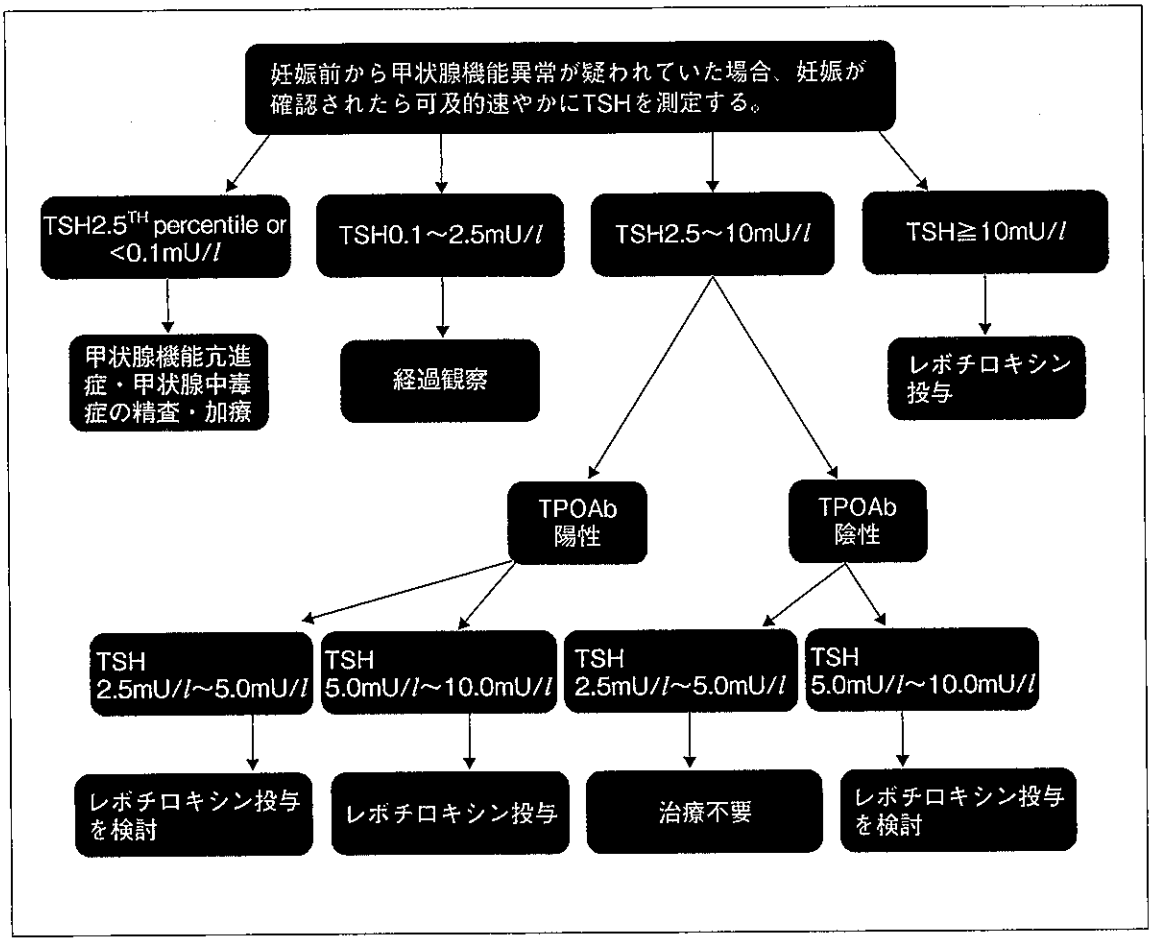


図4 甲状腺機能異常の診断・治療

リン(ヘパリンカルシウム 5,000 単位×2/日)の併用療法を行う。両者とも妊娠成立後すぐ投与開始するが，妊娠前から投与する方法もある。ヘパリンは在宅自己注射が可能であるが，ヘパリンによるアレルギー発生を考慮すると導入時には可能な限り入院での指導が望ましい。ヘパリン療法は陣痛開始まで行う。なお未分画ヘパリンの半減期は1時間程度であるが，出血のリスクを考慮すると帝王切開を行う場合には最終投与から4時間以上あけてから行うのが望ましい。緊急に帝王切開などを行われなければならない場合にはプロタミンを用いて中和する。また，アスピリン療法は妊娠28週～36週まで投与する。欧米では妊娠36週までのアスピリン投与が一般的であるが，わが国では妊娠28週以降の投与は，分娩時の出血増加や動脈管の早期閉鎖などの可能性から禁忌とされているため，患者への説明と同意のもとに行う。

7. 内分泌・代謝異常

甲状腺機能異常はわが国では不育症患者の7%にみられる。甲状腺機能低下症は，黄体機能に影響する結果，流産につながると考えられる。甲状腺機能異常の診断・治療の流れを図4に示した。甲状腺刺激ホルモン(thyroid stimulating hormone；TSH)や抗甲状腺ペルオキシターゼ(thyroid peroxidase；TPO)抗体に異常を有する場合には甲状腺専門医による診断および治療が望ましい。

なおTSHは上昇しているものの潜在性甲状腺機能低下症と診断され経過観察となっているような場合には，妊娠成立後速やかにTSHを検査し，レボチロキシン投与の必要性を確認する必要がある[12]。甲状腺機能亢進症と流産との関連性は指摘されているものの，不育症との関連性については明らかではない。

糖尿病は不育症のリスク因子となりうるが，

コントロールが良好であれば不育症のリスク因子とはしない。また多嚢胞性卵巣症候群(polycystic ovarian syndrome；PCOS)が不育症の原因となるかについても議論の余地がある。さらに，不育症を予防するためのメトホルミンの有効性についても明らかではない。

8. そのほか

最近，ビタミンD欠乏と妊娠糖尿病，子宮内胎児発育不全，妊娠高血圧症候群などの産科合併症との関連について報告されている[13]。ビタミンD欠乏と不育症との関連についてはいまだ結論は得られてはいないものの[14]，免疫異常を起こすことも報告されており[15]，また少なくとも妊娠中のビタミンD内服による副作用は報告されておらず，ビタミンDが低下している場合には内服を検討してもよいものと思われる。また免疫制御薬として免疫グロブリン[16]，イントラリピッド[17]や抗TNF-α製剤[18]などが不育症に対して試みられているが，有効性についてはいまだ明らかではない。

2．トラブル対処法

1 適切な治療法を選択する(リスク因子不明不育症を中心に)

不育症の検査を施行した場合，約60%はリスク因子不明の原因不明不育症と診断される。患者サイドに立って考えてみれば，患者は何度も流産を繰り返し，藁にもすがる思いで病院を受診しているわけであり，不育症に関するリスク因子を検索したものの原因を指摘できないことには納得ができず，患者は何らかの治療を求めてくることが多い。さらに医療者側にとっても何らかの投薬などの治療をしないで経過を見るということは，あまり好まれない。さらに患者からの要望もあれば，“ひとまず”あるいは“とりあえず”アスピリンなどを処方することもあるのではないかと推察される。しかし，原因不明不育症に対するアスピリン療法やヘパリン療法の有効性は否定的であり[19][20]，最も有効であろうとされているのはtender loving careである。

Tender loving careは，包み込むような優しいケアということであり，医療者によるカウンセリングのほか，リスクを十分にスクリーニングして説明すること，治療方針を明確にすること，家族や友人が話を聞いていてあげること，職場や近所で気を使わなくてもよい配慮，妊娠後に超音波で胎児の状態を観察することなどが含まれる。すなわち患者が安心して治療を受けられるように医療者・家族・周囲が心遣いをすることであるといえる。よって，精査を行ってもリスク因子を有さない原因不明不育症であった場合，アスピリンやヘパリンなどを使用するのではなく，十分な結果説明とカウンセリングを行い，次回妊娠に対する不安を取り除いた上で患者および家族の理解を得て，無治療で次回妊娠に臨んでも妊娠は継続する可能性は高いといえる。なお当然のことながら，何らかのリスク因子が認められた場合は，リスク因子に応じた治療を行う。

2 患者の気持ちを理解した対応を意識する

不育症患者は，何度もの流産を繰り返すことにより，次の妊娠への恐怖を感じている場合も少なくない。不妊症患者が妊娠成立したときの喜びとは対照的に，妊娠が成立しても喜べず，妊娠継続への不安と恐怖が先に出てくることが多い。概して不育症患者達は，常にそのような思いを有していると思われる。診療にあたる産婦人科医は，「今回，流産しても大丈夫，すぐまた妊娠するから」，「若いのだからまた頑張ればいいよ」，「流産はよくあること」，「まだ2回目の流産だよ」，「大丈夫，また流産したら来ればいいよ」といった言葉を患者への心遣いのつもりでかけることがあるかもしれない。しかし，不育症患者は常に妊娠継続への恐怖を抱えている。「大丈夫」，「次，頑張れ」，「よくある」など患者を思っての言葉のつもりが，患者にとっては，「何が大丈夫なのか？」，「次はどうしたらいいの？」，「よくあるといわれても…」というように，自分はこの後どうしていったらよいのであろうとの不安を助長していることになるかもしれないということを認識しなければならな

い。まずは，じっくりと患者の話を傾聴し，その患者が抱えている問題を抽出し，上記に述べたtender loving careを行っていく必要がある。

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6 経腟腹腔鏡手術(THL)

福井淳史*1 福原理恵*2 柴原浩章*1
Fukui Atsushi Fukuhara Rie Shibahara Hiroaki

経腟腹腔鏡は，経腟的アプローチによる低侵襲の腹腔鏡手技であり，ダグラス窩からの穿刺のみで腹腔内観察をすることができる。主として不妊症症例に対して施行され，子宮付属器周囲癒着，子宮内膜症，子宮筋腫などの不妊原因検索を行うことができる。また軽度の癒着剝離や子宮内膜症などの病変焼灼，多嚢胞性卵巣症候群に対する卵巣他多孔術も行うことができる。

はじめに

卵管性不妊は不妊原因全体の20～40%を占め，卵管の評価はその後の不妊治療を左右する重要なポイントとなる。卵管性不妊を診断するためには，子宮卵管造影(hysterosalpingography：HSG)あるいは通水検査，通気検査が行われ，卵管性不妊と診断された際の治療としては，体外受精・胚移植(*in vitro* fertilization-embryo transfer：IVF-ET)が選択されることが多くなっているものと推測される。しかし，自然妊娠を希望する患者も少なくなく，卵管性不妊の評価と治療は非常に重要である。

HSGは卵管疎通性を評価するための検査として有用であるが，腹腔鏡検査との比較において卵管通過性の感度は0.65，特異度は0.83であると報告されており，HSGによる診断には限界がある。また，HSGで卵管閉塞と診断された例のうち60%では，その後のHSGで卵管通過性が確認されている[1]。これは粘液栓や剝脱した子宮内膜，あるいは卵管の攣縮によるものであり，1回のHSGで診断確定をするのではなく，HSGを再施行するか選択的卵管造影や選択的通水をする，あるいは腹腔鏡により腹腔内を観察する必要がある。しかし頻回のHSGは被曝量が増え，通常の腹腔鏡検査はもちろん開腹手術に比べれば低侵襲ではあるが，一般に全身麻酔を必要とし，入院期間も数日を要する。また，傷をまったくなくすこともできない。一方，transvaginal

*1 兵庫医科大学産科婦人科
*2 弘前大学医学部産科婦人科

hydrolaparoscopy(THL)は経腟的アプローチによる新しい低侵襲の腹腔鏡であり，経腟的操作のみで通常の腹腔鏡よりもさらに低侵襲に腹腔内を観察することができる。

また，軽度子宮内膜症と診断された場合，腹腔内の内膜症病巣焼灼や腹腔内洗浄はその後の自然妊娠率を上昇させることはよく知られたことである。さらに挙児希望を有する多嚢胞性卵巣症候群(polycystic ovary syndrome；PCOS)の第1選択薬はクエン酸クロミフェン(CC)であるが，CCが無効であるPCOSに対する治療として，低用量漸増法によるFSH療法や卵巣多孔術が選択される。これら子宮内膜症の診断・治療，多嚢胞性卵巣症候群に対する卵巣多孔術は，主として腹腔鏡を用いて行われ，THLでは軽度の内膜症病変の診断，治療や多嚢胞性卵巣症候群に対する卵巣多孔術を施行することができる。

1 THLの適応

経腟内視鏡(transvaginal endoscopy：TVE)は経腟的アプローチによる低侵襲の内視鏡手技であり，子宮鏡検査と腹腔鏡を同一のスコープで行うことにより，同時に双方を観察することが可能である。主として不妊症の症例に用いられ，最初に子宮腔内を観察し(子宮鏡検査)，その後にダグラス窩からスコープを腹腔内に挿入し，通常の腹腔鏡のような気相下ではなく液相下に経腟腹腔鏡(transvaginal hydrolaparoscopy：THL)を施行する。ShibaharaらはTHLの合併症として腸管損傷が0.61%に認められたことを報告している[2]。ただし，経腹超音波ガイド下でTHLを施行することで合併症は防げるとされ，Maらの報告では，経腹超音波を用いたことで合併症が0であったと報告している[3]。われわれも，現在ではTHLを安全に施行するために経腹超音波ガイド下で手技を施行している。

THLの目的は，主として不妊症症例に対して低侵襲に腹腔内を観察して不妊原因を探索することにある。よってTHLの適応は，HSGで卵管通過障害や卵管周囲癒着，卵管采周囲癒着など卵管性病変が疑われるもの，原因不明不妊症，軽度子宮内膜症が疑われるものなどである。また，手術用シースを用いた手術的THLでは，多嚢胞性卵巣症候群に対するレーザープローブあるいは針状バイポーラ電極を用いた卵巣多孔術をダグラス窩からの1カ所の穿刺のみで施行することができる。さらに，手術的THLでは軽度子宮内膜症病変の焼灼や軽度卵管周囲癒着の剝離も行うことができる。なおTHLの要約は，ダグラス窩からスコープを挿入するため，ダグラス窩に異常病変が疑われないこと，前屈子宮であることが挙げられる。これを満たさない場合には，ダグラス窩からの穿刺の際に臓器損傷を引き起こす可能性がある。

THLの利点と欠点を表1に示した。THLは経腟的操作のみで腹腔内を観察するた

表1 THL の利点と欠点

利点	欠点
腹部切開が不要 静脈麻酔あるいは局所麻酔でも施行可能 外来ベースあるいは短期間入院で施行可能 液相での観察のため，卵管などの観察が気相より鮮明	視野が限定される 剥離や切断操作が十分に行えない 手技にやや習熟が必要



図1 THL 翌日の後腟円蓋部（穿刺部）
➡は穿刺部を示す。術創はこの1点のみであり，術後の疼痛はほとんどない。

め腹部切開が不要で，傷は腟壁の穿刺孔のみであり，腹壁にまったく傷を残すことがない（図1）。なお手技は全身麻酔下に行ってもよいが，無麻酔あるいは軽度の静脈麻酔でも手技を進めることができる。このため，外来ベースあるいは短期入院でも手技を行うことが可能となる。また腹腔内に生理食塩水を注水し液相で観察をするため，非常に明瞭な視野で腹腔内を観察することが可能となる。しかし，視野がダグラス窩からの一方向のみに限定され，処置用シースで癒着剝離ができるといっても，把持ができないため剝離は軽度のものに限定され，また卵巣多孔術を行う場合も，卵巣のダグラス窩側しか多孔術を行うことができない。

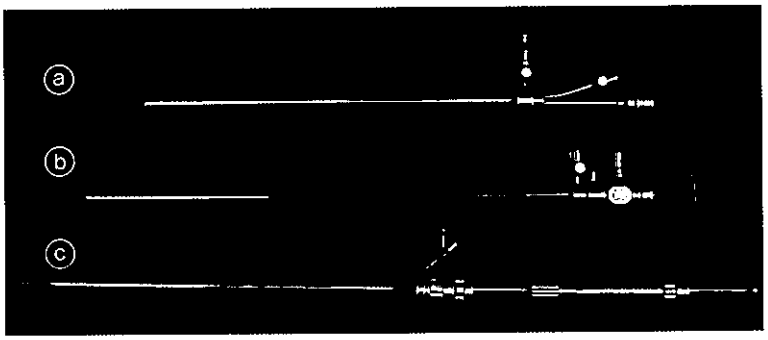


図2 TVEに用いられる機器

a：処置用シース。径は5.6 mm，長さ29 cm。5 Frの処置孔を持ち，卵巣多孔術や軽度癒着剝離に使用可能である。
b：テレスコープを装着した検査用外管。径は2.9 mm，長さ30 cm。30°の斜視鏡であり，スコープを左右に回しながら腹腔内を観察することにより，より広い範囲を観察することが可能である。
c：パンクチャーニードル，ダイレーションシース，トロカーシース。診断的THLでの穿刺時に用いられる。パンクチャーニードルでダグラス窩を穿刺した後にダイレーションシースを進め，トロカーシースを留置した後に腹腔内をテレスコープにて観察する。

2 手術の手技の実際

われわれは，ストルツ社製のTVEを使用しており，以下TVEを用いた手技について述べる。TVEは，無麻酔あるいは局所麻酔のみでも施行可能ではあるが，われわれは観察のみの場合には静脈麻酔で，処置も行う場合(手術的THL)には全身麻酔で手技を施行している。TVEで用いられる機器を図2に示した。図2aは卵巣多孔術や癒着剝離術の際に用いる処置用シース，図2bはテレスコープ，図2cはパンクチャーニードル，ダイレーションシース，トロカーシースである。

1 子宮鏡

体位を砕石位とし，手技を開始する。TVEスコープ(テレスコープ)は2.9 mmの硬性鏡であり，視野角は30°である。スコープを装着した検査用外管は灌流をすることが可能であり，スコープを左右に回すことで容易に子宮腔内を観察することができる。この際，腟口からテレスコープを検査用外管先端から流出する生理食塩水で灌流しながら挿入することにより，腟内を観察することができる。次いでそのまま外子宮口からテレスコープを挿入していくと検査用外管先端から流出する生理食塩水の水圧により頸管が拡張されるため，機械的な頸管拡張操作を行う必要なく，テレスコープは子宮腔内に挿入することが可能である。

この方法で子宮腔内に挿入することが困難な場合には，コリン腟鏡を挿入した上

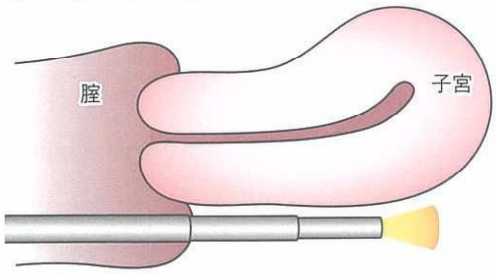
で，単鉤鉗子で牽引するなどして子宮腔内を観察してもよい。テレスコープの画質は良好であり，明瞭に子宮内膜ポリープ，子宮筋腫などの子宮内病変を観察することができる。

2 診断的経腟腹腔鏡(Diagnostic transvaginal hydrolaparoscopy)

子宮鏡検査終了後，コリン腟鏡を挿入し，後のインジゴカルミンによる通水検査のためにヒスキャスを子宮腔内に留置する。クスコ腟鏡ではなくコリン腟鏡を用いるのは，THLを行う際に腟鏡を抜去し，腟鏡によるスコープの可動性制限をなくした上で，腹腔内を観察するためである。次いでダグラス窩が解放していることを確認することとダグラス窩からの穿刺を可能な限り安全に行うことを目的に，100～150 m*l* の生理食塩水による卵管通水を行う。これによりダグラス窩には生理食塩水が貯留し，穿刺をより容易かつ安全に施行することが可能となる。続いて単鉤鉗子にて子宮腟部後唇を横方向に把持し，後腟円蓋より約1 cm背側から腹腔内に向けて穿刺を行う。穿刺の際は腹腔内観察用のトロカーシース(検査用シース)を，ダイレーションシースを装着したパンクチャーニードル(穿刺針)に装着し，穿刺予定部にダイレーションシースの先端を置き，パンクチャーニードルの穿刺深を10～15 mmとして一気に穿刺する。パンクチャーニードルには弾性スプリング機構があるため，ボタン操作で容易かつ一気に腟壁からダグラス窩腹膜まで穿刺することができる。穿刺時には単鉤鉗子を牽引し，また後腟円蓋約1 cm下方に押し当てたパンクチャーニードルをやや腹側(子宮方向)に向け穿刺を行うと穿刺を行いやすい。穿刺後，パンクチャーニードルが動かないようにしながら同様の方向にダイレーションシースと検査用シースを腹腔内に進め，テレスコープで検査用シースが腹腔内に挿入されていることを確認する。**図3**にTHL挿入時の模式図とTHLでの可視範囲を示した。

続いて腹腔内の観察を行うが，腹腔内には500～1,000 m*l* の生理食塩水を注水して観察を行うと，良好な視野で子宮および付属器を観察することができる。またTVEでは，非常に拡大された視野で腹腔内を観察することになるため，スコープの位置を把握することが肝要である。中央部で上方を観察すると子宮後面を観察することができる(**図4**)。子宮後面を確認したら，子宮に沿って左右にスコープを動かすと，卵管，次いで卵巣を確認することができる(**図5**)。卵管に沿って峡部から膨大部，卵管采を確認し，続いて卵巣周囲を観察する。観察により子宮内膜症病変(**図6**)や卵管周囲癒着(**図7**)，子宮筋腫(**図8**)などを確認することができる。十分な観察を終えたら，先に挿入したヒスキャスよりインジゴカルミンを注水し，卵管通過性を確認する。また卵管采より卵管内にスコープを挿入可能であることもあり，このときは卵管内の様子を確認することもできる。

ⓐ 横から見たTHLの挿入イメージ



ⓑ THLの可視範囲

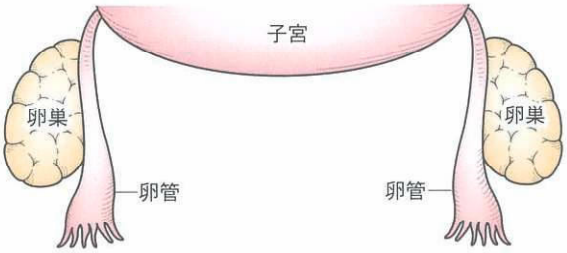


図3 THLの挿入イメージ

a：横からみたTHLの挿入イメージ。
b：THLの可視範囲。斜視鏡を回転させながら観察することにより，子宮後面から両側の付属器までを十分に観察することが可能である。

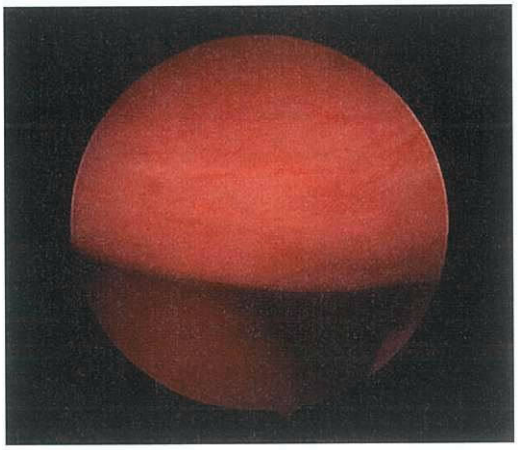


図4 子宮後面

3 手術的経腟腹腔鏡(Operative transvaginal hydrolaparoscopy)

診断的THLを行った後に，癒着剝離術や子宮内膜症病巣除去術を行ったり多嚢胞性卵巣症候群に対する卵巣多孔術を行ったりすることができる。しかし，診断的THL

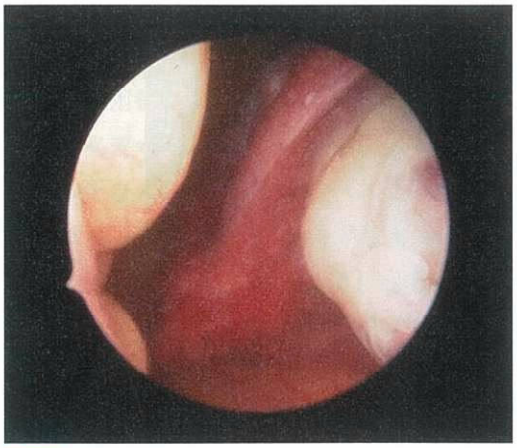


図5 卵巣と卵管



図6 子宮内膜症病変

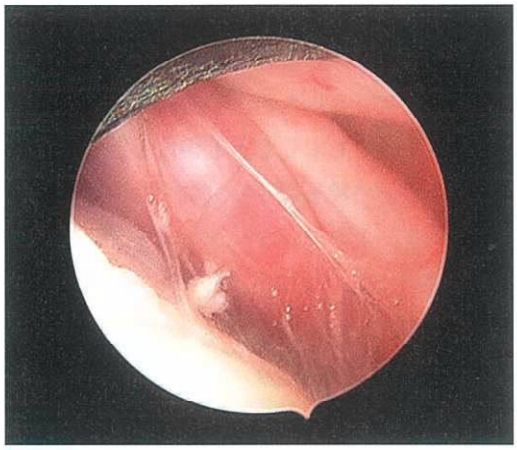


図7 卵管周囲癒着

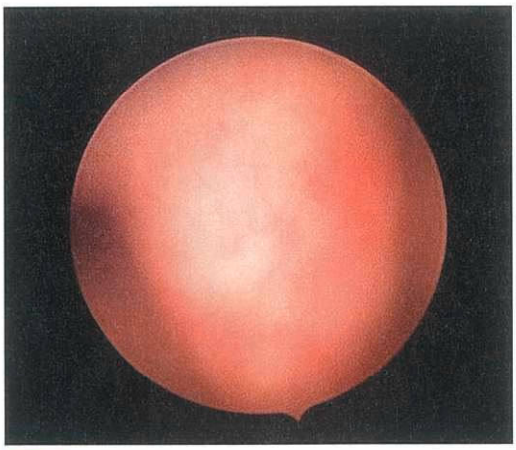


図8 子宮筋腫

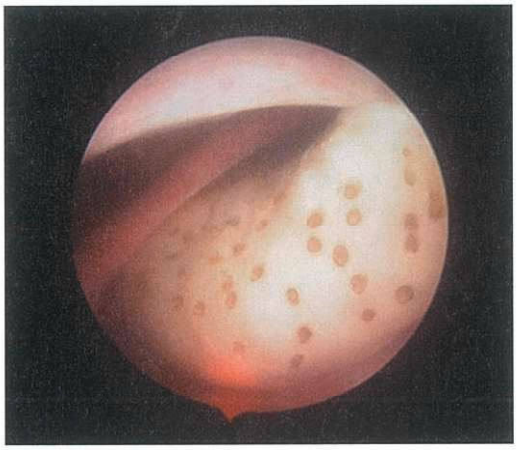


図9 卵巣多孔術

に用いられる検査用外管検査用シースは，剝離操作を行うための鋏鉗子や焼灼術，他行術を行うための電極を挿入する処置孔を有さないため，検査用シースを手術用シースに交換する必要がある。検査用シースを抜去してしまうと再度穿刺が必要となるため，検査用外管とテレスコープを抜去した上で検査用シースにマンドリン(棒)を挿入し，その後に検査用シースを抜去する。その上でマンドリンをガイドに手術用シースを挿入する。手術用シースは5 Frの処置孔を有しており，処置孔より生検鉗子を挿入して生検を行ったり，鋏鉗子を挿入して軽度の癒着剝離を行ったりすることが可能である。また，処置孔よりバイポーラ電極やレーザープローブを挿入することにより卵巣多孔術(図9)を行ったり，子宮内膜症病変の焼灼を行ったりすることができる。なお，いずれの処置の場合にもスコープからの視野はダグラス窩側からの一方向のみで

あり，また処置孔は１カ所しかないため，対象とする癒着組織や内膜症病変，多孔術を行う卵巣を把持・固定することができない。このため通常の腹腔鏡手術のように卵巣全面の多孔術を行うことは困難であり，施行しうるのはダグラス窩側から見える部位のみであることに注意が必要である。同様に剝離操作や焼灼操作もダグラス窩側からみえる部位のみへの処置となる。

手技終了後は，腹腔内に注入した生理食塩水を診断的THLでは検査用外筒の灌流孔から，手術的THLでは手術用シースの処置孔より排出した上で器具を抜去する。通常刺入部からの出血はみられず，縫合などの処置を必要とはしない。

3 留意すべきポイント

THLの合併症の多くはパンクチャーニードルを穿刺する際に発生し，そのなかでも腸管損傷には注意が必要である。これは，THLの腹腔内穿刺がブラインドで行われることが原因の１つであると思われる。THLに伴う腸管損傷発生率は0.61％と報告されており[2]，通常の経腹的腹腔鏡のトロカー穿刺時に発生する大血管・腸管損傷などの合併症発生率0.2～1.5％[4)5)]と比較しても高いものではない。また腸管損傷も多くは腹腔外の直腸損傷であり，またTHLの穿刺径も小さいため，直腸損傷が起こった際でも保存的経過観察のみでよいことが多い[6]。

しかし，合併症をゼロに近づけていくことは私たちに課せられている使命の１つである。われわれは，体外受精・胚移植における採卵の技術を応用し，採卵針を腟壁より腹腔内に穿刺した後にガイドワイヤーを挿入し，ダイレーターを用いて腟壁穿刺部を拡張した後にTHLで用いられる検査用シースを挿入する方法を考案し，現在検討を行っている。

この方法を用いて安全・確実にテレスコープを挿入可能となれば，さらに本法が普及していくものと思われる。

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今日の問題

若年がん女性における卵子・胚・卵巣組織凍結による妊孕性温存の現状と課題

兵庫医科大学産科婦人科学講座 脇本 裕，長谷川昭子，柴原浩章

はじめに

近年，手術・化学療法・放射線治療などのがん治療の進歩により，悪性腫瘍を発症する若年がん女性の完全寛解率は向上し，がんを克服した患者（がんサバイバー）が増加している．一方で，女性のがんの10％程度は45歳以下で発症し，一部の化学療法や放射線療法による卵巣機能障害が報告され，治療によっては妊孕性が消失する．がん治療開始前に予後良好な若年のがん女性に対して，卵子・胚・卵巣組織の凍結保存による妊孕性温存療法をすることで，がんサバイバーのquality of life（QOL）は向上している．2006年にアメリカのWoodruffらは腫瘍学（oncology）と生殖医学（fertility）を融合し[1]，若年がん患者に対する妊孕性温存を目的とする「がん生殖（oncofertility）」という概念を提唱した．

がん生殖は，本邦でも注目され，日本生殖医学会は2013年11月に「未受精卵子および卵巣組織の凍結・保存に関するガイドライン」を発表し，続いて日本産科婦人科学会も2014年4月に「医学的適応による未受精卵子および卵巣組織の採取・凍結・保存に関する見解」を示した．この見解では胚凍結については言及していなかったが，2016年6月に「医学的適応による未受精卵子，胚（受精卵）および卵巣組織の凍結・保存に関する見解」として改訂した[1]．

がん治療に伴う妊孕性温存療法はがん治療医と生殖医療医との間の速やかで緊密な連携が重要であり，双方がエビデンスに基づいた正しい情報や問題点を共有する必要がある．そこで日本がん治療学会から小児，思春期・若年がん患者の妊孕性温存に関する診療ガイドラインが2017年7月に第1版として発刊された．以上のような啓発活動を通しoncofertilityという概念が，がん治療医や生殖医療医に浸透してきている．

がん生殖医療ネットワーク

がん生殖医療の現場では，がんを宣告されて間もない患者に対して限られた時間内に正確な情報を提供する必要があり，がん治療と生殖医療の両チームの速やかで緊密な連携が不可欠である．妊孕性温存により，がん治療に伴う妊孕性喪失を防ぐことは可能となったが，一方で，がん治療の遅れによる予後の悪化が懸念され，妊孕性温存治療に関する情報提供が不十分になるケースがある．このような事態を鑑みて，2012年11月に「日本がん生殖医療研究会（現在は学会）」が設立された．その後，生殖医療医と各科のがん治療医の緊密な連携に向けた医療システムの構築が求められ，2013年2月に岐阜県がん・生殖医療ネットワークを初めとして都道府県単位で設立されている[2]．現在のところ，近畿においては滋賀県，兵庫県，大阪府，京都府でがん生殖医療におけるネットワークが設立されている．これらのネットワークは他府県とも連携しており，それぞれのHPを参照されたい．

◆Current topic◆

Fertility preservation in adolescents and young adults with cancer

Yu WAKIMOTO, Akiko HASEGAWA and Hiroaki SHIBAHARA

Department of Obstetrics and Gynecology, Hyogo College of Medicine

卵子・胚・卵巣組織凍結による妊孕性温存の特徴

妊孕性温存法には卵子・胚・卵巣という3つの凍結法がある．胚凍結は確立された方法で妊娠率が比較的高いが，精子（夫やパートナー）が必要であり，かつ離婚後は無効となる．卵子凍結および卵巣凍結は未婚にも適応があり，精子は不要である．がん治療前の卵子・胚凍結では，原疾患の治療を早急に開始しなければならない場合が多く，一般にARTで行われる月経初期からの卵巣刺激はがん治療の遅延による予後の悪化が懸念される．近年，月経周期のうち卵胞期後期や黄体期から卵巣刺激を開始するランダムスタート法（random-start controlled ovarian stimulation）が一般のARTと遜色なく成熟卵や胚盤胞が得られると報告されている[3]．この月経周期のいつからでも卵巣刺激を開始する方法は，がん治療を早急に開始しなければならない若年がん女性に対する妊孕性温存のための卵巣刺激として有用である．

卵子凍結において融解後の卵子生存率はvitrification法により大きく改善した．ただし，36歳未満の女性の卵子1個あたりの生児獲得率が4～5%であることから，1児を得るために20～25個の卵子を凍結保存する必要性がある．さらに，40歳以上においては，1児を得るために55.5個の卵子の凍結が必要との報告もある[4]．健康女性において卵子を5個，8個，10個凍結保存した場合の生児獲得率は35歳以下ではそれぞれ，15.4%，40.8%，60.5%で，35歳以上では5.1%，19.9%，29.7%と報告されている[5]．ただし，がん患者においては原疾患に伴い卵の質が低下するため，35歳以下で10個の卵子を凍結保存しても生児獲得率は34%との報告もある[5]．したがって，卵巣刺激により多くの卵子を採取（採卵）することが生児獲得率の向上に貢献するため，同一周期に2回の採卵を行うランダムスタート法を応用したDuoStim法という卵巣刺激法が開発された．この方法は，従来法による卵胞期初期からの刺激により1回目の採卵を行い，引き続き同一周期の黄体期から再度卵巣刺激を行い，2回目の採卵を行う方法である．これにより，がん治療開始までにより多くの胚や卵子を凍結保存することが可能である．

しかし，卵巣刺激による採卵数および凍結個数には限界がある．これに対して，卵巣組織凍結保存は理論上一度に多くの原始卵胞を温存することが可能である．卵子凍結と卵巣凍結のコンビネーションにより50～60%の生児獲得率が期待できるとの報告がある[5]．卵巣組織凍結保存は1997年にベルギーのDonnezらにより初めて臨床応用され，2004年に若年ホジキン病患者に対して凍結卵巣組織を自家移植し，世界初の出産例が報告された[5]．卵巣組織凍結による妊娠出産例は年々増加しており，2017年6月までで卵巣組織凍結・移植により世界で約130名の出産例が報告されている[5]．現在のところ移植あたりの妊娠率は約30%で，生児獲得率は約25%とされている．本法は卵巣刺激が不要のため，がん治療に向け迅速な対応ができる．したがって，ランダムスタート法を用いた卵巣刺激でもがん治療開始までに時間的な余裕がない場合，さらには卵巣刺激に反応しない排卵周期が確立していない思春期前の女児に対する妊孕性温存の第1選択として用いられる．また，卵巣組織凍結の際に両側卵巣にある胞状卵胞を穿刺のうえ採卵し，未成熟卵子を体外培養のうえ成熟誘導して卵子凍結が行われている．

一方，卵巣組織凍結は卵巣摘出手術や麻酔管理が必要である．卵巣移植を行う際，移植後の虚血による原始卵胞の死滅や，移植組織における悪性腫瘍細胞の微少残存病変（minimal residual disease；MRD）の混入の可能性の問題点が指摘されている．すなわち，卵巣組織に腫瘍細胞が転移していた場合，移植時に再発のリスクを伴うことになる．このMRDは全身疾患である白血病患者には高リスクとされているが，本疾患は女児が罹患することが多く，また急速に進行することから，卵巣凍結による妊孕性温存が選択されることが多い．2018年1月に完解後で骨髄移植前に凍結保存した卵巣組織を融解移植した白血病患者の妊娠出産例が報告された[6]．この報告では，病理組織学的，免疫不

全マウスへの異種移植，免疫染色（FISH），次世代シーケンサーを用いてMRDの可能性を否定したうえで卵巣組織を移植している．また，白血病患者においては完解期の凍結保存を推奨している．

今後の課題

MRDの問題解決に向け，体外培養，人工卵巣，多能性幹細胞などが考えられる．凍結卵巣組織を融解移植せず，原始卵胞から卵子を培養により成熟卵子にまで誘導できれば原疾患の再発の心配はない．卵子の発育段階に応じたマルチステップ培養システムにより，ヒト卵巣組織切片から成熟卵子の発育誘導の成功例も報告されている[7]．人工卵巣においては，人工臓器をつくるためゼラチンで卵巣構造の足場（scaffold）を構築し，単離した未発育卵胞をこれに適用し人工卵巣を形成する．この人工卵巣を移植して，マウスにおいては産仔が得られている．ヒトにおいては，ヒト原始卵胞を含んだ人工卵巣を免疫不全マウスに移植して前胞状卵胞への発育が観察されている[8]．また，マウスではES細胞，iPS細胞などから成熟卵子を誘導し，次世代を生産できることも明らかになっているが[9]，ヒトへの応用に関しては倫理面・技術面から時期尚早といわざるを得ず，MRD回避のためには，当面，患者の卵巣内に多数存在する発育中の卵胞や休止状態の原始卵胞を培養条件下で有効に発育させる方法の開発がより現実的であろう．

終わりに

がん・生殖医療における医療現場では，何よりもまず原疾患の治療が最優先されるべきである．原疾患の予後に影響がない範囲内で妊孕性温存療法を行うべきであり，その限られた時間のなかで多くの自己決定を行う患者への心理支援体制の充実は不可欠である．さらには，卵子凍結や卵巣組織凍結による妊孕性温存は，特定不妊治療費助成制度の対象外であり，経済的支援体制の構築が必要である．

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コラム　PCOSの多孔術

福井淳史（兵庫医科大学産科婦人科学講座講師）
福原理恵（弘前大学医学部産科婦人科学教室講師）
柴原浩章（兵庫医科大学産科婦人科学講座主任教授）

1 過去のPCOSの多孔術

多嚢胞性卵巣症候群（polycystic ovarian syndrome：PCOS）は，第一選択薬であるクエン酸クロミフェンが無効であることも少なくなく，ゴナドトロピン療法を行うと卵巣過剰刺激症候群を起こすことがあるなど，薬物療法には苦慮することが少なくない。PCOSに対する多孔術は，自然排卵周期の回復とその後の自然妊娠が期待できる外科的治療法である。

1935年，PCOSに対する開腹手術による楔状切除術の有効性が報告され[1]，その後80年代になって腹腔鏡下焼灼術が行われるようになった[2]。

2 現在のPCOSの多孔術

現在では，上記の腹腔鏡下卵巣多孔術（laparoscopicovarian drilling：LOD）に加え（**図1**），より低侵襲に行う経腟腹腔鏡下卵巣多孔術（transvaginal hydrolaparoscopy-ovarian drilling：THL-OD）も施行可能[3]となっており（**図2**），さらには経腟的超音波ガイド下に小卵胞を針で穿刺することにより，排卵機能を回復しうる可能性[4]も報告されている。なお，LODは腹腔鏡下多嚢胞性卵巣焼灼術（K887-4，2万4130点）として保険収載されている。LODは，ゴナドトロピン療法と同等の成績が期待でき，妊娠率は26～48%，生児獲得率は13～32%と報告されている[5]。

多孔術は電気メス（モノポーラ）で行われることが多く，両側施行と片側施行で妊娠率

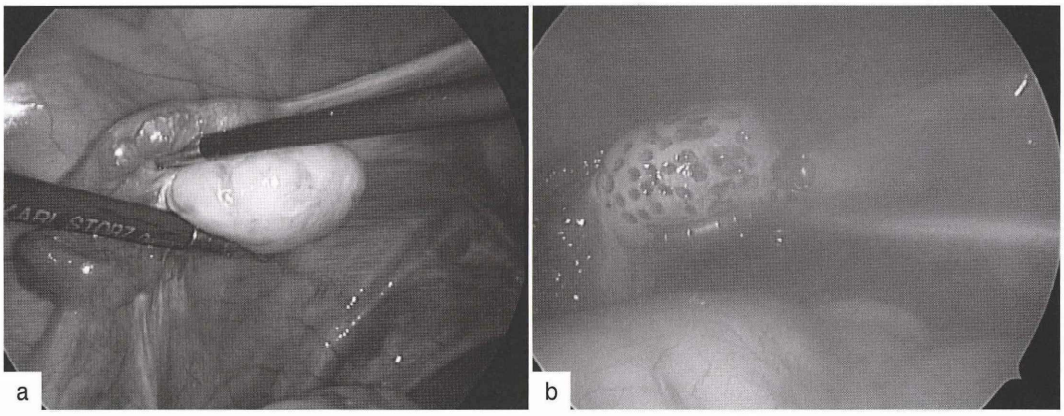


図1　腹腔鏡下卵巣多孔術（LOD）
a：多孔術施行前の卵巣，b：多孔術後の卵巣

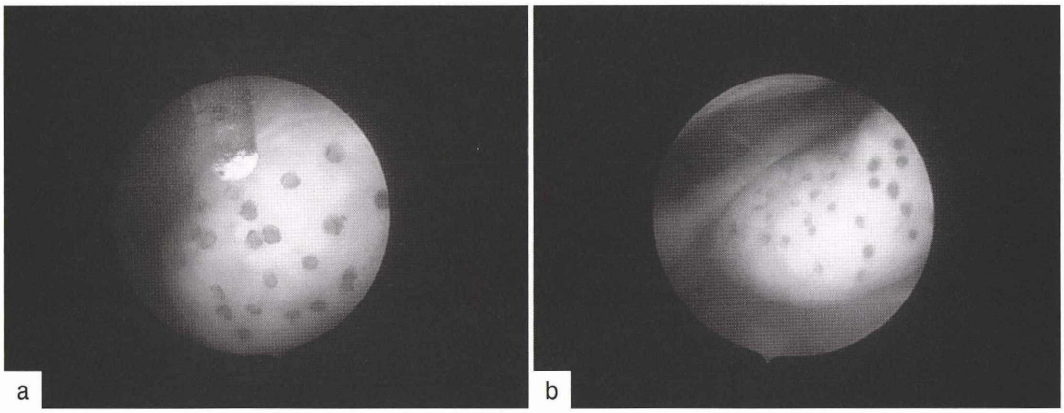


図2　経腟腹腔鏡下卵巣多孔術（THL-OD）
a：KTPレーザーによる多孔術，b：多孔術後の卵巣

や生児獲得率には差はない[5]が，片側卵巣当たりの開孔数は5～10個より15個のほうが妊娠率が良好であり[6]，片側当たり少なくとも15個開孔することが望ましい。また，熱損傷に留意し，モノポーラの電圧を上げる（60～70W）ことにより周囲の熱損傷を防いだり，洗浄水にて卵巣を冷却しながら行うなどの愛護的操作が肝要である[7]。この点では，腹腔内に生理食塩液を注入し，液相下にレーザーによる多孔術を行うTHL-ODは合目的であると思われる。

3 未来のPCOSの多孔術

いったいどのような患者でPCOSに対する多孔術を施行すれば，長期的に有効なのであろうか？　LODの至適症例をピックアップすることが，今後のPCOS診療において重要であると思われる。LOD術後の10年間以上の長期予後を検討すると，術前のFSH値

が6.15mIU/mLより高値である場合，長期効果持続（自然排卵の継続）が期待される可能性が示唆された[8]。

そのほか，LODの効果を予測する因子としては，術前LH値が高値（＞10IU/mL）である場合や肥満がない場合などにLODが効果的であると報告されており[9]，これらを総合的に判断し，手術適応が決定されていくようになるものと思われる。

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コラム　婦人科手術での後腹膜縫合

田畑 務（三重大学医学部産科婦人科学教室准教授）

1 過去の後腹膜縫合

腹腔内臓器は腹膜で裏打ちされているため，手術や炎症などの既往がない限りほとんど癒着しない。子宮は表面を腹膜で覆われた後腹膜臓器であり，子宮を摘出するためには腹膜を切離しなければならない。また，婦人科悪性腫瘍手術では，傍大動脈リンパ節郭清を行うことも多く，切開創は上腹部に及ぶ。手術により腹膜が欠損すればその部位の癒着は必至であり，ひいては腸閉塞に至る可能性も考えられる[1)2)]。

これまで，腹膜欠損部は残存している腹膜を引き寄せ縫合することにより癒着防止を試みてきた。その後，子宮摘出術時には，後腹膜縫合をしてもしなくても術後に発症する腸閉塞などの頻度は変わらないとの報告があり，徐々に子宮摘出後も腹膜の縫合はされなくなってきた。また，腹膜縫合をした場合には，縫合糸に小腸などの周辺臓器が癒着しやすいことが報告されている。

2 現在の後腹膜縫合

現在は，子宮摘出や婦人科悪性腫瘍手術の際にも，後腹膜の縫合は行わない施設が多い。近年，腹腔鏡下子宮全摘出術の頻度が増しているが，腹腔鏡下手術の場合，ほとんど後腹膜縫合は行われない。しかし，後腹膜縫合を行わない部位は，周囲の腸管などと癒着

配偶子保存法の周辺技術

ランダムスタート法による卵巣刺激

浮田 祐司　脇本 裕　柴原 浩章

● 卵胞期後期や黄体期から卵巣刺激を開始する方法をランダムスタート法と呼び，従来法と比較しても同等数の成熟卵や胚盤胞を得ることができる．
● ランダムスタート法はがん治療を早急に開始しなければならない，若年がん女性に対する妊孕性温存のための卵巣刺激に有用である．
● 従来法である卵胞期初期からの刺激による採卵だけでなく，黄体期からの採卵も行う２回採卵（Duostim）法は卵巣予備能力が低下している女性への卵巣刺激法として考慮してよいかもしれない．

はじめに

がん治療の早期診断ならびに集学的治療の進歩に伴い，若年がん患者のがん治療前の妊孕性温存が注目されている．妊孕性温存法には，女性では胚（受精卵），未受精卵子，および卵巣組織の凍結保存が，一方男性には精子の凍結保存が行われている．われわれも女性[1]および男性[2]の妊孕性温存法を積極的に施行し，報告してきた．胚や未受精卵子の凍結保存では，調節卵巣刺激法により少しでも多くの卵子採取（採卵）を目指すことになる．がん治療前の卵子・胚凍結では，原疾患の治療を早急に開始しなければならない場合が多く，一般にARTで行われるように月経初期からの卵巣刺激を待つことができない．このような場合に，月経周期のうち卵胞期後期や黄体期から卵巣刺激を開始しても成熟卵や胚盤胞を得られることが近年報告されている[3]．この月経周期のいつからでも卵巣刺激を開始する方法を，ランダムスタート法（random-start controlled ovarian stimulation）と呼ぶ．これまでわれわれもランダムスタート法の有用性を報告してきた[4]．ランダムスタート法はがん治療を早急に開始しなければならない，若年がん女性に対する妊孕性温存のための卵巣刺激に有用である．

うきた ゆうじ，わきもと ゆう，しばはら ひろあき：兵庫医科大学産科婦人科（〒663-8501　兵庫県西宮市武庫川町1-1）

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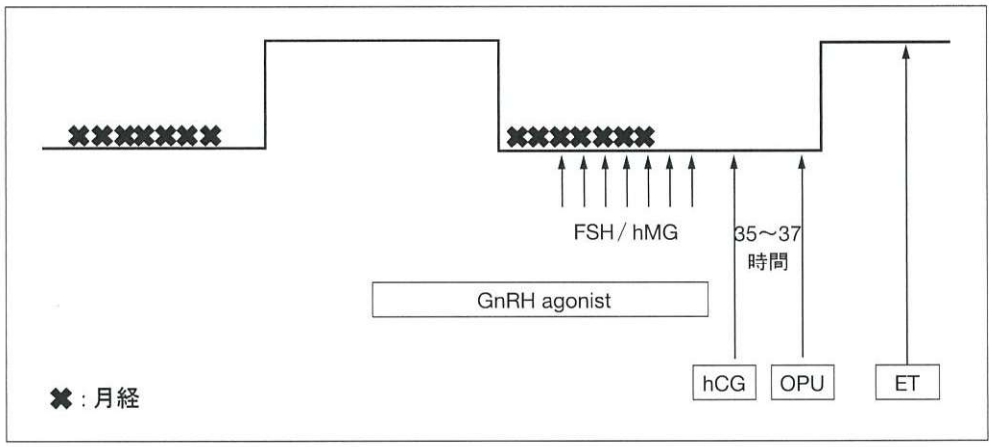


図1　Long 法

ランダムスタート法の原理

ランダムスタート法で成熟卵を得ることができる原理を説明する．従来，卵胞発育は前月経周期の黄体期後期に前胞状卵胞のリクルートがあり，その後，卵胞期中期に 1 個の卵胞が選択されるとその他の卵胞は閉鎖に向かうと考えられていた[5]．しかしながら最近では，同一月経周期のなかで卵胞のリクルートは多くの波があることが示されている[6,7]．そのため，卵胞期後期や黄体期に認める前胞状卵胞は閉鎖に向かうものではなく，卵胞発育の初期の段階であると考えられる．したがって，どの月経周期でも卵巣刺激を行えば，卵胞が発育し採卵は可能になるという原理である．しかし，月経周期と無関係であることから，新鮮胚移植はできないため，全胚凍結が必要である．

従来の卵巣刺激法

ART で用いられてきた卵巣刺激法には，一般に GnRH アゴニストを用いる long 法・short 法と，GnRH アンタゴニスト法がある[8]．

卵巣の反応性が正常である場合は基本的には long 法を用いる．short 法に比して調節性に優れ，成績も良いことが報告されているためである[9]．一方，卵巣の反応性が低下している場合には short 法を用いる．卵胞期初期に GnRH アゴニストの flare up 効果を利用して，卵胞発育を促進させ発育卵胞数の増加を期待するためである．多嚢胞性卵巣や若年女性のように卵巣の反応性が良好な場合には，卵巣過剰刺激症候群（OHSS）の発症や重症化を回避するため GnRH アンタゴニスト法を用いる．

long 法では GnRH アゴニストは黄体期中期から使用を開始する（**図 1**）．

short 法では GnRH アゴニストは消退出血または月経開始時より使用を開始する．ゴナドトロピンの投与は基本的に，月経または消退出血開始の 3 日目から連日行う．long

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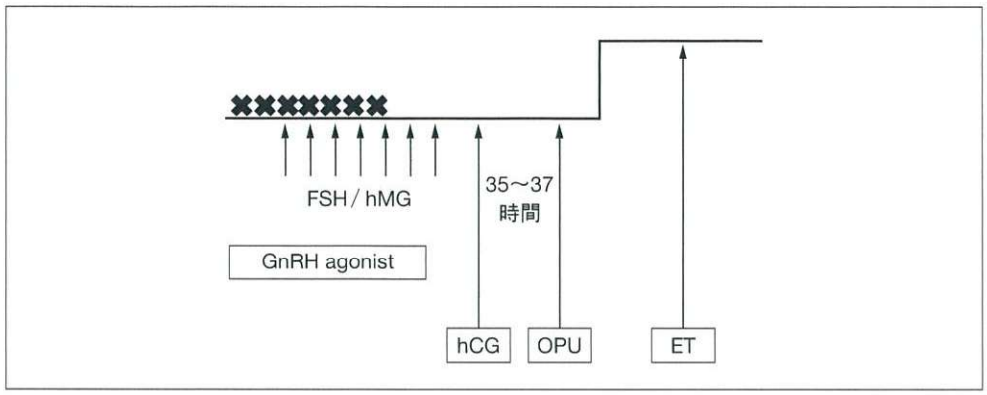


図 2　Short 法

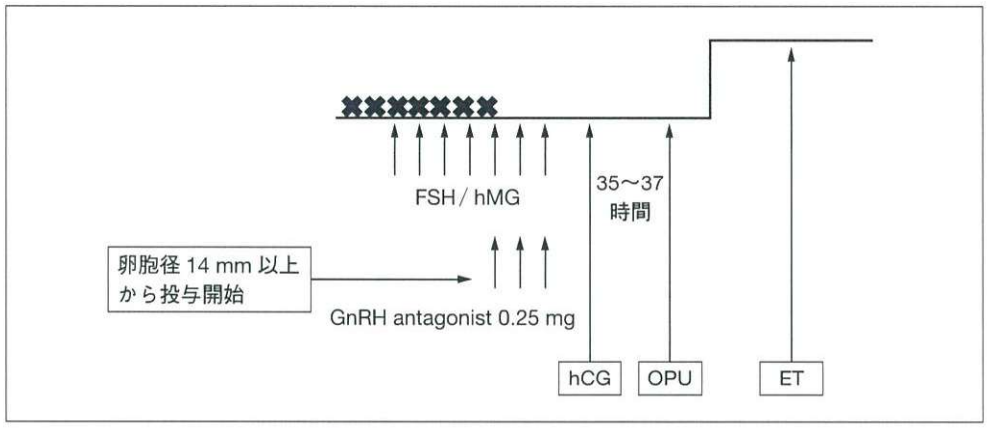


図 3　GnRH アンタゴニストの連日投与法

法・short 法のどちらにおいても主席卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 2**）．

GnRH アンタゴニスト法では，GnRH アンタゴニスト 3 mg の 1 回投与法と，0.25 mg の連日投与法がある．その投与時期は，卵巣刺激開始後 6 日目に投与する固定法と，卵胞径が 14 mm に達した時点で投与する変動法がある．後者のほうが妊娠率が高いとの報告がある[10]．卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 3**）．

ランダムスタート法による卵巣刺激法

ランダムスタート法には月経周期により卵巣刺激法にバリエーションがある．

(1) 卵胞期初期（月経開始後 7 日未満）から刺激開始の場合には，速やかにゴナドトロピンの連日投与を開始する．その後は GnRH アンタゴニスト法と同様に主席卵胞径が 14 mm に到達した時点で，GnRH アンタゴニストを投与開始し，卵胞径

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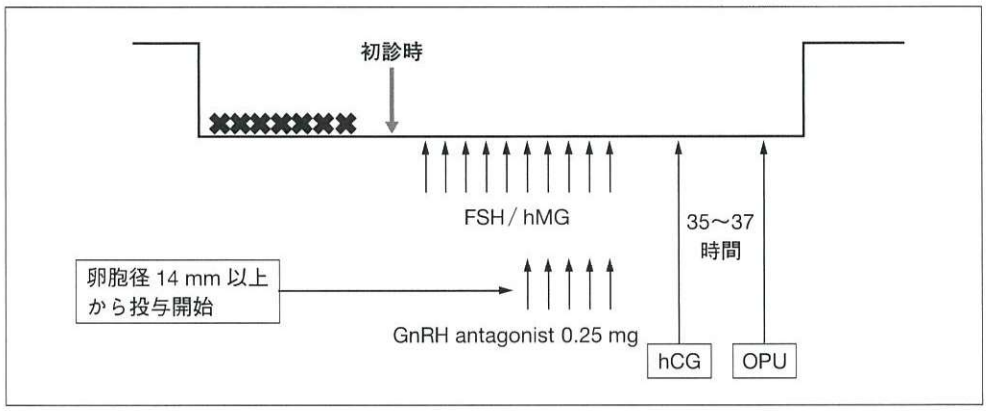


図 4　ランダムスタート法（卵胞期初期開始）

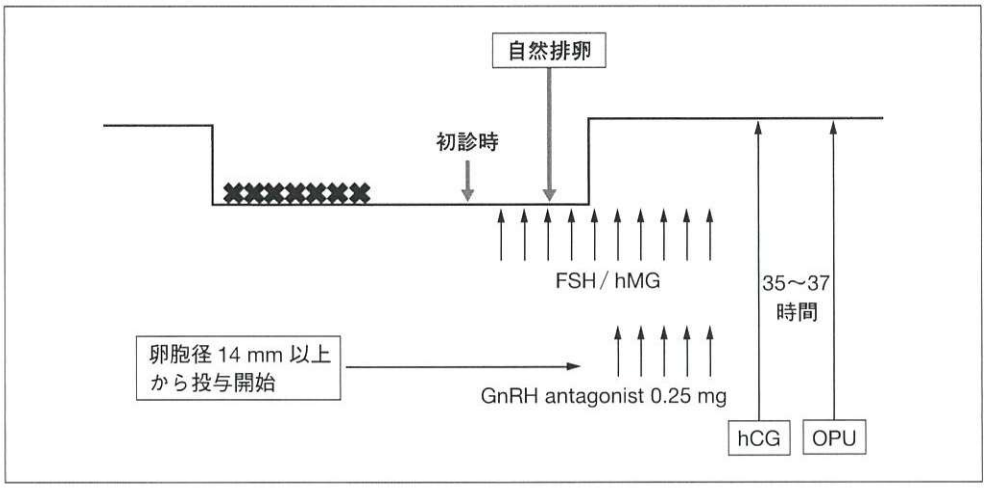


図 5　ランダムスタート法〔卵胞期後期（主席卵胞径 18 mm 未満）開始〕

が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 4**）．

(2) 卵胞期後期（月経開始 7 日以降）から刺激開始の場合には，主席卵胞径が 18 mm 未満と 18 mm 以上で対応が異なる．前者では，主席卵胞以外の卵胞が 12 mm 未満であるのを確認し，ゴナドトロピン投与を開始する．その後，主席卵胞に対しての LH サージ開始後から GnRH アンタゴニストの投与を開始し，2 番目以降の卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 5**）．後者では，hCG 5,000 単位を投与して排卵させ，その 2～3 日後からゴナドトロピンの投与を開始する．その後は GnRH アンタゴニスト法と同様に主席卵胞径が 14 mm に到達した時点で GnRH アンタゴニストの投与を開始し，卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 6**）．

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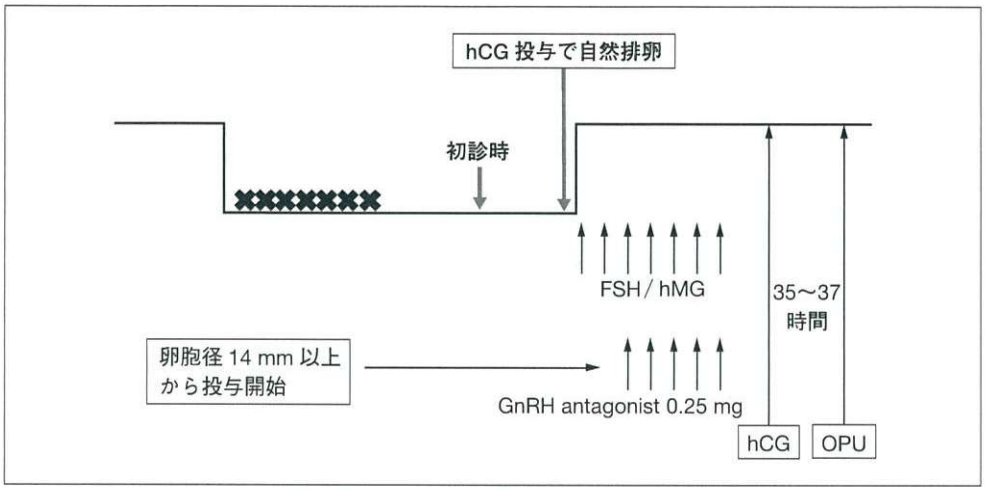


図 6　ランダムスタート法〔卵胞期後期（主席卵胞径 18 mm 以上）開始〕

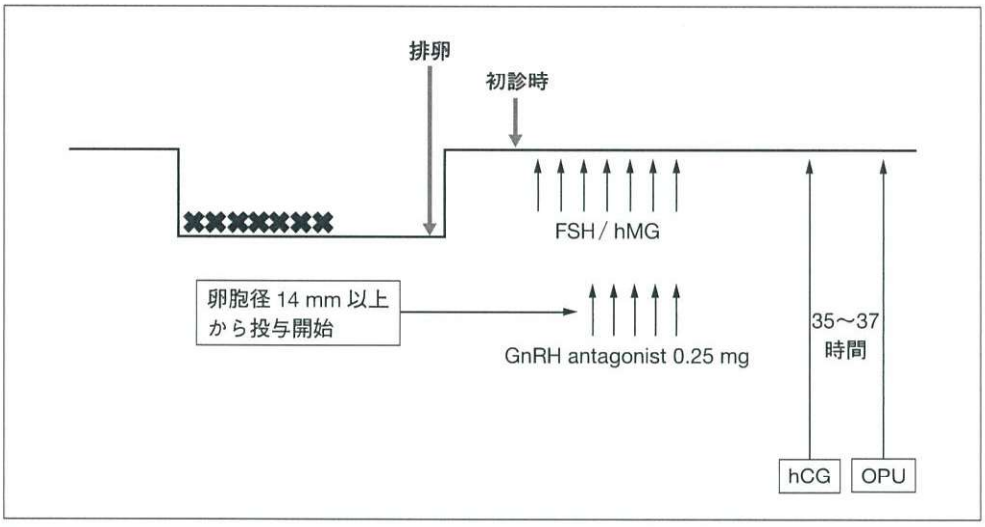


図 7　ランダムスタート法（黄体期開始）

(3) 黄体期から刺激開始の場合には，速やかにゴナドトロピンの連日投与を開始する．その後は GnRH アンタゴニスト法と同様に主席卵胞径が 14 mm に到達した時点で，GnRH アンタゴニストの投与を開始し，卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 7**）．

ランダムスタート法の有効性について

従来法，卵胞期後期からのランダムスタート法，黄体期からのランダムスタート法それぞれの獲得卵子数・成熟卵子数・受精卵数には有意差がないが，ランダムスタート法のほ

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うが有意に総ゴナドトロピン量は多かったと報告されている[1, 11, 12]．われわれも同様の報告を行っている[13]．

妊孕性温存だけではなく，通常のARTで卵巣刺激への反応が低下している症例に対して，ランダムスタート法を応用したDuostim法が報告されている[14]．これは同一周期に2回採卵するという方法で，従来法による卵胞期初期からの刺激により1回目の採卵を行い，引き続き同一周期の黄体期から再度卵巣刺激を行い，2回目の採卵を行う．卵胞期初期と黄体期の獲得卵子数・成熟卵子数・胚盤胞数に有意差を認めなかった．したがって本法は，卵巣予備能が低下している女性への卵巣刺激法として考慮してよいかもしれない．

おわりに

ランダムスタート法は初めて報告されて以来，まだ経験が少ない．そのため妊孕性を温存した女性に対して胚移植を行い，従来法と比べて妊娠・分娩率に影響がないかは今後の課題である．一方がんに罹患していない不妊女性に対し，黄体期から卵巣刺激を開始するランダムスタート法を行い，従来法と変わらない周産期転帰であったとの報告もある[15]．したがって可及的速やかに卵巣刺激を開始しなければならない場合には，ランダムスタート法による卵巣刺激法は積極的に考慮してよいものと考える．

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診断法

抗精子抗体

脇本 裕　杉山 由希子　柴原 浩章

- 抗精子抗体は，女性では同種抗体として，男性では自己抗体として産生され，免疫性不妊症の一因となる．
- 抗精子抗体の検出法として，女性側では精子不動化試験（sperm immobilization test：SIT）が，男性側ではイムノビーズテスト（immunobead test：IBT）が用いられている．
- WHOは精液検査の際に，運動精子上に結合する抗精子抗体の検出を推奨している．

はじめに

不妊症の三大原因とは，内分泌因子，卵管因子，男性因子で，その責任所在は，女性が約65%，男性が約50%（重複は約25%），原因を特定できないカップルは約10%強である．不妊治療において男性因子の検索は重要であり[1]，精液検査は不妊治療開始後早期の実施が望ましい[2]．

男性不妊症の病因として，本邦においては造精機能障害が約80%，精子輸送路の通過障害が約10%，精子機能障害および原因不明不妊症が約10%を占める．

精子機能障害の原因の1つに免疫性不妊の抗精子抗体（antisperm antibody：ASA）がある．抗精子抗体には多様性があり，必ずしも不妊症となるわけではないが，なかには精子不動化作用や受精障害作用をもつ抗体が含まれる[3]．本稿では，抗精子抗体検査の診断法と，治療法について概説する．

抗精子抗体とは

1954年Rumke[4]は不妊男性の血中に高率に精子凝集素を検出し，原因不明男性不妊

わきもと ゆう，すぎやま ゆきこ，しばはら ひろあき：兵庫医科大学産科婦人科学講座（〒663-8501　兵庫県西宮市武庫川町1-1）

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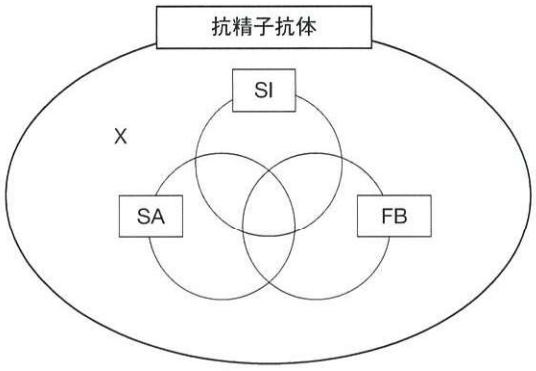


図1　抗精子抗体の精子機能障害作用

X：不妊症と直接関係しない精子結合抗体
SI：精子不動化抗体（sperm immobilizing antibody：SI）
SA：精子凝集抗体（sperm agglutination antibody：SA）
FB：受精阻害抗体（fertilization blocking antibody：FB）

表1　男性不妊患者における抗精子抗体の多様性

抗精子抗体	抗体の多様性
Ig class	IgG, IgA, IgM
精子上の抗体結合部位	頭部・中片部・尾部
力価	IB 結合数
生物活性	精子不動化作用，受精阻害作用
抗体産生部位	局所抗体・循環抗体

症のなかに抗精子抗体が関与するものがあることが臨床的に初めて示唆された．抗精子抗体は男性では自己抗体として産生され，また抗体が示す生物活性は多様である．したがって，抗精子抗体による男性不妊症発症の臨床像も多様で，精子を不動化したり，精子を凝集したりなど，精子の各受精段階に阻害作用を示す（**図1**）．

原因としては，抗体のクラス（IgA，IgG，IgM）またはサブクラス，精子への結合部位や力価，産生部位が造精組織や精路などの局所であるのか，循環血中であるのかなど，これらの相違により抗精子抗体は多様性を示す[5]（**表1**）．このような抗精子抗体の多様性のため，自己が産生する精子細胞に対して抗体が産生される病態および臨床像の詳細はいまだ明らかではない．

なお女性では，抗精子抗体は同種抗体として産生され，1964年FranklinとDukeは不妊女性の血中に高率に精子凝集素を検出し，精子凝集作用のある抗精子抗体と不妊症発症の関係が示唆された[6]．Isojimaら[7]は不妊女性患者血清中の抗体を検出する精子不動化試験（indirect-sperm immobilization test：I-SIT）および精子不動化抗体価であるSI_{50}値（sperm immobilization titer）の測定法を開発し，不妊症との相関を明らかにした．SI_{50}値を計測することで抗体陽性不妊女性の診療指針が決定できる[8]．

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抗精子抗体産生機序

男性にとって精子は自己が産生する細胞であり，精子形成の場である精巣は血液-精巣関門（blood-testis barrier）により，強い免疫抑制機構が誘導されにくい環境（immunologically privileged site）にある[9, 10]．したがって，精子細胞に対する自己抗体の産生は起こらない仕組みになっている．しかし，免疫制御機構の許容範囲以上の精子抗原刺激を受けると精子免疫が誘導され，全身的にも局所的にも免疫応答を引き起こして抗精子抗体を産生し，稀に自己免疫性精巣炎を引き起こす[9]．また，血液-精巣関門の破綻により造精器官局所において抗精子抗体が産生される可能性や，臓器特異性の自己免疫疾患を罹患した男性患者の場合では，自己免疫応答の異常によるポリクローナルB細胞の活性化により抗精子抗体を有意に産生しやすいことが報告されている[11, 12]．なお，精巣に病変をもたない健康な不妊男性においても，血中および精液中に抗精子抗体が検出されることがある．

抗精子抗体の検出法

不妊男性の抗精子抗体の検出法として多数の測定法が報告されているが，不妊症との関係では少なくとも運動精子と反応し，精子に抗体が結合することにより精子の運動性あるいは受精能に影響を及ぼす抗体を検出する必要がある[9]．射出運動精子細胞膜上に付着している抗精子抗体の検出法として，Bronsonら[13]の方法による直接イムノビーズテスト（direct-immunobead test：D-IBT）が繁用されてきたが，現在のところIBTの検査キットは，製造中止に伴い入手不能である．そこで，IBTと同じ原理による抗精子抗体検出法のイムノスフェア®（ImmunoSpheres®：IS）（Bioscreen Inc.）を用いる．なお，Centolaら[14]はIBTとISの検討で，強い相関性を報告している．ISの検査手順を取り扱い説明書に従って**図2**に示す．

D-IBTではウサギ抗ヒトIgG・A・M抗体を結合させたポリアクリルアミドビーズを用いて運動精子細胞膜に結合している抗精子抗体を網羅的に検出する．これにより，抗体のイムノグロブリン（Ig）の各クラス（IgG，IgA，IgM），精子の結合部位（頭部，中片部，尾部），抗体結合運動精子が運動精子全体に占める割合を判定する[13]．ただし，この方法によって検出される精子結合抗体のすべてが，不妊症を引き起こすわけではない．したがって，1次スクリーニングとして用いるにはよいが，検出された抗精子抗体の運動精子に対する生物学的な障害作用を調べる必要がある．抗精子抗体が生物作用を示すのは，患者のもつ射出精子上の抗体が精子不動化抗体の場合である．このためD-IBT陽性すなわち運動精子に抗精子抗体が結合している場合は，直接精子不動化試験（direct-sperm immobilization test：D-SIT）[15]を実施する必要がある．

D-SITは運動精子に結合した抗精子抗体に，補体依存性の精子不動化作用という生物活性があるかを確認する検査法である（**図3**）．D-SITは運動精子細胞膜上に付着する抗精子抗体の中で，精子の運動能と関連する抗体の存在を予知しうる検査法であり，D-SIT

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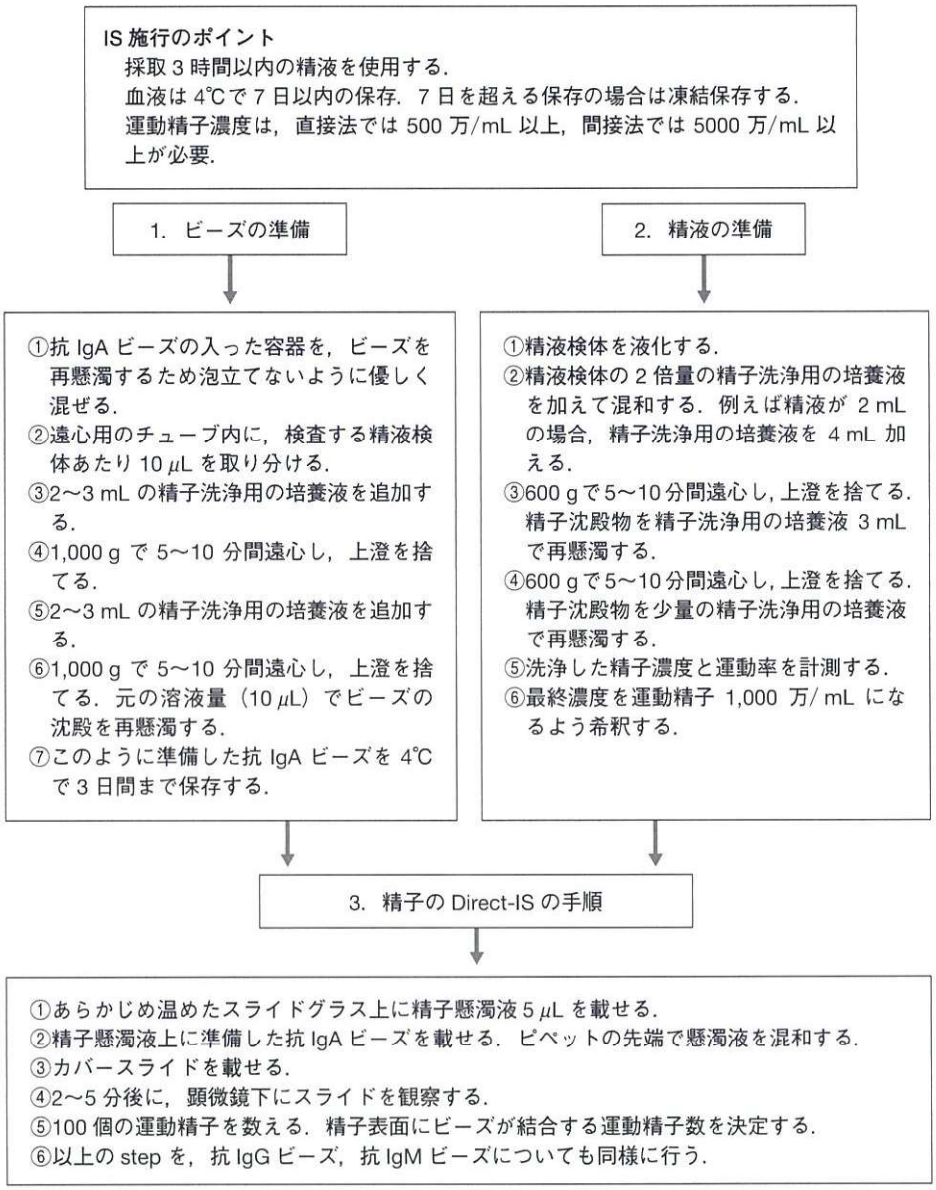


図 2　ImmunoSpheres® (IS) の実施法

陽性患者において, 程度の差はあるものの, 有意に精子無力症を発症すると報告されている[15].

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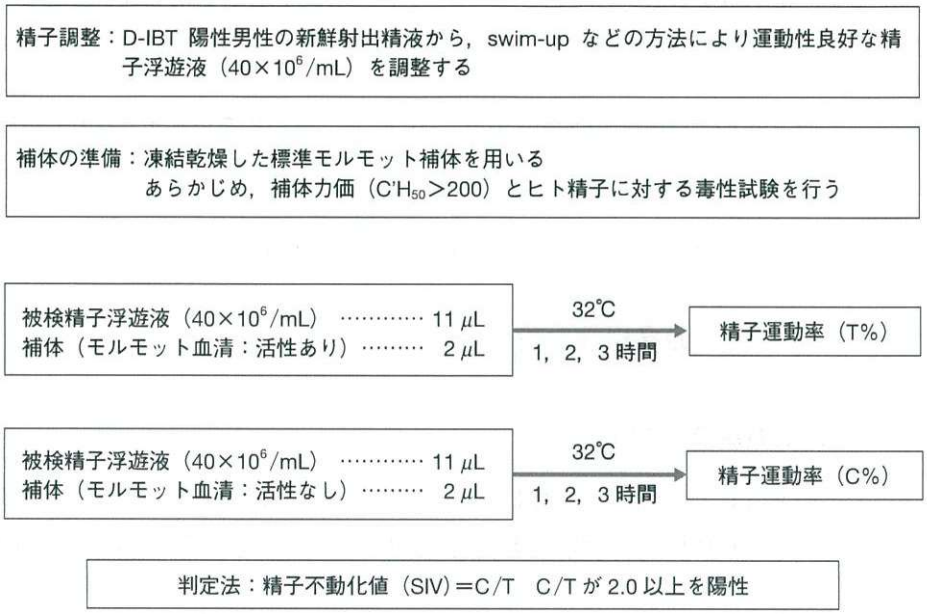


図3　直接精子不動化試験の方法

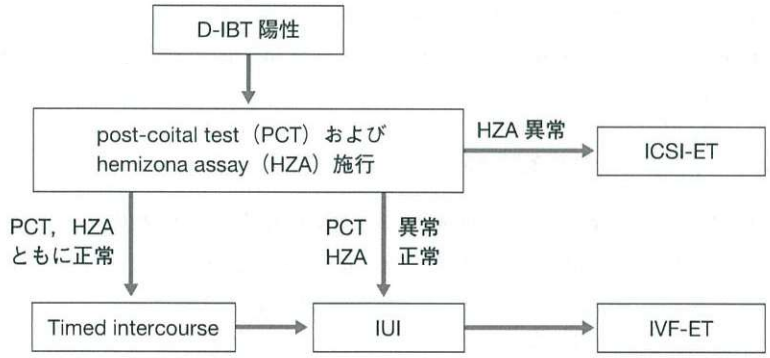


図4　抗精子抗体陽性不妊男性の治療指針

抗精子抗体陽性不妊男性の治療指針

上述したように，抗精子抗体には多様性があるため，D-IBT の結果からは検出された抗体が不妊症と関係があるかどうかを判定することはできない．そこで Shibahara ら[16, 17]は**図4**に示すような治療指針を考案した．1次スクリーニングとして D-IBT を実施し，陽性者に対しては性交後試験（post coital test：PCT）により頸管粘液内での精子貫通能を評価する．次いで，ヘミゾナアッセイ（hemizona asszy：HZA）[18]を行

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い，精子の受精能を診断する．PCT および HZA が良好であれば，抗精子抗体は射出精子に結合していても不妊症とは関連性がないと判定し，タイミング療法から治療を開始できる．PCT が不良かつ HZA 良好であれば，頸管粘液内精子貫通障害という理由で人工授精（intra-uterine insemination：IUI）による治療を選択する．もし数周期の IUI が奏効しない場合，体外受精による治療へ step-up する．HZA が異常である場合は精子による受精障害があると判断し，PCT の結果にかかわらず卵細胞質内精子注入法（intracytoplasmic sperm injection：ICSI）による治療の適応となる．

抗精子抗体の多様性と臨床成績

IBT の基準値について

IBT の cut-off 値については，従来はイムノビーズを 1 個でも結合する精子が全体の 20%以上存在すれば，抗精子抗体陽性と判定していた[13]．しかし，1999 年および 2010 年に改訂[19]された WHO マニュアルでは cut-off 値は 50%に修正されたが，この変更の根拠に関する記載はない．一方，Koriyama ら[20]は，PCT の分析結果から，cut-off 値は 20%とするほうが妥当であると結論し，これを基準値とすべきと報告している．

イムノグロブリンのクラスと男性における免疫学的不妊症に関して

De Almeida[21]らは抗精子抗体の IgG クラスは受精障害と強く相関することを報告し，Hjort と Hansen[22]や Parslow[23]らは IgA クラスが免疫学的不妊症との相関が強いことを報告している．

抗精子抗体の運動精子への結合部位とその生理作用に関して

Bronson ら[24]は抗精子抗体が運動精子の頭部に結合していた場合，精子の卵子への結合を有意に障害すると報告し，Mandelbaum ら[25]は有意に受精障害を発症すると報告している．また，Witkin[26]らは抗精子抗体が運動精子尾部に結合していた場合，精子の運動能を有意に障害すると報告している．

抗精子抗体と精子運動率や精子濃度との関連性について

Dimitrov ら[27]は精子無力症のある不妊男性は，精子無力症のない不妊男性と比べ有意に精子細胞膜上に抗精子抗体を付着すると報告している．また，精子運動率が 60%未満の場合，抗精子抗体が陽性となる頻度が高いことを報告している．一方，精子濃度に関しては，抗精子抗体陽性率との相関性はないと報告している．

抗精子抗体と受精障害との関連性について

Shibahara ら[16]は精子不動化抗体陽性不妊男性の臨床上の特徴を検討し，D-IBT 陽性不妊男性において，運動精子へのイムノビーズ結合率が 80%以上と 80%未満を比較し

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て前者で有意に受精障害を発症することを報告している．

抗精子抗体陽性不妊男性のICSIによる治療成績について

Nagyら[28]によると，ヒトにおける抗精子抗体の受精卵への影響に関して，精子結合抗体のIgAクラスとIgGクラスの精子への結合率が80%以上と80%未満の場合，前者でICSI後の胚の質が低下すると報告している．またイムノグロブリンのクラスに関係なく，抗精子抗体の精子への結合率80%以上において，結合部位が尾部および中片部より頭部で高率であった場合，ICSI後の胚の質が低下するとも述べている．一方で，Estevesら[29]は抗精子抗体陽性不妊男性をICSIで治療した場合，抗精子抗体の結合率は受精率，分割率，胚の質，臨床妊娠率に影響を与えないと報告している．

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精液検査／抗精子抗体検査

脇本 裕　杉山由希子　柴原浩章

検査のポイント

◇ 不妊症の三大原因とは，内分泌因子・卵管因子・男性因子であり，簡便に実施可能な精液検査は不妊治療の早期に実施する必要がある．

◇ 免疫性不妊症の一因となる抗精子抗体は，女性では同種抗体として，男性では自己抗体として産生される．

◇ 精液検査の際に，運動精子上に結合する抗精子抗体の検出が推奨されている（WHO）．

目的と適応

不妊症の三大原因とは，内分泌因子，卵管因子，男性因子である．不妊原因の責任所在は，一般に女性が約65%，男性が約50%，（重複は約25%），原因を特定できないカップルは約10%強に存在する（WHO，1996）．したがって，男性因子の検索は不妊治療を実施するうえで重要である．なかでも簡便に実施可能な精液検査は，不妊治療開始後早期に実施するのが望ましい[1]．

男性不妊症の病因として，本邦においては造精機能障害が約80%，精子輸送路の通過障害が約10%，精子機能障害および原因不明不妊症が約10%を占める．精子機能障害のうち，精子免疫による抗精子抗体（antisperm antibody：ASA）が原因となる男性不妊症の存在も指摘されている．

本稿では，精液検査および抗精子抗体検査について概説する．

検査の種類

◇精液検査

男性不妊症の診断のための基本的な手段は精液検査である．ただし施設間・検者間でも精液の採取法や測定法はさまざまで，さらに被検者の禁欲期間による精液所見の変動，個人内変動，検者による測定バイアスなど，精度管理を考えるうえで配慮すべき点は多い[2]．精液検査の標準化を目的に，WHOラボラトリーマニュアル[3]や日本泌尿器科学会監修の『精液検査標準化ガイドライン』[4]が発刊されている．WHOマニュアル（2010）による

わきもと ゆう，すぎやま ゆきこ，しばはら ひろあき
兵庫医科大学産科婦人科学講座（〒663-8501　兵庫県西宮市武庫川町1-1）

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表1　WHOマニュアル（2010）による精液所見の正常下限値

精液量（mL）	1.5
総精子数（$\times 10^6$）	39
精子濃度（$\times 10^6$）	15
総運動率（前進＋非前進）（%）	40
前進運動率（%）	32
生存精子率（%）	58
正常形態率（%）	4

（WHOマニュアル，2010）

表2　主な精子機能検査法

1. optional tests
   hypo-osmotic swelling（HOS）test：精子尾部膨化試験
   computer-aided sperm analysis（CASA）：精子自動分析装置
   zona-free hamster egg sperm penetration test：ハムスターテスト
2. reserch tests
   reactive oxygen species（ROS）：活性酸素
   human zona pellucida binding test：透明帯結合試験
   hemizona assay（HZA）
   sperm-zona binding assay（differentially labeling）
   assessment of the acrosome reaction：先体反応評価

（WHOマニュアル，2010）

精液所見の正常下限値を表1に示す．

◇精子機能検査

IVFに際しての受精障害の原因は必ずしも精子側に限定されない．しかしながら，精液所見が正常であるにもかかわらず，精子自身の受精機能に障害がある症例は，臨床上確かに存在する．そこで精子の受精機能検査法としてWHOマニュアルにも表2に示すように記され，また臨床現場で比較的よく用いられる方法を紹介する[5,6]．

1 hypo-osmotic swelling（HOS）test（精子尾部膨化試験）

射出精子に低浸透圧負荷をかけ，精子尾部細胞膜の形態学的変化を観察し，間接的に精子の機能面を判定する検査法である．

2 zona-free hamster egg sperm penetration test（ハムスターテスト）

先体反応を終了した精子だけが，あらかじめ透明帯を除去したハムスター卵子に侵入でき，一般精液所見からでは予測困難なヒト精子の受精能力を評価する．ハムスターテスト不良，すなわち精子侵入率15%未満の症例はIVF受精率が不良と報告されている．

3 hemizona assay（HZA）

ハムスターテストと異なり，HZAでは同種のヒト卵透明帯を用いる点で，信頼度が高い．

4 内分泌学的検査

内分泌学的検査としては，血中FSH，LH，テストステロン値，ならびにプロラクチン値を測定する．FSH値がおおむね10 mIU/mLを超える場合，造精機能障害を認める．

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◇その他の特殊精液検査

1 精子運動能解析装置

客観的かつ国際的に通用する検査法として，精液量，精子濃度，精子運動率に関してはcomputer-aided sperm analysis（CASA：精子自動分析装置）がある．CASAでは，顕微鏡画像をCCDカメラが捕捉し，その情報をコンピューター処理することにより，精子濃度，精子運動率はもちろんのこと，肉眼的な顕微鏡検査では測りえないさまざまなパラメーター（精子の平均速度や精子頭部振動の平均幅など）を自動的，客観的，簡便かつ迅速に算定することができる[5]．ただし，非運動性精子と他の細胞成分（白血球，赤血球，精子細胞など）との識別能は完全ではない[2,5]．

2 抗精子抗体検査

免疫性不妊の原因として抗精子抗体がある．精子結合抗体には多様性があり，必ずしも不妊症の原因となるとは限らないが，なかには精子不動化作用や受精障害作用をもつ抗体も含まれる[2]．

男性が自己抗体としてもつ精子細胞膜に結合する抗精子抗体の検出法として，直接イムノビーズテスト（direct-immunobead test：D-IBT）がある．ウサギ抗ヒトIgG・A・M抗体を結合させたポリアクリルアミドビーズを用いて精子細胞膜に結合している抗体を検出する．

緩衝液Ⅰとして0.3%ウシ血清アルブミン添加Tyrode's solution（またDulbecco's PBS）を作製し，使用時には25～35℃に加温しておく．検査に必要な精液の量は精子濃度や運動率に応じて加減するが，約5×10^6個の運動精子が含まれるようにする．イムノビーズ保存液は1回，精液は2回（用いる精液量が1 mLを超えるような場合は3回）緩衝液Ⅰ約10 mLを加えて遠心（500 g，5分間）洗浄する．各沈渣に緩衝液Ⅱ 0.1 mLを加えて再浮遊させる．イムノビーズ浮遊液5 μLと精子浮遊液5 μLをスライドグラス上でよく混和し，カバーグラス（20～24 mm四方）で覆う．室温で湿潤箱に10分間静置後，位相差顕微鏡400倍下で鏡検し，運動精子200を観察してイムノビーズが結合している精子の割合（%），および結合している場合は免疫グロブリンの種類（IgG・A・M）と結合部位（頭部，中方部，尾部）を記録する．

[基準値] IBTのcut-off値については，従来はイムノビーズを1個でも結合する精子が全体の20%以上存在すれば，抗精子抗体陽性と判定していた[7]．ところが1999年改訂のWHOマニュアルでcut-off値は50%に修正された．この変更の根拠に関する記載はないまま，2010年の改訂版[3]でもこの数値が踏襲されている．最近Koriyamaら[8]は，PCTの分析結果から，D-IBTにおけるスクリーニングテストとしてのcut-off値は20%とするほうが妥当であると結論し，これを基準値とすべきと報告している．

実施法／評価法

◇精液採取法

検査に用いる精液は，最低2日以上で最高7日間以内の禁欲期間後，用手法（マスターベーション法）による射出液全量を採取する．採取後の精液は室温から20～37℃に保温

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した状態で搬送し，1時間以内に検査を実施するのが望ましい．滅菌したプラスチック製またはガラス製で，口径が広く自立し透明の採取容器を用いる．精液の検体を受け取る際に，禁欲期間，採取日時，全量採取の有無，搬送状態などを把握しておく．

◇一般精液検査法と基準値

1 外観と液化

採取直後の精液はゲル状を呈することから，室温～37℃で約15～60分静置してから検査を行う．通常，精液の外観は不透明な乳白色である．血精液症や膿精液症の有無を確認する．前立腺からの蛋白質分解酵素（セリンプロテアーゼ）分泌の作用により60分以内に液化する．

2 精液量

精液をシリンジに吸引して液量を計る容量法では，採取容器の壁面に精液が残った場合には誤差が大きく[3]，計量器を用いる重量法が推奨されている．秤量単位0.1 gまで測定可能な計量器で，測定した重量を1.0 g＝1.0 mL（比重1.0）として精液量に換算する．

[基準値] 1.5 mL以上

[基準値を下回る場合] 前立腺や精嚢の異常（欠損・閉塞），部分逆行性射精を疑う．なお，精液採取ミスがなかったかは，問診で確認する．

3 pH

均一化した精液一滴をブロムチモールブルー（BTB）試験紙やクレゾールレッド（CR）試験紙に載せ，30秒後に判定する．BTBの変色域はpH6.0～7.6（黄～青），CRの変色域はpH7.2～8.8（黄～赤）である．なお，精液を放置しておくとpHは経時的に上昇するので，採取後1時間以内に測定する．

[基準値] 7.2以上

[基準値を下回る場合] 慢性の前立腺炎・精嚢炎・精巣上体炎，射精管の閉塞を疑う．

4 精子運動率

スライドグラスに均一化した精液を載せ，気泡が入らないようにカバーグラスで覆う．計算上の液厚が約20 μmとなるよう，精液の量を調整する．鏡検に用いる光学顕微鏡はステージ保温盤付きの位相差顕微鏡が望ましい．まず，100倍下で観察し，精子が均一に分散していることを確認する．精子の凝集や粘液糸の混入などを認める場合は標本を作製し直す．次に400倍下で数か所の視野から200個以上の精子を観察し，運動性を以下のa～dに分類する．

a：速度が速く直進

b：速度が遅い，あるいは直線性が不良

c：頭部あるいは尾部の動きを認めるが前進運動していない

d：非運動

精子運動率はa＋bの割合（%）で表す．基本的に1検体につき3回測定を行い，その平均値を測定値とする．

[基準値] a＋bが40%以上，またはaが32%以上

[基準値を下回る場合] 精子無力症（asthenozoospermia）と診断する．

5 精子濃度

改良型NeubauerまたはBurker-Turk血球計算盤を用いて，表3の精子数に応じた希

表3　精子濃度の測定における希釈倍率

1視野あたりの精子数	希釈倍率	精液量	希釈液量
15以下	5倍	100 μL	400 μL
15～40	10倍	100 μL	900 μL
40～200	20倍	100 μL（10倍希釈済）	100 μL
200以上	50倍	100 μL（10倍希釈済）	400 μL

釈倍率で測定する．

精子運動率測定に用いた標本を，400倍下で観察したときの1視野あたりの精子数に基づいて希釈する．希釈液には0.1% Triton-X-生理食塩液や，1.0%ホルマリン（炭酸水素ナトリウム5 g＋ホルマリン1 mLを精製水100 mLに溶解）を用いる．

計算盤にニュートンリングができるようにカバーグラスを載せ，毛細管現象を利用して固定・希釈した精液を上下の計算室に入れる．湿潤箱のなかに約5分静置してから鏡検する．位相差顕微鏡の使用が望ましい．はじめは100倍下で精子が均一に分散していることを確認し，次に400倍下で頭部と尾部をもつ精子の数を測定する．分画線上に精子の頭部がある場合，任意の2辺（例：下辺と右辺）のみ数える．上下の計算室を測定して，その平均値を測定値とする．差が大きすぎる場合，希釈から測定をやり直す．

原精液中に精子を認めない場合，精液量全量を遠心（500 g，15分間）し沈渣を鏡検する．

[基準値] 15.0×10^6 個/mL以上

[基準値を下回る場合] 乏精子症（oligozoospermia）と診断する．なお，遠心による沈渣中にも精子を認めない場合，無精子症（azoosperima）と診断する．

6 精子形態

均一化した精液5～20 μL（精子濃度に応じて加減）をスライドグラスに載せ，引きガラスを用いて塗沫標本を作製する．風乾後ディフ・クイック染色法で染色する．鏡検は，光学顕微鏡1,000倍下で200個以上の精子を観察し，正常形態率を算定する．頭部形態はKurugerらのstrict criteriaに基づいて分類する．

[基準値] 正常形態率が4%以上

[基準値を下回る場合] 奇形精子症（teratozoospermia）と診断する．

7 精子生存率

精子の運動率が低い場合，Eosin Y染色法を用いて非運動精子の生死を鑑別する．均一化した精液とEosin Y染色液を等量混和し，スライドグラス上に1滴載せカバーグラスで覆う．30秒ほど静置したのち，光学顕微鏡400倍下で鏡検する．生存精子は染色されずに無色であるが，死滅精子は赤色に染色される．精子中の生存精子の割合（%）で表す．

[基準値] 58%以上

[生存精子をまったく認めない場合] 精子死滅症（necrozoospermia）と診断する．

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Trend Check

従来IBTを行っていたが，製造中止に伴いIBTと同じ原理による抗精子抗体検出法としてImmuno Spheres®（IS）（Bioscreen Inc.）を行っている．なお，Centolaら[9]は両者の相関性に関して検討し，ISによる抗精子抗体の検出結果はIBTによる結果と一致していたと報告している．

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特集◉生殖医療の最新知識

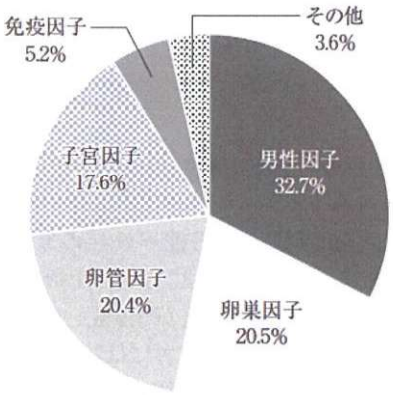
不妊症の原因診断と治療法の選択

杉山由希子*・柴原浩章*
Yukiko Sugiyama, Hiroaki Shibahara**

Key words：不妊症，原因診断，原因別治療

はじめに

30歳の女性の75%，35歳の女性の66%，40歳の女性の44%が1年以内に妊娠する[1]。不妊症とは妊娠を望む健康な男女が避妊なく性交をしているにもかかわらず，一定期間妊娠しない状態をいう。わが国でも晩婚化が進み，平成29年の平均初婚年齢は，夫31.1歳，妻29.4歳で，婚姻件数も減少していることから[2]，この一定期間については最近1年に改定された[3]。頻度については，6組に1組のカップルが不妊症である[4]。女性の年齢が上昇すると，妊孕能は低下する。



（文献[6]を一部改変）

図1 不妊原因の割合

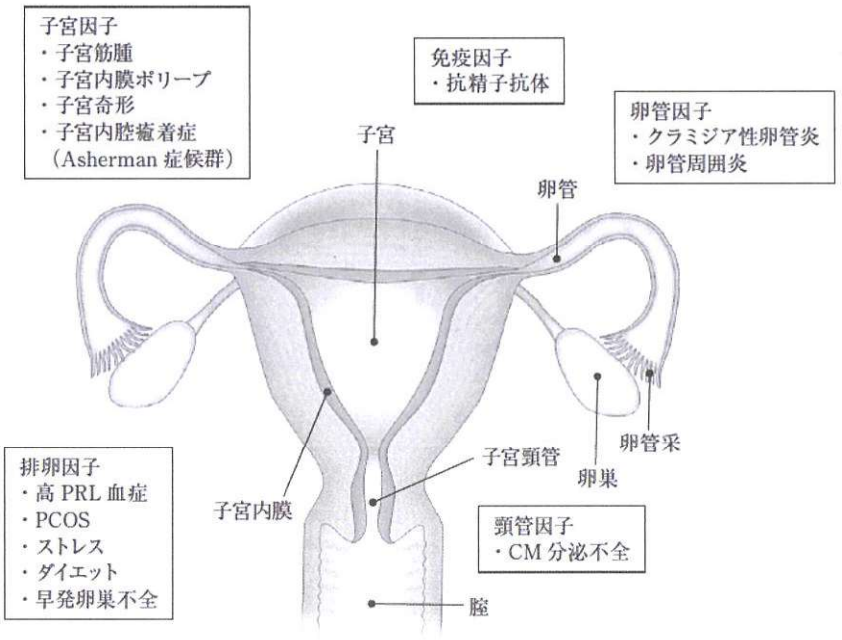
I 不妊症の原因

排卵因子，卵管因子，男性因子が三大不妊原因として知られているが，女性の社会進出に伴い晩婚化が進んだ影響で不妊原因として年齢による影響が大きく，35歳以上では不妊原因が不明と診断される頻度は上昇する[5]。一方，2003年（平成15年）に日本受精着床学会が行った不妊治療患者に対するアンケート調査では，男性因子32.7%，卵巣因子（排卵因子）20.5%，卵管因子20.4%，子宮因子17.6%，免疫因子5.2%，その他3.6%であった[6]（図1）。女性側

Diagnosis and treatment of infertility

*兵庫医科大学産科婦人科学講座 *Department of Obstetrics and Gynecology, Hyogo College of Medicine*

Presented by Medical*Online



（文献[7]より）

図 2　女性側の不妊原因

の不妊原因を**図 2** に示す[7]。

1. 男性因子

表 1 に示すように男性不妊症の原因としては，造精機能障害が 82.4%，性機能障害が 13.5%，閉塞性精路障害が 3.9%，その他 0.2% であった[8]。造精機能障害には特発性，精索静脈瘤などがあり，性機能障害には勃起障害，射精障害がある。

2. 排卵因子

卵管因子とならび女性側の不妊原因として多い。原因として視床下部－下垂体－卵巣系のいずれかの機能障害で発症する。代表的な疾患として，多嚢胞性卵巣症候群（PCOS），高プロラクチン血症，早発卵巣不全，体重減少性無月経などがある。

3. 卵管因子

原因として，性器クラミジア感染症や淋菌感染症による卵管閉塞や卵管周囲の癒着，子宮内膜症による卵管周囲の癒着，子宮筋腫や子宮内膜ポリープによる卵管子宮口の閉鎖や卵管への圧迫による卵管閉塞，骨盤内手術既往による卵管癒着などがある。

4. 子宮因子（頸管因子を含む）

先天的な子宮奇形，子宮筋腫や子宮内膜ポリープ，アッシャーマン症候群など子宮内腔へ影響を及ぼす器質的疾患，子宮内膜炎が原因となる。また頸管粘液の分泌不全や，頸管の先天奇形や手術操作による頸管狭窄等は，精子の子宮内への侵入を妨げる。

5. 免疫因子

男性では自己抗体，女性では同種抗体として産生された抗精子抗体によって，女性生殖器官内における精子通過障害，受精障害，着床障害の原因となる[9]。

6. そ の 他

子宮内膜症の女性ではサイトカインなどの生理活性物質により骨盤内の免疫異常を来し，排卵障害や胚の質の障害，着床障害に関与する可

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表1 男性不妊症の原因

造精機能障害（82.4%）	①特発性	42.1%
	②精索静脈瘤	30.2%
	③染色体・遺伝子異常	
	・クラインフェルター症候群	1.8%
	・その他の染色体異常	1.2%
	・AZF 欠失	1.4%
	④薬剤性	
	・抗がん剤治療後	1.3%
	・その他の薬剤	0.5%
	⑤停留精巣	1.6%
	⑥低ゴナドトロピン性性腺機能低下症	1.0%
	⑦上記以外	1.3%
性機能障害（13.5%）	①勃起障害	6.1%
	②射精障害	7.4%
閉塞性精路障害（3.9%）	①原因不明	1.2%
	②精巣上体炎	0.7%
	③精管結紮後	0.7%
	④鼠径ヘルニア術後	0.6%
	⑤先天性精管欠損	0.5%
	⑥上記以外	0.2%
その他（0.2%）		

（文献[8]を一部改変）

能性がある[10]。不妊症例における子宮内膜症の合併率は，25～50%と高頻度とされているが[11]，自験例では11.4%であった[12]。

なお，甲状腺疾患は月経異常や排卵障害による不妊症の原因となる[13]。

II 原因診断

男性因子，女性因子ともに，問診はとても重要である。男女ともに嗜好品，不妊期間，性交障害があるか，職業などの問診を行う。男性には性感染症，ムンプス，抗癌剤や放射線治療，精巣の手術，鼠径ヘルニアなどの既往，ならびに高温環境への暴露のチェックが必要である。女性には月経歴・妊娠分娩歴，既往歴（性感染症，子宮筋腫，子宮腺筋症，子宮内膜症，子宮や卵巣の手術既往，その他腹部や鼠径部の手術既往，抗癌剤や放射線治療など），体重の大幅な増減・ダイエットの有無，乳汁分泌の有無，分娩後であれば断乳しているかなどの問診が必要である。

1. 男性因子

外陰部の診察では，外性器や恥毛の発育の状態を確認する。次に精索・精管・精巣・精巣上体の触診を行う。オーキドメーターを使用して精巣容積を簡易的に測定する。陰嚢の超音波検査も有効である。禁欲期間を約2～7日もうけて精液検査を行う。精液検査の正常下限値を**表2**に示す[14]。一方，低ゴナドトロピン性性腺機能低下症や高プロラクチン血症などによる不妊症が存在するため，内分泌検査も有用である。

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表2 精液検査における正常下限値

精液量（mL）	1.5
総精子数（個）	39×10^6
精子濃度（/mL）	15×10^6
総運動率（%）	40
前進運動率（%）	32
生存精子率（%）	58
正常形態率（%）	4

（文献[14]より）

1）造精機能障害

①精索静脈瘤

精索の静脈である精巣静脈の蔓状静脈叢の停滞や逆流により生じる[15]。左精巣静脈は左腎静脈に流入し，合流点で血流が停滞したり，静脈圧が上がりやすくなるため，左側に多い[15]。うっ滞する静脈血による陰嚢内温度上昇を来し，精液所見異常を来す。診察時の注意点として，立位や腹圧をかけての視触診が重要である。また診察室が寒い場合は診断が困難になることがあり，暖かい室内での診察を心がける。陰嚢超音波検査時は，ドップラー法を用いて怒張した静脈を描出する。

②染色体異常・遺伝子異常

男性不妊患者の染色体異常で最も頻度が高いのはクラインフェルター症候群である。頻度は男性1,000人に1～2人とされ[16]，このうち47,XXYが最多である。外性器の発育は正常であることも多いが，精巣容積は小さく，不妊を契機として診断されることが多い。無精子症・乏精子症を示し，血中ゴナドロトロピン高値，血中テストステロン値は正常～低値である。ロバートソン転座も男性不妊症例で認める。

その他，Y染色体微小欠失（AZF欠失）がある。無精子症の10.4%，精子濃度100万/mL未満の男性の10.1%にAZF欠失を認める[17]。

③停留精巣

発生頻度は1歳時で1.0～1.7%である[18]。無治療のまま経過すると，片側性で約43～83.5%，両側性で88.6～100%に乏精子症や無精子症等の精液所見異常を認める[18]。触診，超音波検査やMRI検査などの画像検査が有用である。性分化異常の疾患との鑑別のために，内分泌検査や染色体検査が必要である[18]。

④低ゴナドトロピン性性腺機能低下症

先天性遺伝子疾患（カルマン症候群），下垂体腫瘍や下垂体炎などによる後天性ゴナドトロピン分泌不全が起こり，二次性性腺機能低下を来す。

2）閉塞性精路障害

精巣容積が正常で，内分泌検査異常もなく，無精子症を呈した場合に疑う。診断には精巣生検を行うこともある。閉塞部位としては精管，精巣上体および射精管がある。精管閉塞の原因には先天性精管欠損症，避妊手術としての精管切断術後，あるいは鼠径ヘルニア術後などがある。精巣上体閉塞の原因には，淋菌やクラミジア・トラコマティスなどを起因菌とした精巣上体炎，精巣上体頭部閉塞による無精子症と慢性呼吸器疾患を合併するヤング症候群などがある。射精管閉塞の原因には先天的な開口不全や炎症に伴う場合がある[19]。

3）性機能障害

勃起障害，腟内射精障害，逆行性射精などがある。性機能障害の原因として，薬剤の副作用（向精神薬，前立腺肥大・前立腺癌治療薬）や骨盤内悪性腫瘍術後（膀胱癌，前立腺癌，直腸癌などの根治手術後），肥満，運動不足，動脈硬化，糖尿病神経障害，内分泌疾患，うつ症状・うつ病が関連する[20][21]。逆行性射精では精液量の減少や尿の白濁などについての問診と，射精後に排尿させ，尿中の精子の存在を確認する。

2. 排卵因子

ストレス，体重の増減や過度の運動により視床下部－下垂体－卵巣系の機能異常を来すため，問診は重要である。また基礎体温の測定の意義として，排卵や黄体機能不全の有無などを診断できる。

卵胞期にLH，FSH，PRLを，排卵期および

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黄体期にエストラジオール，プロゲステロンを測定する。PCOSの診断のため，テストステロンを測定する。甲状腺機能異常や耐糖能異常は排卵障害を来しうる。卵巣予備能のチェックとして，血中抗ミュラー管ホルモン（anti-Müllerian hormone：AMH），経腟超音波検査で卵胞期初期の胞状卵胞数（antral follicle count：AFC）の測定が有用である。

1）多嚢胞性卵巣症候群（PCOS）

生殖年齢女性の5～8%に認める[22]。診断基準は①月経異常，②多嚢胞卵巣，③血中男性ホルモン高値，またはLH基礎値高値かつFSH基礎値正常である[23]。PCOSはメタボリック症候群のハイリスク群と位置付けられ，耐糖能異常，脂質代謝異常，2型糖尿病，高血圧，心血管系疾患などの合併が多い[22]。

2）高プロラクチン血症

一般女性の約0.4%に認める[24]。原因として，薬剤性（向精神薬，抗潰瘍剤など），視床下部・下垂体病変，原発性甲状腺機能低下症などがある。

3）卵管因子

卵管の役割は卵管采での卵子の捕捉，卵子や精子の輸送，膨大部における受精の場の提供，受精卵の分割の場の提供，子宮内への受精卵の輸送などがある[10]。まずクラミジア感染の有無をチェックする。診断としては血中クラミジア・トラコマティス抗体価，あるいは子宮頸管分泌物からクラミジア・トラコマティス抗原検査を行う。クラミジア感染があれば，抗菌剤による治療を行う。続いて子宮卵管造影法（HSG）により，卵管通過性，卵管留水腫や卵管周囲癒着の有無を診断する。また子宮鏡検査により，両側卵管口の開口を確認できる。二次検査として，腹腔鏡検査により直視下に骨盤内の状態を観察する。インジゴカルミンによる通色素検査を行い，卵管の疎通性を確認する。

4）子宮因子（頸管因子を含む）

粘膜下子宮筋腫や子宮内膜ポリープなど，子宮内腔へ影響を及ぼす疾患の診断には，経腟超音波検査，HSG，sonohysterography，子宮鏡検査が有用である。子宮内膜炎を疑う時は，細菌培養検査を行う。

頸管因子に関しては，排卵日直前～排卵日に頸管粘液を採取し，量や牽糸性，およびシダ状結晶形成を検査する。またHuhnerテストを行い，精子の頸管粘液中への侵入の有無を確認する。

5）免疫因子

抗精子抗体の検出のため，血中の精子不動化抗体を検出する。精子不動化試験（SIT）を行う。SITが陽性の場合，治療方針を決定するため，必ず定量的精子不動化試験によりSI_{50}値を測定する。

6）そ の 他

子宮内膜症の診断には，超音波検査やMRI検査などの画像検査，腹腔鏡検査が有用である。

甲状腺機能異常については，TSH，FT4，FT3等のホルモン検査，各種抗体検査，甲状腺超音波検査を行い診断する。

III 治　療

1. 男性因子

1）造精機能障害

無精子症の場合，閉塞性無精子症と非閉塞性無精子症に分類する。閉塞性無精子症では，主に手術療法が有用である（下記「2）閉塞性精路障害」参照）。非閉塞性無精子症の精巣内でも，部分的には精子形成を認めることがある[25]ため，精巣内精子採取術（TESE）が有用である。非閉塞性無精子症患者の約10%にAZF欠失を認める[25]。AZFa，AZFb，AZFb+c欠失では精子回収は困難であるが，AZFc単独の欠失患者の71.4%がTESEで精子を回収できたと報告されている[17]。ただしAZFc領域の欠失は次世代の男児へ継承する[26]。

特発性の造精機能障害では原則的に薬物療法を行う。精子形成に要する期間を考慮して少なくとも3カ月程度は投与する[27]。抗酸化作用のあるビタミンEやビタミンCや亜鉛，精細胞

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のDNA合成を促すビタミンB_{12}，男性ホルモン増強作用のある補中益気湯，内分泌療法などがある。なお男性型脱毛症の治療に用いる5αリダクターゼ阻害剤（フィナステリド）など，薬剤性に造精機能に影響を及ぼす可能性のある薬剤[28]を中止する。また生活習慣が男性の妊孕性へ影響を及ぼすので[27]，是正を心がける。

①精索静脈瘤

治療として手術がある。手術適応としては，不妊，鼠径部や陰嚢部の不快感や鈍痛がある，精巣の萎縮や発達障害を伴う，両側例，美容上の問題がある場合である[7][29]。小児期に発見された精索静脈瘤に対しては，妊孕性に対する評価が困難であり，治療や術式に対しての明確な基準は定まっていないが，Grade 2以上の静脈瘤で疼痛や不快感などの症状を有する場合は手術適応となる[30]。不妊の場合は，Grade 2以上の静脈瘤で精液所見に異常を認めるなどが手術適応である[7]。

②停留精巣

hCG投与により，精巣のLeydig細胞からのテストステロン分泌を亢進させ，精巣の下降は促進する[18]。手術による精巣の移動も有効であるが，精巣固定術の手術時期は父性獲得率（最終的に正常妊娠と挙児を得る率）が低下するため，1～2歳までに行うことが望ましい[18]。

③低ゴナドトロピン性性腺機能低下症

ゴナドトロピン補充療法が有効な治療である。

2）閉塞性精路障害

精路再建術，またはTESEにより回収した精子でICSIを行う。このうち精路再建術については，精管切断術後，鼠径ヘルニアによる精管閉塞では精管－精管吻合術を行う。精巣上体炎やヤング症候群では精管－精巣上体吻合術を行う。

3）性機能障害

勃起障害に対してはphosphodiesterase-type 5（PDE5）阻害薬を使用する。射精障害のうち，早漏には選択的セロトニン再取り込み阻害薬を用いる場合がある[31]。逆行性射精に対しては三環系抗うつ薬や[31]，排尿後にマスターベーションさせて膀胱内精子を回収し，AIHまたはARTを行う方法がある[32]。

2. 排卵因子

排卵障害に対する治療として，シクロフェニルやクロミフェンの内服，あるいはゴナドトロピン投与による排卵誘発を行う。黄体機能不全に対しては，hCGによる黄体刺激やプロゲステロンによる黄体補充を行う。

1）PCOS

挙児希望がある場合は，肥満があれば減量を勧め，排卵誘発としてクロミフェン療法を行う。肥満，耐糖能異常，インスリン抵抗性のいずれかを認め，クロミフェン単独で卵胞発育を認めなければ，メトホルミンを併用する。クロミフェン抵抗性ではゴナドトロピン療法（低用量漸増法）または腹腔鏡下卵巣多孔術を行う[33]。

2）早発卵巣不全

エストロゲン療法やGnRHアゴニスト療法などを行う。視床下部－下垂体－卵巣系のホルモン動態の正常化を目的として，周期的にカウフマン療法を行うこともある。

3）高プロラクチン血症

薬剤性の場合は主治医と相談し，原因薬剤の休薬もしくは変更を依頼する。原発性甲状腺機能低下症による場合は甲状腺ホルモンを投与する。プロラクチノーマではドパミンアゴニスト療法が無効な場合，手術療法（Hardyの手術）を選択する。

3. 卵管因子

クラミジアなどの感染症が存在する場合は，HSGや不妊治療開始前に薬物治療を行う。最近では卵管鏡下卵管形成術（FT）が普及しているが，FT不成功例も含め一般にIVF-ETを選択する傾向にある。腹腔鏡下の卵管開口術や癒着剥離術が有効な場合もある。

4. 子宮因子

子宮内膜ポリープは低用量や中用量ピルによるmedical curettageによりポリープが消失することがある。消失しない場合や粘膜下筋腫に対しては，子宮鏡下に経頸管的切除術（TCR）を行う。筋層内筋腫に対しては，子宮筋腫核出

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術が有効となる場合がある。

頸管粘液分泌不全に対しては，AIH が有効である。

5. 免疫因子

精子不動化抗体陽性女性に対しては，抗体価が低い場合には AIH または反復 AIH で妊娠に成功するが，抗体価が常に高い場合は AIH では妊娠は困難であり，IVF-ET を行う。

6. そ の 他

子宮内膜症の治療としては，軽症子宮内膜症（R-AFS stage I, II）では，年齢が 30 歳以下，不妊期間 3 年以内，他の不妊因子合併なしの場合は待機療法やタイミング療法をまず考慮する。年齢が 30 歳以上，不妊期間が 3 年以上，他の不妊因子合併例では早期に排卵誘発，AIH 併用排卵誘発治療を考慮する。軽症であっても，卵管癒着が高度であれば早期の ART への移行を考慮する[11]。

重症子宮内膜症（R-AFS stage III, IV）では，3～4cm 以上のチョコレート嚢胞が存在する場合は嚢胞摘出のうえ 37 歳以下では排卵誘発剤使用（AIH 併用）を，38 歳以上では早期に ART を考慮する[11]。

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性腺分化の異常

ライディッヒ細胞低(無)形成症

Leydig cell hypoplasia(Leydig cell aplasia)

Key words：ライディッヒ細胞低形成，LH 受容体遺伝子

嶋谷公宏[1]
兼松明弘[1]
井上佳代[2]
柴原浩章[2]
山本新吾[1]

1. 概念・定義

ライディッヒ細胞低形成症(Leydig cell hypoplasia: LCH)，ライディッヒ細胞無形成症(Leydig cell aplasia: LCA)は，LHCGR (luteinizing hormone/choriogonadotropin receptor)遺伝子の突然変異により LH 受容体が機能を喪失し，低男性化を来す性分化疾患(disorder of sex development: DSD)である．本症は Berthezène ら[1]が 1976 年に最初に報告した疾患で，疫学としては 100 万人に 1 人と非常にまれである．性染色体は 46,XY で，常染色体劣性遺伝の遺伝形式をとる．

2. 病因・病態

46,XY DSD の一つである本症は，性腺は精巣に分化しているが LH 受容体異常によりアンドロゲン合成が障害され，結果として胎児期，思春期における男性化が障害される．

1992 年に Toledo[2]は，LHCGR 遺伝子の不活化の程度により LCH を 2 つのタイプに分類した．すなわち，type 1 は LHCGR の完全な不活性化により起こる重症例で，外陰部は女性型で通常女子として養育される．乳房発達はなく，腟は盲端で思春期に原発性無月経の精査で判明する．精巣は腹腔内あるいは鼠径部に存在する．

Type 2 は LHCGR 遺伝子の不完全な不活性化により起こる軽症例で，外陰部は女性型に近いものや，マイクロペニスから重度の尿道下裂までさまざまなスペクトラムを呈し，新生児期に精査を要する．いずれのタイプにおいても Sertoli 細胞から AMH (anti-Müllerian hormone)は分泌されるため，子宮・卵管は認めず，Müller 管構造物は退縮している．

内分泌血液検査では，思春期以降で血清テストステロン低値，LH 高値，FSH 正常から高値となり，幼少期で血清テストステロン低値，LH 正常，FSH 正常から高値となる．LH-RH 試験は正常だが，hCG 負荷試験は陰性となる．

精巣の病理組織像としては，Sertoli 細胞は認めるが，間質での Leydig 細胞の増生はみられない．病理学的に Leydig 細胞を認めない場合でも精管や精巣上体が存在する症例があり，胎生期に部分的，一時的に Leydig 細胞が機能していたことが推察される[3]．Leydig 細胞機能を喪失させた LH 受容体遺伝子ノックアウトマウスにおいても，わずかな男性化徴候を認めることから，胎生期の Leydig 細胞にはゴナドトロピン非依存性のテストステロン分泌機能があることが予想される[4]．

1995 年に Kremer ら[5]により，LCH が LH 受容体遺伝子の不活性型変異によって起こることが報告された．その後も LH 受容体遺伝子変異は細胞外ドメイン，膜貫通領域の遺伝子欠失，点突然変異などが同定されている[6]．近年にも exon 10 におけるナンセンス変異 Gln303Ter とミスセンス変異 Asn312Ser のホモ接合体が同一の遺伝子に存在していたこと[7]や，その他の

[1]Kimihiro Shimatani, [1]Akihiro Kanematsu, [2]Kayo Inoue, [2]Hiroaki Shibahara, [1]Shingo Yamamoto: [1]Department of Urology, Hyogo College of Medicine 兵庫医科大学 泌尿器科 [2]Department of Obstetrics and Gynecology 同 産科婦人科

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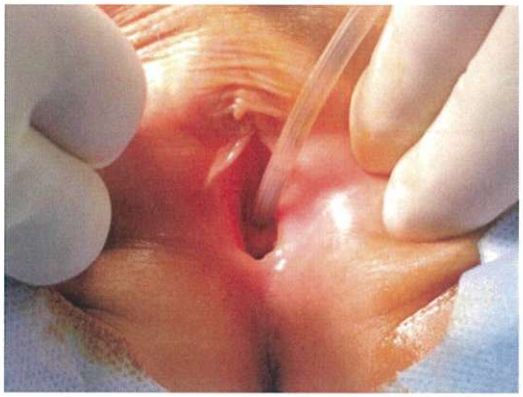


図 1　成人後に患者主導で受診された症例の外陰部
外陰部は女性型だが恥毛はなく，腟口の 6 時方向に軽度の癒合を認め，腟は盲端．

変異[8]が報告されている．

3. 鑑別診断

46,XY DSD の鑑別としては，性腺分化異常，アンドロゲン合成または作用の異常，その他（高度尿道下裂や総排泄腔外反など）に大きく分類できる[9]．アンドロゲン不応症では血中テストステロンが上昇しているので鑑別は容易となる．hCG 負荷後のテストステロン上昇を認めない場合の鑑別は，MRI または腹腔鏡による内性器の精査，性腺生検による組織診断で行う．自験例の LCA においては，原発性無月経を主訴に思春期後に患者主導で婦人科を受診，外陰部は女性型（**図 1**）だが MRI で子宮・卵管を認めず，DSD を疑い臨床遺伝部および当科に紹介となった．染色体は 46,XY，LH 高値，テストステロン低値，エストラジオール低値であった．MRI で両側鼠径部に性腺を認め，腹腔鏡検査で子宮卵管は認めず，癒痕的な精管様構造が内鼠径輪に連続するように存在していた（**図 2**）．腹腔鏡では性腺は確認できず，両側鼠径部より性腺を摘除した（**図 3**）．性腺は Sertoli 細胞で構成され，間質に Leydig 細胞を認めないことを組織学的に確認し，本症の診断が確定した（**図 4**）．

4. 治療と予後

男子として養育する場合，尿道下裂，停留精巣の手術後，テストステロン製剤の投与が必要となる．女子として養育する場合，性腺摘除後，思春期にエストロゲンの投与および Müller 管構造のない CAIS（complete androgen insensitivity syndrome）や MRKH（Mayer-Rokitansky-Küster-Hauser）症候群に準じた腟の拡張療法が必要となる場合が多い[10, 11]．

自験例のように思春期後に患者主導で受診される例もあり，社会的性がすでに決定しているため，対応は慎重かつきめ細かく行う必要がある．社会的性の選択と適応は個人のアイデンティティーに大きな影響を与え，対応の不統一や重複は患者・家族にとっての負担が大きくなるため，専門科によるチームアプローチが原則



図 2　腹腔鏡所見
子宮，卵管は認めず，癒痕的な精管様構造（➔）が内鼠径輪に連続するように存在．



図3 両側鼠径部より摘出した性腺

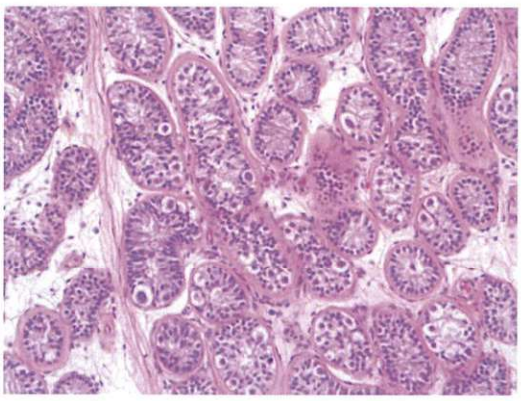


図4 摘出した性腺の組織像(LCA)
Sertoli細胞で構成され，間質にLeydig細胞を認めない．

となる．自験例では多科共同診療システムを構築し，患者への対応は産婦人科と臨床遺伝部のみに限る一方で，小児科，産婦人科，泌尿器科，病院病理部，小児外科，臨床遺伝部が患者，家族の情報を共有し，それぞれの診療科が専門領域に対応しながら，横断的に診断・治療・健康管理を行った．

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特集　新時代の子宮鏡診療

［III．手術］

12．子宮鏡を用いた卵管閉塞の手術 —子宮鏡補助下卵管鏡下卵管形成術の適応，術式，意義

福井淳史（ふくい あつし）
兵庫医科大学産科婦人科

要　旨

卵管鏡下卵管形成術は，近位卵管閉塞や狭窄に対する再疎通あるいは拡張手術である．手技が成功すれば卵管閉塞患者も自然妊娠が可能となり，非常に有用な手術である．しかし卵管鏡のみの画像では卵管口へのカテーテルの挿入に難渋することがある．この問題を解決するために子宮鏡補助（観察）下に卵管鏡下卵管形成術を行うと，カテーテルの卵管内挿入を非常にスムーズに行うことができ，手技の確実性は格段に増し，成功率が高まる．

KeyWords：卵管鏡下卵管形成術，卵管閉塞，子宮鏡補助

はじめに

卵管鏡下卵管形成術（falloposcopic tuboplasty：FT）は，主として近位卵管閉塞や狭窄など近位卵管病変に対して行われる卵管の再疎通あるいは拡張手術である．本術式は，本来卵管鏡単独で行うものであり，卵管鏡で卵管口を確認した後に卵管内へカテーテルを挿入し，卵管の閉塞部位の解除あるいは狭窄部位の拡張を行う．しかし卵管鏡はその細さゆえ，画素数も高くはなく，画像の解像度は高くはない．さらに焦点距離も短いため，卵管口を確認するのは容易ではなく，卵管内へのカテーテル挿入に苦慮することがある．この問題点を解決するために子宮鏡を併用すると，卵管口は容易に同定でき，簡便・安全・確実にFTを施行することが可能となる．

子宮鏡補助下卵管鏡下卵管形成術とは

子宮鏡補助下FT（hysteroscopically assisted FT：HAFT）は，卵管口からFTカテーテルを挿入する際に行われるFTカテーテルのウェッジ（FTカテーテルの先端を卵管口のところにもっていき固定する操作）をより解像度の高い子宮鏡を用いて子宮腔を観察しながら行うものである．卵管鏡のみの画像では卵管口を同定するこ

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とが難しいということはよく経験することである．子宮鏡で補助することによりこの問題は解決し，さらには子宮腔が変形している場合，子宮底部が平坦ではない場合，子宮後屈の場合など，FTカテーテルのウェッジが困難な場合でも子宮鏡下にFTカテーテルを卵管口に誘導することにより，格段に操作性は向上し，不安なく，卵管口にカテーテルをウェッジすることができる．よって，その後は安心して卵管開通あるいは卵管拡張操作を行うことができる．

子宮鏡補助下卵管鏡下卵管形成術の適応

FTの適応は，子宮卵管間質部あるいは卵管峡部といった近位卵管の閉塞や狭窄を有する自然妊娠を希望する不妊症症例である．HAFTはFTを行うすべての症例に行うことが可能である．卵管疎通性を確認し，卵管閉塞や狭窄を診断するために子宮卵管造影(hysterosalpingography：HSG)が第一選択の検査法として行われる．HSGは，卵管疎通性を評価するための検査として非常に有用であるが，腹腔鏡検査との比較において卵管通過性の感度は0.65，特異度は0.83であると報告[1]されており，HSGによる診断には限界がある．またHSGで卵管閉塞と診断された例のうち60%では，その後に再施行したHSGで卵管通過性が確認されている[2]．これは粘液栓や剝脱した子宮内膜，あるいは卵管のれん縮によるものであり，1回のHSGのみで卵管閉塞や狭窄の診断を行うと，本来異常のない卵管を異常として扱ってしまう可能性があるので注意が必要である．よって卵管閉塞や卵管狭窄が疑われた場合にはHSGを再施行するか選択的卵管造影や選択的卵管通水を施行するなどして，卵管の状態を再度評価することが肝要である．また片側性の病変には不向きであるが，両側卵管狭窄や卵管閉塞が疑われる場合には通気検査を行ってもよい．なお複数回のHSGを行う場合には，複数回の被曝が起こっているということも忘れてはならない．

術　式

1．卵管鏡下卵管形成術で用いられる手術システム

FTシステムは卵管鏡(0.6 mm径，6,000画素，焦点距離0.5 mm)，卵管閉塞の解除や卵管拡張を行うFTカテーテル(6 cm，10 cm)，FT灌流ポンプ，カメラヘッドと卵管鏡をつなぐアイピースアダプタ，光源装置，ビデオシステムからなる(図1)．光源装置やビデオシステムは通常の内視鏡システムが用いられている．FTカテーテルを卵管内に挿入するためには卵管口を同定し，卵管口にFTカテーテルをウェッジさせ，そこからカテーテルを押し出しながら卵管内に挿入していく必要がある．卵管鏡は6,000画素と決して画素数が高いわけではなく，さらには焦点距離が0.5 mmと非常に短いため，卵管鏡は卵管内の観察には適しているが，子宮腔内で卵管口を確認することが容易ではないことも多い．しかし，子宮鏡を併用することによりこの問題は完全に解決される．

2．HAFTの実際

1）麻酔法

無麻酔でも施行可能であるが，一般的には静脈麻酔下に行われる場合が多い．もちろん全身麻酔下でも施行可能である．

2）エバージョンテスト

FTを行う前にはバルーンの破損がないことを確認するために，カテーテルを4気圧に加圧したうえでカテーテルを進めるエバージョンテストを行う．

3）子宮鏡による子宮腔観察

FTの際には専用の腟鏡(スペキュラム)と単鈎鉗子(タナキュラム)を用いる．スペキュラムを挿入し，腟内を消毒した後に，タナキュラム



図1 FTシステム

で子宮腟部後唇を把持し，腟鏡に固定する．続いて子宮鏡を挿入し子宮腔を観察する．HAFTに用いる子宮鏡は硬性鏡でも軟性鏡でもよいが，FTカテーテルと子宮鏡を2本同時に挿入するため細径のもののほうが操作を行いやすい．

4）FTカテーテルの子宮腔への挿入

子宮鏡で卵管口を同定したら，子宮鏡の側方からFTカテーテルを挿入する．通常，FTを行う場合には頸管拡張は不要であるが，子宮頸管が狭く，子宮鏡と卵管鏡を同時に挿入することが困難である場合には，Hegar 8番くらいまで頸管を拡張するとスムーズに挿入することが可能となる．前処置としてラミナリア桿などで頸管拡張を行うと頸管が拡張しすぎて，子宮鏡と卵管鏡を同時挿入した場合，灌流液が逆流してしまい子宮腔の拡張が十分に得られないことがあるので，筆者らは前処置を行わず，手術時に頸管拡張を行っている．

5）FTカテーテルのウェッジと卵管内への挿入

子宮腔へ挿入したFTカテーテルを処置側卵管方向に90°回転し，子宮鏡観察下に先端を卵管角へウェッジさせる．その後にカテーテルを陰圧にして灌流しながら卵管鏡をカテーテルの先端まで進め，卵管口を確認する．なお卵管鏡で観察した時，卵管口がわかりづらいこともあるが，子宮鏡補助下に手技を行っている場合，間違いなくFTカテーテルは卵管口にウェッジされているので，安心してその後の操作を進めてよい．卵管口を確認したら，灌流しながら卵管鏡を0.5～1 cmほど引き戻し，カテーテルを6気圧まで加圧し，カテーテルを進める．この時カテーテルに連動して卵管鏡も前進するが，卵管鏡はカテーテル内のバルーンの2倍の距離前進する構造となっており，卵管鏡により卵管穿孔を起こさないようにするため，卵管鏡がバルーンの先端まで達したら，カテーテルを2気圧に減圧し，灌流を行いながら卵管鏡を0.5～1 cm後退させる．この操作をくり返し，6～10 cmバルーンを進めていくことにより卵管の閉塞解除もしくは拡張を行う．なおバルーン内に卵管鏡がある時，画面は緑にみえ（図2a），卵管内に卵管鏡が出ると卵管内が映るため画面はピンクにみえる（図2b）．カテーテルが閉塞部あるいは狭窄部に到達すると，カテーテルに抵抗を感じる．この時は少しずつカテーテルを進めることが肝要である．閉塞部を通過すると，抵抗が消失するのを感じる．この時，卵管鏡は卵管内に存在しているので，卵管のピンクの像がみえ

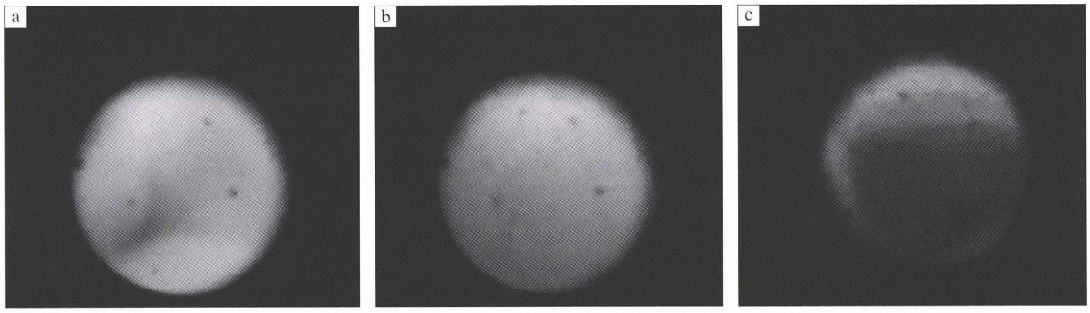


図2 卵管鏡画像
a：画面にはFTカテーテルが映っており，卵管鏡はFTカテーテル内に存在する．
b：画面には卵管内腔が映っており，卵管鏡は卵管内に存在する．
c：緑色にみえるFTカテーテルの先は暗くなっている．このような画面がみえた場合，卵管穿孔が起こっていることが推測される．
[カラー口絵⑮]

なければならない．もし閉塞部を解除することができず卵管穿孔が起こった場合には，カテーテル先端は卵管間膜内あるいは腹腔内に存在しており，卵管鏡画像は卵管のピンクの像ではなく暗い像となるので穿孔を認識することができる(図2c)．なお穿孔が起こった場合でも穿孔の穴は非常に小さいため，特に追加の処置は必要としない．

6）レトログレードイメージング

カテーテルを最後まで進めたら，続いて卵管内の観察に移る．これをレトログレードイメージングという．カテーテルの圧を2気圧に減圧し，灌流をしながらカテーテルを引き戻す．この時，カテーテルを進めるのとは逆に卵管鏡は2倍の距離後退するので，カテーテルの引き戻しに協調させながら卵管鏡を常にカテーテルの先端におくようにして卵管内を観察する．この時卵管内所見が正常であれば卵管ひだが全周性に確認される．卵管ひだの減少や消失，卵管内の発赤所見や血管像は異常所見であり，卵管機能の低下を意味する．

3．子宮鏡下FTのコツとトラブルシューティング

1）子宮収縮

FTカテーテルを挿入することが卵管口への刺激となり，片側のFTが終了した後に子宮収縮が起こることがある．子宮が収縮してしまうと子宮鏡での卵管口確認が困難となることが多いので，対側卵管への処置に移る際は，可能な限り速やかに操作を行う必要がある．片側終了前に対側卵管口を子宮鏡で観察しておき，片側終了後に速やかに対側卵管口にウェッジさせるようにするとよい．また子宮収縮が起こってしまった場合には，子宮が弛緩するまで数分間待つか，子宮腔内の灌流圧をあげることにより子宮腔の拡張を得るとよい．

2）人　員

HAFTの際は子宮鏡担当と卵管鏡担当の2名で手技を行う必要がある(図3)

意　義

FTを施行する最大の意義は，卵管鏡手術により卵管の再疎通が得られ自然妊娠が可能となることである．この卵管再疎通手術の成功率をあげることが子宮鏡補助下にFTを行う意義である[3)~5)]．FTは子宮鏡を併用することなく卵管鏡単独で施行することが可能である．FTシステムは非常によくできており，通常の子宮腔形状をしている患者では，子宮鏡補助下にFT

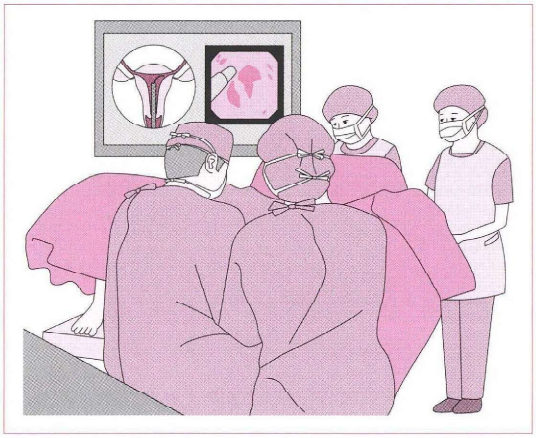


図3　HAFTの施行例
左が卵管鏡担当で右が子宮鏡担当.



図4　閉塞部から押し戻されているFTカテーテル
［カラー口絵⑯］

を施行していると，FTカテーテルを右側あるいは左側にかたむけるだけで，容易に卵管口にFTカテーテルがウェッジされることが確認される．ただしこれはあくまでも子宮鏡で子宮腔を観察しているから卵管口にウェッジされていることが確認されるのであり，卵管鏡のみの画像で卵管口へのウェッジを認識するのはそれほど容易なことではない．

1．卵管口へのウェッジ操作が確実・容易にできる

卵管鏡で子宮腔を観察してみると，卵管口と類似してみえる部分が散見され，卵管口を確実に卵管口として認識することは非常に難しい．しかし子宮鏡を挿入することにより容易に卵管口を探し出すことができ，子宮形態に問題がなければ，極めて容易に，そして確実に卵管口にカテーテルをウェッジさせることができる．卵管口へのウェッジが確実であるので，卵管穿孔が起こる確率も激減する．当然のことではあるが，医療者側にとっても患者側においても手技の成否はとても重要なことであり，またFTの最も重要かつ成否を握るポイントがFTカテーテルのウェッジ作業であると思われる．もし卵管口にウェッジされないまま手技が進んでしまった場合，卵管口以外の子宮壁にFTカテーテルが押しつけられてしまった場合には，子宮穿孔やFTカテーテル，卵管鏡の破損が起こる可能性があり，また子宮腔でカテーテルが進められてしまった場合，FTカテーテルが子宮腔でとぐろを巻く状態になってしまい，もしかすると一見，FTの手技が完遂したように思っても，全く卵管内にカテーテルが挿入されていない可能性もないわけではない．

2．確実な操作・診断ができる

卵管閉塞の程度が強い場合，通常はFTカテーテルを進めようとしてもカテーテルを進めることができないというのが通常であるが，ときにウェッジした卵管口からカテーテルが押し戻されてくることがある．この時，子宮鏡で観察していない場合，カテーテルが押し戻されていることに気付かず，挿入操作が進んでいるものと誤認してしまう可能性がある．しかし子宮鏡で観察していれば，カテーテルが押し戻されていることを容易に知ることができる（図4）．この時はカテーテルを卵管口に押しつけるようにしながら挿入を行うと閉塞を解除できることが多い．また卵管閉塞の程度が強い場合，子宮鏡補助下に手技を行い，確実に卵管口にウェッジできているとしてもFTカテーテルはより抵抗の少ない方向に進むため閉塞部位を通過せず，子宮穿孔や卵管穿孔を起こしてしまう可能性もある．しかし，これはFTの限界であり，



図5 子宮底部のうねりにより誤った部位にウェッジされたFTカテーテル

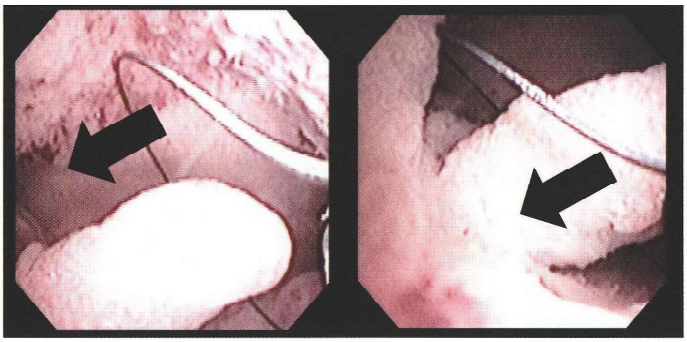
このような場合には，体外受精・胚移植を選択するなどほかの方法で妊娠成立を目指すことが望ましい．FTが成功した際には自然妊娠を期待することができるし，穿孔の際には体外受精・胚移植を選択するというようにHAFTを行うことにより，その後の治療方針が明確になる．

3．子宮形態異常があっても安全・確実に対応できる

子宮腔が変形しているような場合や子宮底部が平坦でないような場合には，子宮鏡補助下であってもFTカテーテルをウェッジすることが容易ではない場合がある．子宮底部が平坦ではなく，うねりが存在する場合，FTカテーテルがうねりの部分にはまってしまい，卵管口へのウェッジが困難な場合がある(図5)．この時，子宮鏡補助がないと，うねりの部分にFTカテーテルがはまった状態を卵管口にウェッジしたものと誤認してしまい本来卵管ではない部分でカテーテルを進めてしまい子宮穿孔を起こしてしまう可能性がある．このような場合でも子宮鏡で観察にすることにより卵管口へ確実にウェッジすることができ，その効果は計り知れない．このように安全に，確実に，簡便に，そして安心して卵管口へFTカテーテルを導くことができることがHAFTの最大の意義であると思われる．なおどうしても手術担当医数や施設に設置されているシステムの都合により子宮鏡と卵管鏡を同時に施行することが難しい場合もあると思われる．このような場合でも，先に述べたように子宮腔形状に問題なければ通常は容易に卵管口にFTカテーテルはウェッジされるため，事前に子宮鏡を施行し子宮腔形状を確認しておくと，卵管鏡単独で行ったとしても，安心度は格段に高まる．

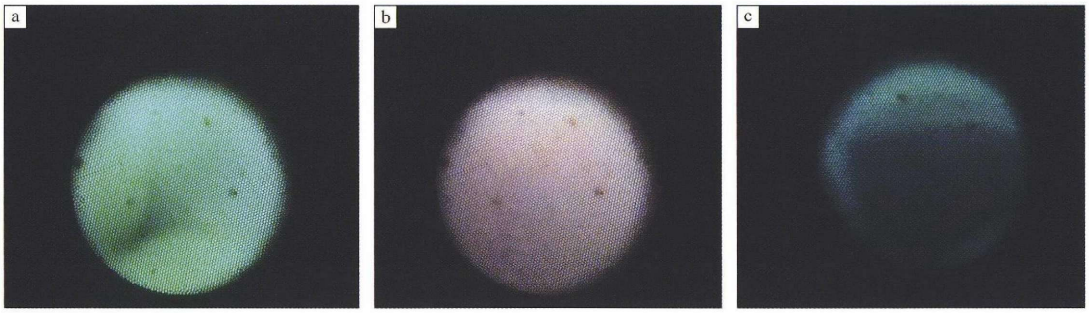
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スネアをポリープにかけているところ

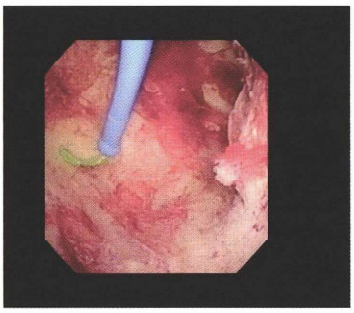
口絵⑭［p.1368　図14］



卵管鏡画像

a：画面にはFTカテーテルが映っており，卵管鏡はFTカテーテル内に存在する．
b：画面には卵管内腔が映っており，卵管鏡は卵管内に存在する．
c：緑色にみえるFTカテーテルの先は暗くなっている．このような画面がみえた場合，卵管穿孔が起こっていることが推測される．

口絵⑮［p.1384　図2］



閉塞部から押し戻されているFTカテーテル

口絵⑯［p.1385　図4］

体外受精における卵巣刺激

ART卵巣刺激における日程調整法

浮田祐司　浮田美里　柴原浩章

- 月経周期のうち卵胞期後期や黄体期から卵巣刺激を開始しても成熟卵や胚盤胞が得られ，従来法と比較しても同等数を得ることができる．
- ランダムスタート法は，がん治療を早急に開始しなければならない若年がん女性に対する妊孕性温存のための卵巣刺激に有用である．
- 従来法である卵胞期初期からの刺激による採卵だけでなく，黄体期からの採卵も行う2回採卵（Duo stim）法は卵巣予備能力が低下している女性への卵巣刺激法として考慮してよいかもしれない．

はじめに

若年者のがん罹患率は近年増加傾向を示す一方で，集学的治療や早期診断の進歩に伴いその治療成績は向上し，生存率が増加している．そこで若年がん患者のQOL向上のため，がん治療前の妊孕性温存が注目されている．われわれも女性[1]および男性[2]の妊孕性温存法を積極的に施行し，報告してきた．

1983年に世界初のヒト凍結融解胚を用いた妊娠・出産例が，1986年にはヒト凍結融解未受精卵を用いた妊娠・出産例が報告された．以降，妊孕性温存にもARTの技術が応用されている．

若年がん患者のがん治療前の妊孕性温存（胚や未受精卵子の凍結保存）では，原疾患の治療を早急に開始しなければならない場合が多く，一般にARTで行われるような月経初期からの卵巣刺激を待つことはできない．このような目的に対して，卵胞期後期や黄体期からでも卵巣刺激を開始して成熟卵や胚盤胞を得ることのできることが近年報告されている[3]．この月経周期のいつの時期からでも卵巣刺激を開始する方法を，ランダムスタート法（random-start controlled ovarian stimulation）と呼ぶ．当然のことながら，胚や

うきた ゆうじ，うきた みさと，しばはら ひろあき：兵庫医科大学産科婦人科（〒663-8501　兵庫県西宮市武庫川町1-1）

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未受精卵子を少しでも多く凍結保存するために，同一月経周期で2回採卵するDuo stim法という卵巣刺激方法も報告されている[4]．

ランダムスタート法の原理

ランダムスタート法で成熟卵を得ることができる原理を説明する．従来，卵胞発育は前月経周期の黄体期後期に前胞状卵胞のリクルートがあり，その後，卵胞期中期に1個の卵胞が選択されるとその他の卵胞は閉鎖に向かうと考えられていた[5]．しかし最近では同一月経周期の中で，卵胞のリクルートには多くの波が存在することが示されている[6,7]．そのため，卵胞期後期や黄体期に認める前胞状卵胞は閉鎖に向かうのではなく，卵胞発育の初期段階と考えることができる．したがって月経周期のいつの時期からでも卵巣刺激を行えば卵胞が発育し，採卵は可能になるという原理である．ただし，月経周期と無関係であることから，新鮮胚移植はできないため，全胚凍結が必要である．

Duo stim法の原理

ランダムスタート法の卵胞期スタートと黄体期スタートを組み合わせるのがDuo stimである．同一月経周期の中で卵胞のリクルートは多くの波があることが示されているため，一度卵胞期から卵巣刺激を開始したとしても，採卵後に再度卵巣刺激を行えば卵胞の発育を認め，黄体期に再度採卵することができる．ランダムスタート法と同様に，新鮮胚移植はできないため全胚凍結が必要である．

ランダムスタート法による卵巣刺激法

ランダムスタート法には月経周期により卵巣刺激法にバリエーションがある．

1）卵胞期初期（月経開始後7日未満）から刺激開始の場合には，速やかにゴナドトロピンの連日投与を開始する．その後はGnRH antagonist法と同様に首席卵胞径が14 mmに到達した時点で，GnRH antagonistを投与開始し，卵胞径が18 mmに到達した時点でhCG 5,000単位を投与し，35～37時間後に採卵する（**図1**）．

2）卵胞期後期（月経開始7日以降）から刺激開始の場合には，首席卵胞径が18 mm未満と18 mm以上で対応が異なる．前者では，首席卵胞以外の卵胞が12 mm未満であるのを確認し，ゴナドトロピン投与を開始する．その後，首席卵胞によるLHサージ開始後からGnRH antagonistの投与を開始し，2番目以降の卵胞径が18 mmに到達した時点でhCG 5,000単位を投与し，35～37時間後に採卵する（**図2**）．後者では，hCG 5,000単位を投与し，その2～3日後からゴナドトロピンの投与を開始する．その後はGnRH antagonist法と同様に首席卵胞径が14 mmに到達した時点でGnRH antagonistの投与を開始し，卵胞径が18 mmに到達した時点でhCG 5,000単位を投与し，35～37時間後に採卵する（**図3**）．

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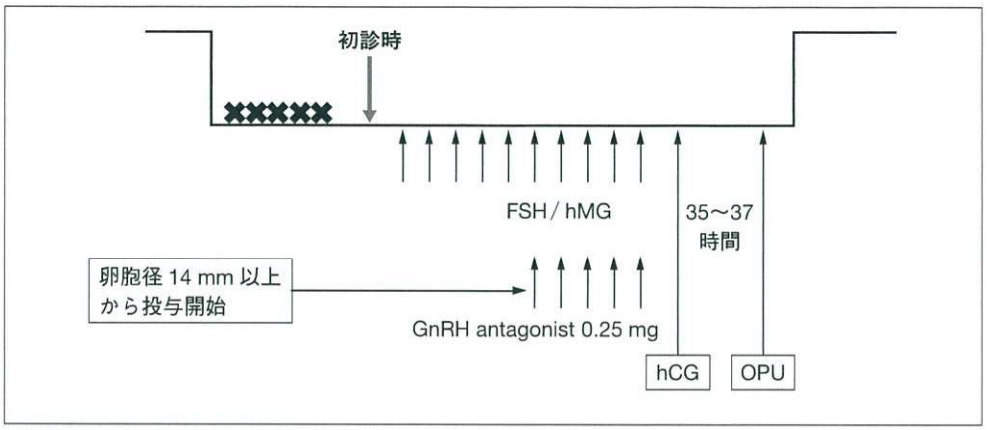


図1　ランダムスタート法（卵胞期初期開始）

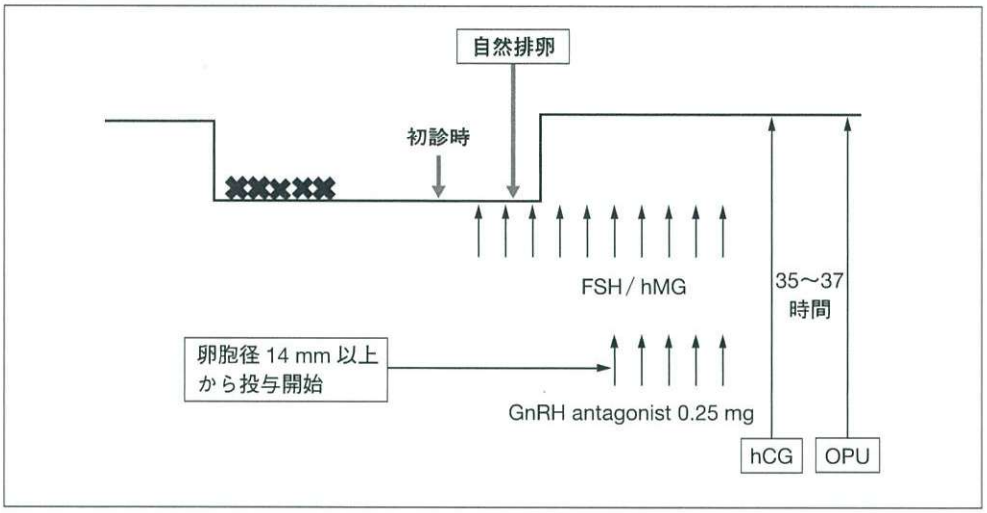


図2　ランダムスタート法〔卵胞期後期（首席卵胞径 18 mm 未満）開始〕

3）黄体期から刺激開始の場合には，速やかにゴナドトロピンの連日投与を開始する．その後は GnRH antagonist 法と同様に首席卵胞径が 14 mm に到達した時点で，GnRH antagonist の投与を開始し，卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図4**）．

Duo stim 法による卵巣刺激法

まず，卵胞期から刺激を開始する．首席卵胞径が 14 mm に到達した時点で，GnRH antagonist を投与開始し，卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する．

次に，1 回目の採卵後 5 日目より再度刺激を開始し，首席卵胞径が 14 mm に到達した

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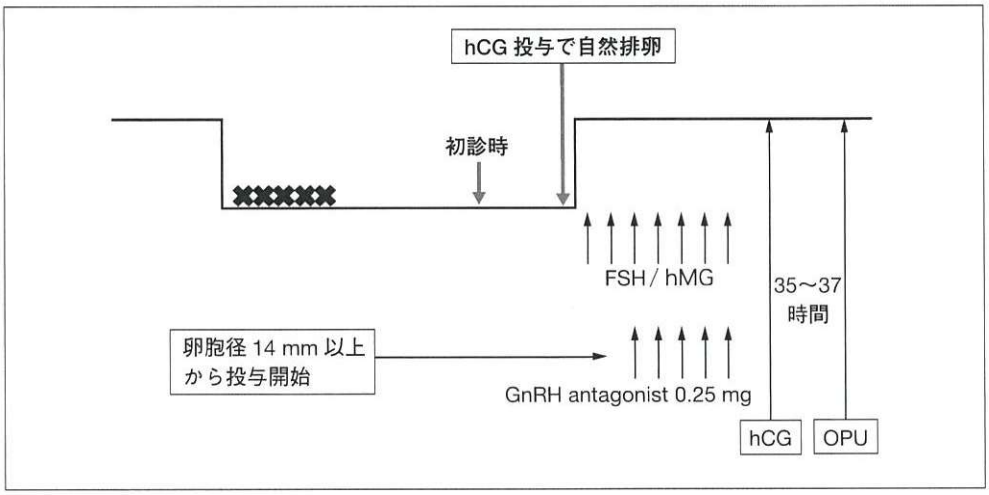


図3　ランダムスタート法〔卵胞期後期（首席卵胞径 18 mm 以上）開始〕

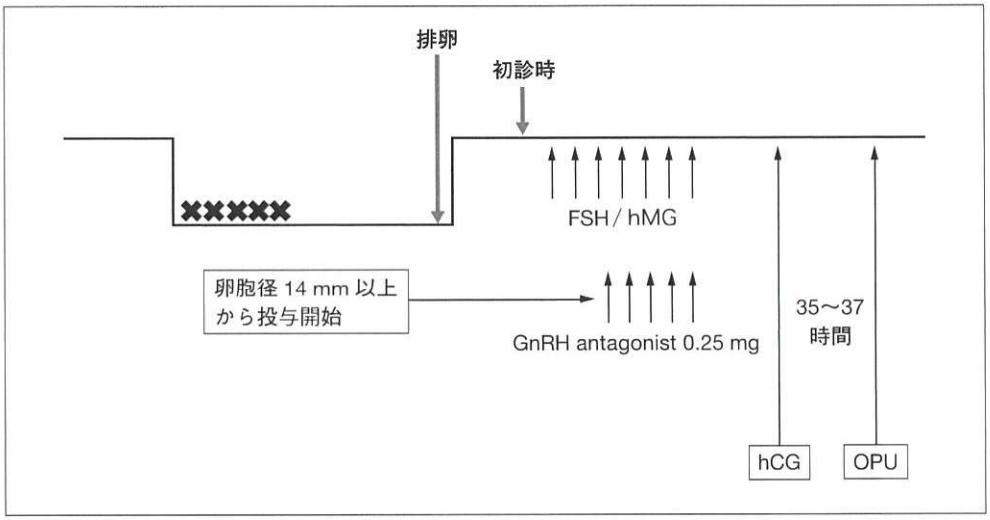


図4　ランダムスタート法（黄体期開始）

時点で，GnRH antagonist を投与開始し，卵胞径が 18 mm に到達した時点で hCG 5,000 単位を投与し，35～37 時間後に採卵する（**図 5**）．

ランダムスタート法および Duo stim 法の有効性について

従来法，卵胞期後期からのランダムスタート法，黄体期からのランダムスタート法それぞれの獲得卵子数・成熟卵子数・受精卵数には有意差がないが，ランダムスタート法のほうが有意に総ゴナドトロピン量は多かったと報告されている[8, 9]．われわれも同様の報告を行っている[10, 11]．

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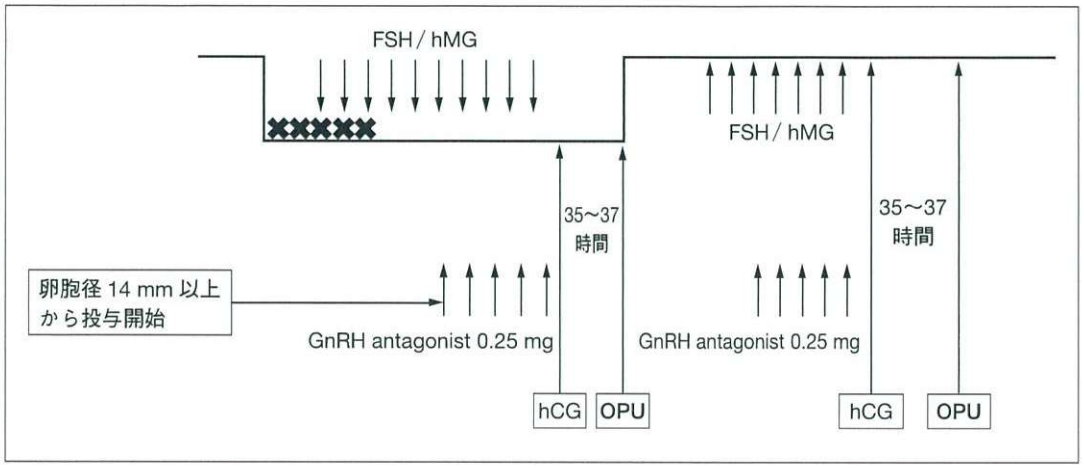


図 5　Duo stim 法

Duo stim 法においては，卵胞期と黄体期の獲得卵子数・成熟卵子数・胚盤胞数に有意差を認めなかったと報告されている[4]．したがって本法は，卵巣予備能が低下している女性への卵巣刺激法として考慮してよいと考えられる．実際，この報告では低反応性の不妊女性に対する検討であった．

おわりに

ランダムスタート法や Duo stim 法が報告されて以来，まだ経験が少ない．そのため妊孕性を温存した女性に対して胚移植を行い，従来法と比べて妊娠・分娩率に影響がないかは今後の課題である．一方がんに罹患していない不妊女性に対し，黄体期から卵巣刺激を開始するランダムスタート法を行い，従来法と変わらない周産期転帰であったとの報告もある[12]．したがって可及的速やかに卵巣刺激を開始しなければならない場合には，ランダムスタート法による卵巣刺激法は積極的に考慮してよいものと考える．また，Duo stim 法による卵巣刺激法を組み合わせることでより多くの卵子を獲得することができる．

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臨床の広場

卵管病変と卵管鏡

福井淳史，佐伯信一朗，柴原浩章

兵庫医科大学産科婦人科学講座

はじめに

卵管閉塞あるいは卵管狭窄と診断されたとき，できることであれば，通常の性交により挙児を得たいと願うものも少なくはない．これらの方達の妊娠を可能とするのが卵管形成術である．卵管形成術には卵管の障害部位により腹腔鏡下に行うもの，卵管鏡下に行うものが存在する．卵管遠位側の閉塞や卵管周囲癒着に対しては腹腔鏡手術が，卵管近位側の閉塞や狭窄に対しては卵管鏡手術が行われる．卵管鏡手術の適応は近位側の卵管性病変，すなわち子宮卵管間質部や卵管峡部の閉鎖・狭窄を有するもので，自然妊娠を希望するものである．

卵管鏡下卵管形成術の現状

産科婦人科領域の内視鏡には，腹腔鏡，子宮鏡，卵管鏡，胎児鏡がある．それぞれ腹腔，子宮腔，卵管腔，胎児を観察するために用いられる．このうち卵管腔へのアプローチには，子宮腔側から，卵管采側からの2種類がある．卵管采および卵管采近傍の卵管腔の確認は，3 mmほどの細径スコープを用いると通常の腹腔鏡でも確認可能である．さらに経腟的アプローチによる液相下での腹腔鏡（経腟腹腔鏡；Transvaginal hydrolaparoscopy；THL）を用いると，腹壁上に傷を残すことなく，卵管采および卵管采近傍の卵管腔を確認可能である[1,2]．

一方，子宮腔側からのアプローチで用いられるのが，卵管鏡下卵管形成（FT）システムである．FTシステムは主として近位卵管閉塞や狭窄など近位卵管病変に対して行われる病的卵管に対する再疎通あるいは拡張手術である．一般に卵管閉塞や卵管狭窄など卵管性不妊と診断された場合，その後の治療法として体外受精・胚移植（IVF-ET）が選択される機会が多いものと推定される．現在，FTの普及度は高いとはいえず，FTカテーテルの出荷施設から類推するに全国でFTを施行可能な施設数は100施設ほどと思われ，年間3700件程度のFTが行われている．なおFT施行施設のうち1/3が関東，1/4が関西であるが，施行件数は関西が約50％を占めている．なおFTは保険が適応（片側46410点，両側の場合は92820点）されているため，IVF-ETと比較しても，患者の費用負担が抑えられるといえる．

卵管鏡下卵管形成術

FTシステム（図1）は卵管鏡（0.6 mm径，6000画素，焦点距離0.5 mm），卵管再疎通を行うFTカテーテル（6 cm，10 cm），FT潅流ポンプ，カメラヘッドと卵管鏡をつなぐアイピースアダプタ，光源装置，ビデオシステムからなる．FTカテーテルを卵管内に挿入するためには卵管口を同定し，卵管口にFTカテーテルを

◆Clinical view◆

Tubal infertility and falloposcopy

Atsushi FUKUI, Shinichiro SAEKI and Hiroaki SHIBAHARA

Department of Obstetrics and Gynecology, Hyogo College of Medicine



図1　FTシステム

ウェッジさせ，そこからFTカテーテルを押し出しながら卵管内に挿入していく必要があるが，卵管鏡は6000画素と決して画素数が高いわけではないため，卵管口は確認しづらいことも多く，その際には次に示す幾つかの卵管口確認操作を行って，確実に卵管内にカテーテルを挿入する必要がある．

FTを行う際には専用の腟鏡（スペキュラム）と単鈎鉗子（タナキュラム）を用いる．腟鏡を挿入し，腟内を消毒した後に，専用単鈎鉗子で子宮腟部後唇を把持し，腟鏡に固定する．続いてFTカテーテルを子宮腔内に挿入し，カテーテルを再疎通させる卵管方向に90度回転し，先端を卵管角へウェッジさせる．その後にカテーテルを陰圧の状態でフットスイッチを押して潅流を行いながら卵管鏡をカテーテルの先端まで進め，卵管口を確認する．卵管鏡でみた卵管口を図2に示す．卵管口が確認できたら，卵管鏡を潅流しながら0.5～1 cmほど引き戻し，カテーテルを6気圧まで加圧し，カテーテルを進める．この時カテーテルに連動して卵管鏡も前進するが，卵管鏡はカテーテル内のバルーンの2倍の距離を前進する構造となっているため，卵管鏡により卵管穿孔を起こさないようにするた

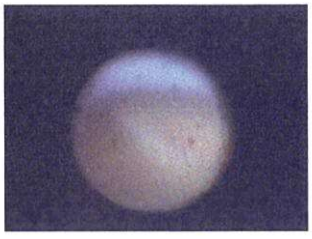


図2　卵管口

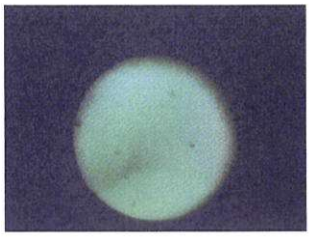


図3　カテーテル内

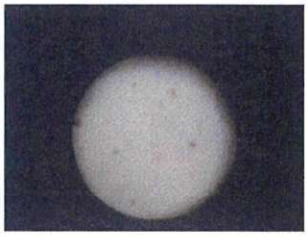


図4　卵管内

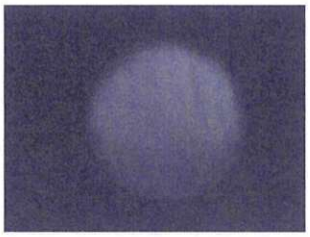


図5　穿孔

め，卵管鏡がバルーンの先端まで達したら，カテーテルを2気圧に減圧し，潅流を行いながら卵管鏡を0.5～1 cm後退させる．この操作を繰り返し，6～10 cmバルーンを進めていく事により卵管を再疎通させる．なおバルーン内に卵管鏡があるとき，画面は緑色に見え（図3），FTカテーテル先端に卵管鏡が達すると卵管内が見えるため画面はピンク色にみえる（図4）．このとき卵管内のピンク色の像が見えたらそれ以上カテーテルを進めないことが肝要であり，これを怠りさらにFTカテーテルを進めた場合や勢いに任せてカテーテル進めてしまった場合，容易に卵管穿孔を起こしてしまう．卵管鏡を前進させる際には手元に神経を集中し慎重にカテーテルを進める必要がある．カテーテルが閉塞部あるいは狭窄部に到達すると，カテーテルを進めるときに抵抗を感じる．この時は一気にカテーテルを進めないで少しずつカテーテルを進めることが肝要である．閉塞部を通過すると抵抗が消失するのがわかる．なお，閉塞部の開通操作あるいはFTカテーテルの前進操作中に卵管穿孔が起こるとFTカテーテルは腹腔内や卵管間膜内に存在しているため，卵管鏡がFTカテーテルの先端に達した際に卵管内のピンク色の像ではなく非常に暗い像が確認されるので容易に穿孔を認識することができる（図5）．なお穿孔が起こった場合でも穿孔の穴は非常に小さいため，出血が起こることは通常考えられず，とくに追加の処置は必要としない．

カテーテルを最後（6 cmあるいは10 cm）まで進めたら，続いて卵管腔の観察に移る（レトログレードイメージング）．すなわちFTカテーテルの圧を2気圧に減圧し，潅流をしながらカテーテルを引き戻す．このときカテーテルを進めるのとは逆に卵管鏡は2倍の距離を後退するので，カテーテルの引き戻しに協調させながら卵管鏡をFTカテーテルの先端におくようにして卵管鏡を進め卵管腔を観察する．

卵管が強固に閉塞している場合には6気圧ではカテーテルを進めることができなくなってしまうことがある．これは閉塞部でカテーテルがパンチングを生じているためであり，そのままカテーテルを進めるとカテーテルの破損や卵管穿孔を起こしてしまう可能性が高くなる．カテーテルが進まないときには，まずカテーテルを2気圧に減圧し潅流を行ってみる．この時灌流液が流れるのであれば再度圧を6気圧として挿入を試みる．それでもカテーテルが挿入されない場合には圧を1気圧ずつあげながら（最高9気圧まで）同様の挿入を試みる方法もある．灌流液が流れない場合にはパンチングを起こしている可能性が高いので，まずはカテーテルを2気圧に減圧した後に潅流液が滴下するところまでカテーテルを引き戻したうえで，圧を1気圧ずつあげながら（最高9気圧まで），カテーテルの挿入を試みる．

卵管鏡下卵管形成術の成功率を高めるための対策

FTカテーテルの確実な挿入法（卵管口の探

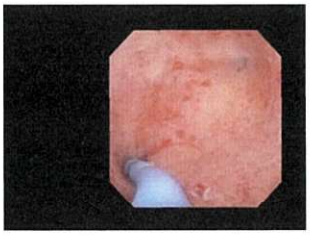


図6　子宮鏡補助下FT

し方，卵管口確認操作）

ブラインドエバージョン；卵管口が判然としない場合にはブラインドにFTカテーテルを2 cmほど進めた後にレトログレードイメージングを行って卵管内の管腔構造を確認しFTカテーテルが卵管内に挿入されていることを確認しても良い．

子宮鏡補助下FT；卵管口へのFTカテーテルのウェッジをより解像度の高い子宮鏡を用いて行うものである（図6）．卵管鏡のみの画像では卵管口を同定することが難しいことはよく経験することである．さらには子宮腔が変形している場合，子宮底部が平坦ではない場合，子宮後屈の場合など，FTカテーテルのウェッジが困難な場合でも子宮鏡下にFTカテーテルを卵管口に誘導することにより，安心してその後の開通操作を行うことができる．

腹腔鏡補助下FT；腹腔鏡を用いてFTカテーテルと卵管鏡の挙動を確認しながら開通操作を行うものである．FTカテーテル卵管口を確認することはできないが，腹腔鏡の光源を落とすことにより卵管鏡の光を透見することができる（図7）．これによりカテーテルのおおよその位置を知ることができ，卵管内への挿入や閉塞部位でのカテーテルの挙動，穿孔の有無などを肉眼的に確認することができる．また開通が得られると卵管采から潅流液が流れ出ることを確認できるため，手技の完遂度を知るためにも有用な方法である．

透視下FT；FTカテーテルの外筒はX線透視で確認できるため，FTカテーテルの向きを術中透視により確認することができる．潅流液の流れるルートに造影剤を流すことにより，手技中にリアルタイムで卵管通過性を確認する方法もある．また術直後に透視を行うことにより，手技の成否を確認する事ができる．卵管閉塞部位は1カ所のみではない場合もあり，開通操作部より遠位に閉塞部位が存在するか否か，卵管水腫の有無（もともとは近位閉塞と診断されている例がほとんどであるため超音波で確認できるような卵管水腫以外の卵管水腫を事前に診断することはできない）も確認する事ができる．

卵管鏡で確認される所見

レトログレードイメージングで卵管腔を観察すると，卵管のヒダや卵管粘膜の様子を確認することができる．閉塞部位以外には卵管内に異常を認めないような場合，すなわち卵管内所見が正常であれば卵管ひだを卵管内全周性に確認することができる（図8）．一方，卵管ひだの減



図7　腹腔鏡補助下FT



図8　卵管内のひだ

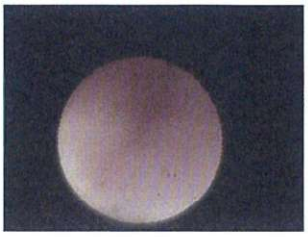


図9　浮腫状のひだ

少や消失，卵管内の浮腫状変化（図9），発赤所見や血管像は異常所見であり，卵管機能の低下を意味すると思われる．

FT後の妊娠率

FT後の自然妊娠率は，約25～40％程度[3-5]であり，妊娠は術後1年以内に成立することが多い．卵管閉塞例では自然妊娠が全く望めないわけであり，このうち約3割が自然妊娠できることから考えてもFTを行う意義は大いにあると考えられる．

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寄稿◆研究者の最新動向

セロトニンによる新たな男性不妊治療法の開発

Development of a novel male infertility treatment using serotonin

1）兵庫医科大学 産科婦人科
Department of Obstetrics and Gynecology
Hyogo College of Medicine
2）獨協医科大学 医学部 生理学（生体制御）
Department of Physiology, School of Medicine
Dokkyo Medical University
3）獨協医科大学 先端医科学統合研究施設
実験動物センター
Laboratory Animal Research Center
Dokkyo Medical University

すぎやま ゆきこ
杉山 由希子 1)・ふじのき まさかつ 藤ノ木 政勝 2) 3)
Yukiko Sugiyama　Masakatsu Fujinoki
しばはら ひろあき
柴原 浩章 1)
Hiroaki Shibahara



杉山 由希子
2012年 兵庫医科大学 医学部 卒業
兵庫医科大学 大学院在学中
'19年 兵庫医科大学 産科婦人科 助教

Key words セロトニン，精子，超活性化運動，体外受精

Abstract

哺乳類では受精能獲得した精子のみが受精できる。受精できる精子は超活性化運動と先体反応を示し，これらは卵管内ホルモンによって惹起される。セロトニンは卵管内にも分泌され，超活性化運動と先体反応を惹起する。ところで，超活性化運動の惹起率は体外受精の成績と正の相関をする事から，私達はセロトニンで超活性化を惹起する事で体外受精の成績を上げられないか検討を始めた。マウスを用いてセロトニンの超活性化運動と体外受精の成績への影響を検討したところ，セロトニンは超活性化運動を惹起し，体外受精の成績を上げた。今後はヒトでも同様の結果を得られるか検討し，セロトニンを用いた新たな男性不妊治療法の開発を目指したい。

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はじめに

ヒトを含め哺乳類精子は，卵管内において受精能獲得とよばれる質的な変化を経て卵子と受精する。受精能獲得の過程で精子に見られる反応として運動の超活性化と先体反応が知られている[1,2]。これら反応はどちらも卵管内に分泌されるホルモン等によって惹起される[3]。先体反応は，卵丘細胞層や透明帯を消化するヒアルロニダーゼ等の消化酵素を放出する膜輸送であると共に卵との結合装置を表出させる反応で，受精に必須とされる[1]。一方の超活性化運動は，粘性の高い卵管内液中を遊泳し，卵丘細胞層や透明帯を通過するための推進力を出す特別な運動とされるが，受精に必ずしも必須ではない[1,4]。しかし，少なくともヒトにおいては精子の超活性化運動の惹起率と体外受精の成績との間に正の相関を示すとされる[5]。

精子が受精能獲得を起こしたか否かの確認には，先体反応が起こったか否か，もしくは超活性化運動が起こったか否かを判断することになる。先体反応の確認は，細胞の染色が必要で，intact な状態で判断することは容易ではないが，超活性化運動は精子が泳いでいるところを観察すればよいため intact な状態で判断することは容易である。加えて，超活性化運動の惹起率と体外受精の成績との間に正の相関がある事から[5] 精子の運動状態（超活

性化をしているか，していないか）を判断し，体外受精の実施の判断の参考にする事は有意義であると考えられる。さらに，一歩踏み込んで超活性化運動は卵管内に分泌されるホルモン等によって惹起される事から[3]，人為的に超活性化を惹起させる事で体外受精の成績を向上させる事が出来るだろうと期待できる。しかしながら現在までに明確に人為的に超活性化を惹起させることで体外受精の成績が向上したという報告はなく，残念ながらヒト精子を用いた報告では超活性化運動はプロゲステロンで惹起されるもののプロゲステロンで人為的に惹起された超活性化は体外受精の成績に影響することはないとされている[5]。

最近，プロゲステロン以外にも実験動物精子において超活性化運動を惹起する生理活性物質が複数見出されている[3]。その中でセロトニン（5-hydroxytryptamine，5-HT）はハムスター精子において先体反応と超活性化運動を惹起する物質として見出され[6][7]，ヒト精子においてもその受容体が検出され，かつセロトニンにより遊泳速度が増加すると報告されている[8]。

セロトニンは中枢神経系において働く主要な神経伝達物質の一つとして良く知られ，その受容体は5-HT_1，5-HT_2，5-HT_3，5-HT_4，5-HT_5，5-HT_6，5-HT_7の7つのファミリーから構成されている[9][10]。さらに，5-HT_1受容体は5つの，5-HT_2受容体は3つのサブファミリーから構成される。これら受容体は5-HT_3受容体を除きGタンパク質共役型受容体であり，5-HT_1と5-HT_5受容体はアデニル酸シクラーゼを抑制しcAMP濃度を下げる作用を示し，5-HT_4，5-HT_6，5-HT_7受容体はアデニル酸シクラーゼを活性化しcAMP濃度を上げる作用を示す。5-HT_2受容体はホスホリパーゼCを活性化させIP_3を産生し，細胞内Ca濃度を上昇させる。5-HT_3受容体はリガンド作動性陽イオンチャネルで，一般的にNaチャネルとして働き脱分極を引き起こす。これら受容体を介してセロトニンは様々な生理機能の調節を行っている。生殖機能に関して，セロトニンおよび受容体がマウスおよびヒトの卵母細胞，卵丘卵母細胞複合体（COCs），卵胞液，卵管，胚から検出されており，卵成熟や発生のほかステロイド合成の調節に関与しているとされる[10~13]。精子については，ハムスターにおいて先体反応と運動の超活性化を5-HT_2と5-HT_4受容体を介して惹起することがわかっている[6][7]。また，ヒトとウマ精子から5-HT_1，5-HT_2，5-HT_3受容体が検出され，かつヒト精子の遊泳速度をセロトニンが上昇させると報告されている[8][14]。

上述のような背景を基に，私達はセロトニンを培地に添加して体外受精を実施する事でヒト体外受精の成績の向上が図れるのではないかとの着想に至り検討を行った。これまでの報告において，ヒト精子の運動超活性化と体外受精の成績の間に正の相関があるが，プロゲステロンを用いてヒト精子超活性化を人為的に惹起する条件下で体外受精を行っても，成績の向上に寄与しないとされている[5]。プロゲステロンは古くから良く知られている先体反応惹起物質であり，卵胞液に含まれる[15]。一方でステロイドホルモンであるため，アルコール等の有機溶媒を用いないと培地に溶かす事ができない。受精は精子，卵子，卵丘細胞の三者が関与する現象であり，仮に精子の機能亢進を惹起しても卵子や卵丘細胞に毒性があれば受精は成立しないと考えられる。従って，用いる添加物はでき得る限り水溶性の生体物質である方が良いことは言うまでもない。セロトニンは私達の体内ではトリプトファンから作られる生体物質であり，水溶性である。その為，培地への添加において溶媒を必要とすることはなく，私達の設定する条件に合致する物質である。加えて，ヒト精子において受容体が検出され，かつ運動性も向上させると報告されている事[8]から，検討する物質として第一候補と考えた。しかしながら，セロトニンの超活性化運動への影響についてはハムスター精子を用いた私達の報告[7]があるのみであり，体外受精における影響は管見の限りにおいては報告を見つけることができなかった。

ヒト体外受精への応用を最終目標として，手

始めにモデル動物での検討を行った。ハムスター精子においてセロトニンの超活性化運動への影響を調べた私達の検討が既にあるが[7]，ハムスターでの体外受精の実施は容易ではない。体外受精の実施の容易さを考慮するとマウスでの検討が望ましいが，マウス精子超活性化へのセロトニンの影響に関して知見がなく，一からの検討を行わなければならなかった。しかし，現状においてハムスターでの体外受精の実施は現実的ではなくマウスを用いて検討する事が最も良いと判断できたので，マウスを用いてまず超活性化運動へのセロトニンの影響を調べ，次に体外受精の成績の向上につながるか検討を行うこととした[16]。

1. 研究方法

精子は 8 ～ 20 週齢の成熟した雄 ICR マウスの精巣上体尾部より採取し，mTALP 溶液[17]に懸濁し，通常の培養環境下で培養した。運動はビデオ位相差顕微鏡で記録した。運動率と超活性化率は，総精子数，運動精子数，超活性化精子数を数えて求めた。

体外受精の実施において，精子は 10 ～ 20 週齢の成熟した雄 ICR マウスの精巣上体尾部から，卵子は過排卵処置した 8 ～ 12 週齢の成熟した雌 ICR マウスの卵管膨大部から採取した。体外受精は熊本大 CARD の方法に基本的に従って行った[18]。体外受精の評価は 2 細胞胚の発生率を受精率として行った。

2. 現在までの研究成果

ハムスター精子での検討で，セロトニンは fM ～ pM のレベルで 5-HT_2 受容体を刺激し，nM レベルでは 5-HT_4 受容体を刺激して超活性化運動を惹起する事を見出している[7]。そこでマウス精子に対して 100 fM，100 pM，100 nM の 3 種類の濃度でセロトニンを作用させてみた。図 1 に示す様にマウス精子の運動率にセ

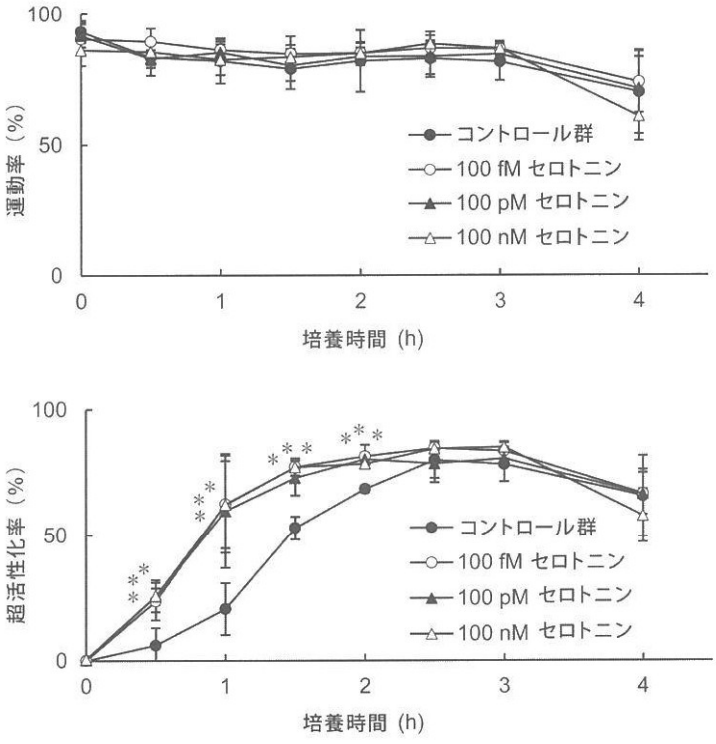


図 1　マウス精子運動率および超活性化率へのセロトニンの影響
＊は有意差を示す（$P < 0.05$）

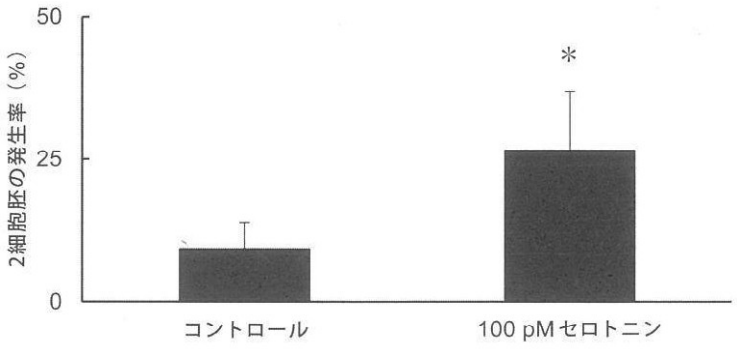


図2　セロトニン存在下でのマウス体外受精の結果
＊は有意差を示す（$P < 0.05$）。

ロトニンは影響を及ぼさなかったものの，超活性化率は有意に増加・促進させた。

セロトニンでマウス精子超活性化の増加・促進作用が確認されたので，体外受精を行い，その成績を向上させるか検討した。マウスでの体外受精は精子前培養を1時間行ったのち媒精し5時間静置するのは通常の方法であるが，媒精5時間ではセロトニンを作用させても2細胞胚の発生率に差はなく，受精率に影響しなかった。しかし，セロトニンが超活性化率を有意に上昇させるのは，精子の遊泳開始後2時間までであるので（図1），精子前培養を1時間行い，その後媒精時間を30分と短くして行ったところ，図2に示すようにセロトニンを作用させると有意に2細胞胚の発生率が上昇し，受精率が上がることを確認した。

3. 考察

今回の検討で，セロトニンはマウス精子の超活性化運動を惹起し，体外受精の成績を上昇させる作用があることが分かった。本稿では結果を示していないが，この作用は5-HT_2，5-HT_3，5-HT_4，5-HT_7の各セロトニン受容体を介して起こっていた[16]。これらセロトニン受容体のうち，5-HT_2受容体はCa濃度を，5-HT_4と5-HT_7受容体はcAMP濃度を上昇させる。CaとcAMPは運動性や受精能獲得の調節に関わるシグナル分子である事から[9][10]，超活性化運動の惹起につながったものと考えらえる。一方，5-HT_3受容体はリガンド作動性ナトリウムチャネルで脱分極を惹起する受容体である[9][10]。脱分極により電位依存型カルシウムチャネルの開口が起こることが考えられ，その場合細胞内Ca濃度を上昇させるので超活性化の惹起につながり得る。また，細胞外ナトリウム濃度の変化やNa/K-ATPaseが運動性や超活性化運動の調節に関与する事が示唆されているので[19][20]，これらの仕組みとの関連で超活性化を惹起するのかもしれない。

繰り返しになるが，5-HT_1，5-HT_2，5-HT_3の各セロトニン受容体がヒト精子においても存在し，セロトニン添加により運動能が向上すると報告されている[8]。ヒト精子に存在するセロトニン受容体のうち5-HT_2と5-HT_3受容体はマウスにおいても存在した[16]。そしてこれら受容体を刺激する事で，マウス精子超活性化は惹起され，体外受精の成績は向上した。従って，セロトニン添加によりヒトにおいても体外受精の成績を向上させる可能性がある。

4. 今後の展望

ヒト精子受精能獲得に関して明確にセロトニンが惹起するかどうかまでの検討はなされていない。したがって，今後の検討課題として，マウス精子で見られた現象がヒト精子でも見られるのか，具体的にはヒト精子でも超活性化運動の惹起をセロトニンが引き起こすのか，を検討する事が必要である。さらに，引き起

こすとすれば至適濃度はどの位か，そして複数存在するセロトニン受容体の内のどのタイプの受容体を刺激して惹起を引き起こすのか，基礎的な知見の収集を行う事を計画している。そしてヒト精子でもセロトニンで超活性化が惹起されるならば，体外受精を行った時の成績を向上させるのか検討する事を計画するべきであるが，ヒト卵子を用いた研究に行くには超えるべき課題は多いので，体外受精はまずは透明帯を除いたマウス卵子やハムスター卵子での検討から始めたいと考えている。

おわりに

我々のハムスターやマウスでの研究成果の発展として，今後はヒト精子を用いたセロトニンの精子運動性への影響を検討し，ハムスターテスト等を経て，男性不妊治療への応用を目指したい。

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[教育講演]

女性不妊症の現状と課題

柴原　浩章

Key Words：少子化，卵巣機能，女性不妊症，生殖補助医療，妊孕性温存

「Current status and problems of female infertility」

Department of Obstetrics and Gynecology, Hyogo College of Medicine, and Center for Reproductive Medicine, Hyogo College of Medicine College Hospital

１．はじめに

現在わが国は深刻な少子化にあり，年々人口は減少している。図１に示すように，平成30年における出生数92.1万人に対し，死亡数は136.9万人であり，この１年間の人口減少数は約45万人であった[1)]。この人口減少の傾向は平成17～18年頃から始まり，政府による積極的な少子高齢化への政策も十分には奏功していないことが伺える。

近年の価値観の多様化から，男女とも未婚率が増加し，平均初婚年齢が増加し，結婚後も特に女性側が妊活より仕事に専念し，生活の安定やキャリア向上を目指す傾向にある。その結果，妊活開始年齢は著しく上昇し，ようやく妊活を開始したとしても，高年齢による卵巣機能低下により妊孕性は著しく低下し，容易に妊娠・分娩可能な時期を逸することが多い。従って生殖医療や周産期医療への期待度は年々増加している。すなわち挙児を希望するのに妊娠できない不妊症や，流産や死産を反復する不育症などのため通院するカップルに対し，より確実にその希望を叶えることが可能となれば，現在

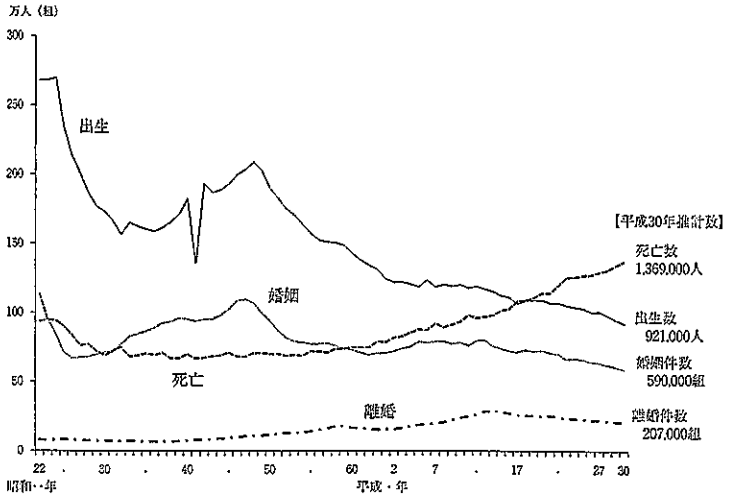


図１．人口動態の年次推移[1)]

（平成31年１月13日　第62回兵庫県医師会学術セミナー「少子化に立ち向かう ～不妊・不育診療の最前線～」於　兵庫県医師会館　講演内容）

兵庫医科大学産科婦人科学講座　主任教授
兵庫医科大学病院　生殖医療センター長

のわが国における深刻な少子化問題の解決の糸口が見える。

２．女性不妊症の現状

最近では不妊症の定義が見直され，これまでは結婚後２年間で妊娠できなければ不妊症と定義されてきたが，女性の平均初婚年齢が30歳に迫り，この晩婚化による影響で「年齢因子」による不妊症が増加したことから，結婚後１年間で妊娠できなければ不妊症とすると改められた。

不妊外来の進め方としては，まず従来から指摘されてきた三大原因である「内分泌」「卵管」「男性」を始めとする諸因子に対する基本検査を速やかに行い，必要に応じて二次検査を追加する。不妊治療の原則は，判明した原因に基づく最適な手段の提供であるが，必ずしも妊娠できるわけではない。原則として３回の治療周期で妊娠不成立の際の step up については，妊娠・分娩歴，年齢，卵巣予備能検査としての anti-Mullerian horomone（AMH）値，不妊原因の重症度等をカップル毎に考慮し，可能性が低いと判断した治療法からは早目に撤退し，次の治療 step に進む決断を要する。

ところで不妊カップルがより安心して検査や治療に立ち向かうにあたり，精神的なケアを必要とすることがある。例えば不妊症の女性は，自分は本当に妊娠できる身体なのかという不安と常に向かい合っている。通院してからまだ間もない時期には，特に女性側はこれまで未経験の検査を受けたり，時にはまさか自分が受けることになろうとは想像もしなかった高度な不妊治療の話を目の当たりにし，その受け入れに戸惑いが生じる。中には夫の協力を得にくい，あるいは親族からプレッシャーをかけられる，などの思わぬ苦労や苦痛もある。以上のような精神的・肉体的負担だけでなく，人工授精（AIH：artificial insemination with husband's semen）や体外受精（IVF：in vitro fertilization），顕微授精（卵細胞質内精子注入法；ICSI：intracytoplasmic sperm injection）などの生殖補助医療（ART：assisted reproductive technology）は自費診療であり，経済的な負担も大きい。また治療期間が長期化した場合には，まるで出口のないトンネルの中にいるような感覚に陥るという。

一方生殖医療においては，しばしば倫理的な判断を求められる場面に遭遇する。わが国では法的な規制は存在しないが，日本産科婦人科学会等による会告を遵守するというルールがある。例えば非配偶者間の不妊治療に関しては，現在行われている治療法は提供精子による人工授精（AID：artificial insemination with donor's semen）だけであるが，その妊娠・分娩率は極めて低い。そこで提供精子による ICSI を行えば多くの希望者が妊娠できることは明白であるが，現状として配偶子や胚の提供による ART あるいは代理懐胎はわが国では行われていない。時代による価値観の変化や国際的な趨勢にも視点を向け，定期的に議論する必要性はあろうが，会告を無視するスタンスは容認されていない。

３．将来の妊孕性温存のための不妊治療の応用

これまで晩婚化による特に女性側の妊孕性低下と，わが国における少子化との関係につき述べてきた。すなわち最近では様々な理由から，若いうちに妊活をスタートできないため，例えば40歳を過ぎたら区切りをつけて本格的に妊娠・出産を考えたい，という願望を有する女性が増加傾向にある。ところが一般に女性は35歳を境に卵巣機能は著しく低下し，ましてや40歳を過ぎてからの妊娠を望むことは成功率の点から非現実的と言わざるをえない。

そこで卵子の数や質が低下しないうちに，少しでも若いうちに不妊治療の技術を利用して凍結保存しておき，将来の妊孕性を担保するという妊孕性温存法が注目されている。ただしこれに対して関連学会では，これらの方法は現時点では決して非常に高い確実性のある妊孕性温存手段とは言えず，あくまで適切な時期に普通に妊娠することを推奨するという見解を公表している。

一方，これまでは若年者ががんに罹患した場合，手術や抗がん剤，あるいは放射線治療をできる限り早急に進め，生命予後の改善を最優先してきた。これらの集学的治医療により生存率は著しく改善したが，性腺毒性の強い治療を選択する場合には妊孕性の低下や消失などを生じたことから，がんサバイバーの QOL の低下

が問題視されてきた。海外ではこの領域を，oncology（腫瘍学）と妊孕性（fertility）を結合して“oncofertility”と呼び，2000年代初頭から若年者のがんの診断後に，精子・卵子や受精卵，あるいは卵巣の凍結保存の機会の選択を推奨してきた。

そこで兵庫県においても，兵庫県がん診療連携協議会と兵庫県産科婦人科学会が合同で，2016年に「兵庫県がん・生殖医療ネットワーク」を開設し，がん治療施設と生殖医療施設が密接に連携できる体制が整った[2]。腫瘍専門医が若年者のがんの診断後，速やかに生殖医療専門医と本ネットワークを通じて連携し，クライアントに対して生殖医療専門医が妊孕性温存に関する情報提供を行い，希望があれば精子・卵子や受精卵，あるいは卵巣の凍結保存を実施している[3-5]。

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〈総論〉

ARTに関する基礎知識

柴原浩章　加藤徹　福井淳史

▶ポイント

- **女性の平均初婚年齢の上昇により，卵子の老化に伴う「年齢因子」による不妊症が占める割合は年々増加している．**
- **初診のカップルに対して不妊治療をすぐに開始するのではなく，まず原因を診断するため一連の検査を行い，診断に基づく治療の提案を原則とする．**
- **ARTの適応は，それ以外に妊娠できる方法がないと判断されたカップルに限定されている．**
- **ただし，カップルの年齢，不妊期間，妊娠・分娩歴，合併症の有無，過去の不妊治療，家族計画の意向なども勘案し，標準治療からの開始でよいか治療法選択のうえで，ARTを含め特別な配慮を要するかなどを個別に判断することが重要である．**

Keyword　生殖補助医療（ART），受精，着床

はじめに

わが国では近年著しく晩婚化が進み，女性の平均初婚年齢は直近のわずか四半世紀の間に，およそ25歳から30歳へと約5歳も上昇した．しかも若いカップルにとって，わが国は経済的にも社会的にも安心して産み・育てる環境が必ずしも整備されているとはいえず，晩婚にもかかわらず，妊活の開始はしばらくの期間様子をみて決意する傾向にある．そこでこのような現状を鑑み，不妊症の定義は最近，「努力しても1年以上にわたり子どもを授かることができないカップル」と，従来より1年短縮された．このトレンドは先進国では共通であり，WHOでも同様に不妊症の定義を1年と見直している．

女性は35歳を超える頃から一気に妊孕能が低下する[1]．20～24歳の女性の妊孕率を100%として対比した，年齢層別の妊孕力の変化を**図1**に示す．加えて，年齢の上昇とともに流産率も上昇することから，分娩まで至る可能性はますます低下する．最近はわれわれの施設においても，不妊外来への女性側の初診時平均年齢は約36歳である．したがって，これまで周知の三大不妊原因（排卵因子・卵管因子・男性因子）に加え，この卵子の老化に伴う「年齢因子」による不妊症が占める割合は年々増

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Basic knowledge for modern ART
Hiroaki Shibahara, Toru Kato and Atsushi Fukui：兵庫医科大学産科婦人科学講座（〒663-8501 兵庫県西宮市武庫川町1-1）

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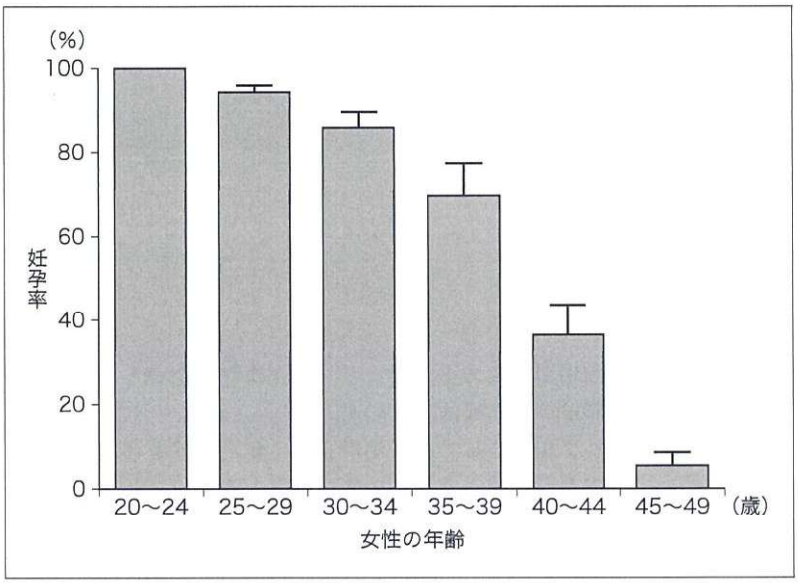


図1　女性の年齢による妊孕力の変化
(http://www.jsrm.or.jp/public/funinsho_qa18.html より引用改変)

加している.

このような晩婚化による不妊症の増加は，これまで社会的にはあまり認識されてこなかった．一方でこの間に，体外受精・胚移植（*in vitro* fertilization-embryo transfer：IVF-ET）や卵細胞質内精子注入法（intracytoplasmic sperm injection：ICSI，いわゆる顕微授精）などをはじめとする，生殖補助医療（assisted reproductive technology：ART）は進化・発展し，わが国における深刻な少子化への対策として大いに貢献している.

不妊症カップルへの診療の質的向上と社会からのサポート

不妊治療を受けるカップルは，できる限り最善の生殖医療を受けたい，なるべく少ない負担で治療を続けたい，とにかく早く結果を望みたいという希望をもつ．そのためには，専門職集団の努力と社会からのサポートが必要である.

生殖医療はチーム医療であり，従事する職種には医師（主として産婦人科医，泌尿器科医），コーディネーター（主として看護師），胚培養士，ならびにカウンセラー（心理，遺伝）がある.

日本生殖医学会は生殖医療の水準を向上させる目的で生殖医療従事者資格制度委員会を設け，生殖医療専門医，生殖医療コーディネーター，認定研修施設，研修連携施設を指定している．また日本卵子学会では，生殖補助医療胚培養士，ならびに管理胚培養士を指定している．これらの有資格者たちが，生殖医療の質的向上に大いに貢献してきた.

カウンセリングに関しては，日本生殖心理学会は生殖心理カウンセラー，生殖医療相談士，がん・生殖医療専門心理士を指定している．また，日本遺伝カウンセリング

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学会は臨床遺伝専門医，認定遺伝カウンセラーを指定している．不妊治療の過程において，これらのカウンセリングが有用な場面にしばしば遭遇する．

ところで，不妊症の診療には保険診療でまかなえない部分があり，これに対し各自治体では不妊治療費助成事業を行っている．具体的にはARTによる治療を受けたカップルに対して，上限を30万円とする特定不妊治療費の助成が行われている．男性不妊治療に対しても，精巣または精巣上体から精子を採取する手術を行った場合に，上限を15万円とする助成がある．所得制限や年齢制限があるものの，このような制度がなかった時代と比べ，前述のように少子化にあるわが国では，不妊治療を希望するカップルに対し，よりサポート体制は整備されてきたといえる．

ARTによる不妊症治療法の進歩

不妊症治療は，種々の検査結果に対する診断に基づく治療の提案を原則とする．ただし，カップルの年齢，卵巣予備能を反映する抗ミュラー管ホルモン（anti-Mullerian hormone：AMH）値，不妊期間，妊娠・分娩歴，合併症の有無，過去の不妊治療，家族計画の意向なども勘案し，標準的治療からの開始でよいか治療法選択のうえで，ARTを含め特別な配慮を要するかなどを個別に判断することが重要である．

ARTの適応

検査の結果から自然妊娠が可能な場合，経腟超音波所見などから排卵期を予想し，性交のタイミングを指導するタイミング療法から開始する．各不妊治療法は一般に3周期ごとを目途に，妊娠不成立の原因について検討し，次の治療法へステップアップするが，本稿では不妊治療の最終ステップであるARTの適応を中心に述べる．

日本産科婦人科学会による会告では，わが国におけるARTの適応は，それ以外に妊娠できる方法がないと判断されたカップルに限定している[2]．その場合，基本的にはIVFを実施するが，受精障害を伴う場合（主として男性因子，あるいは男性側の抗精子抗体による免疫性不妊など）にはICSIを選択する[2]．

以下にARTの4つの適応因子について解説する．

▶卵管因子

卵管因子の検査としては，卵管性不妊症の発症と関連が深いクラミジア抗体，および子宮卵管造影検査（hysterosalpingography：HSG）を行う．子宮頸管のクラミジア核酸増幅検査は偽陰性があるため，過去に治療歴のない血中クラミジア抗体陽性女性には，卵管病変の進行や将来の産道感染を予防するという観点から，カップルともに治療後，HSGを施行する[3]．

クラミジアの既往感染の有無にかかわらず，スクリーニング検査であるHSGで卵管閉塞や卵管周囲癒着を疑う場合，**図2**を参考に診断・治療を進める．二次検査としての経腟的腹腔鏡（transvaginal hydrolaparoscopy：THL）[4]，あるいは経腹的腹腔鏡検査の実施が可能な施設であれば，確定診断に基づき診療を行いうる．また経腹法であれば，診断と同時に卵管形成術や癒着剝離術も行うことができる．ただし高年齢女性などでは，可及的早期の妊娠・出産を考慮し，すみやかにIVF-ETを適用

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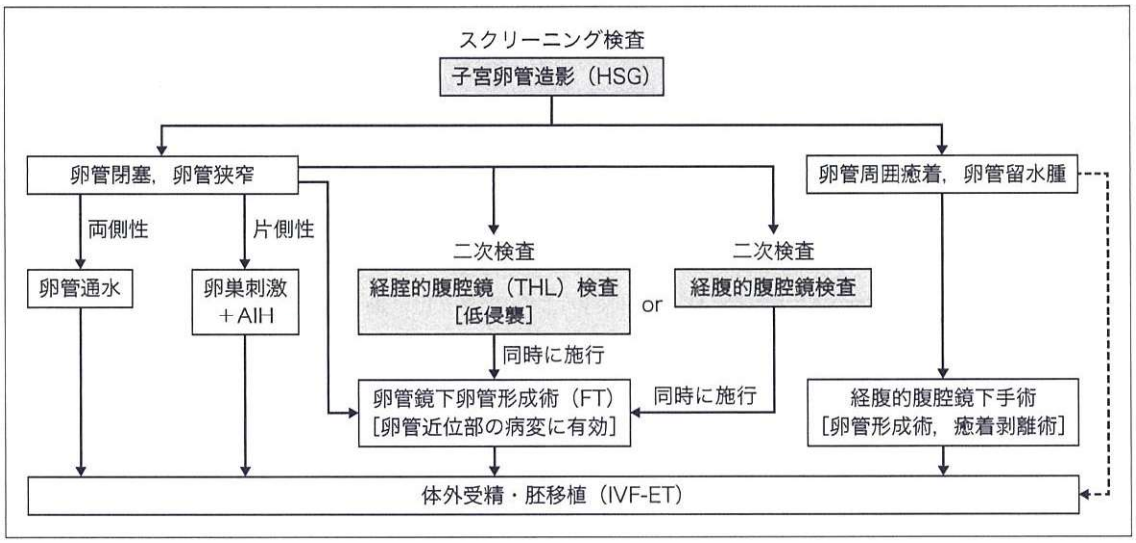


図2　卵管性不妊症の診療指針

することが多い．

特に卵管近位部での病変（卵管間質部や卵管峡部の閉塞や狭窄）に対しては，卵管鏡下卵管形成術（falloposcopic tuboplasty：FT）が有用な症例を経験するが[5]，術後妊娠に至らなければ，IVF-ETの適応とする．

一方，卵管遠位部での病変（卵管膨大部閉塞・狭窄や卵管采周囲癒着）ではクラミジア感染が多く，骨盤内観察のため腹腔鏡による診断や癒着剝離などの手術，あるいはIVF-ETの適用を考慮する．

▶男性因子

男性側の不妊症については，生殖医療に通じた泌尿器科医による診断・治療が原則である．男性因子の診断として，精液検査，ホルモン検査，精索静脈瘤に対する検査，造精関連遺伝子（azoospermia factor：AZF）の検査を行うが，本稿では割愛する．閉塞性無精子症には精路造影検査，非閉塞性無精子症には末梢血の染色体検査も考慮する．精巣生検は最近では診断法としてより，不妊治療法としてmicro TESEがICSIを前提に行われている．

薬物療法や手術療法で造精機能を改善しても妊娠に至らなければ，配偶者間人工授精（artificial insemination by husband：AIH）を行う．AIH不成功の場合はIVFまたはICSIの適応とする．

非閉塞性無精子症の男性には，AZFの検査結果により精子採取の可能性が高い場合，micro TESEにより精巣精子の採取を試みる．無精子症で，かつ精巣精子も獲得できない場合には，提供精子を用いる人工授精（artificial insemination by donor：AID）が行われる．ただし，凍結・融解精子を用いるAIDによる妊娠率は非常に低率であり，提供精子によるICSIの認可が待たれている[6]．

なお精液検査の際に，男性側の免疫因子の診断として抗精子抗体の検出を行う．男性では射出運動精子を用い直接イムノビーズテスト（direct-immunobead test：D-IBT）を行うが，IBTの販売停止により，現在は代替としてImmunoSpheres®を実

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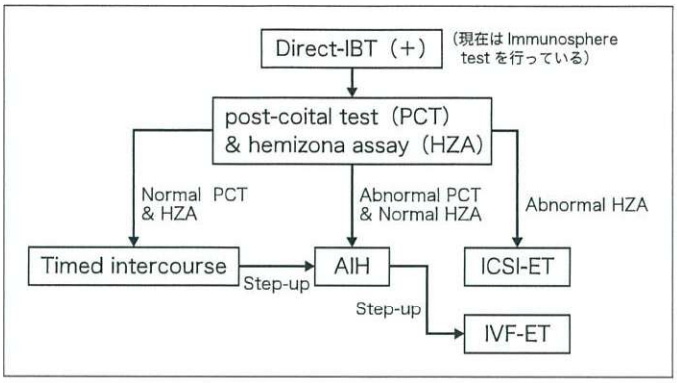


図 3　抗精子抗体保有不妊男性の診療指針
（Shibahara H, et al, 2005[7] より引用改変）

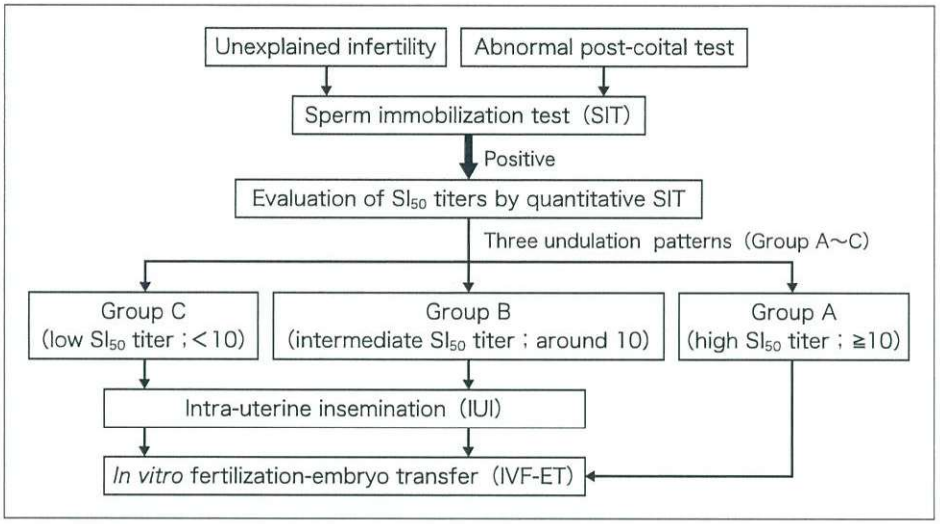


図 4　精子不動化抗体保有不妊女性の診療指針
（Shibahara H, et al, 2009[8] より引用改変）

施している．抗精子抗体が陽性の場合は，**図 3** に示すように精子の頸管粘液内通過能を性交後試験（post-coital test：PCT）で，受精能を hemizona assay（HZA）により検査し，治療方針を検討する．すなわち，精子結合抗体を有する男性には，精子の受精障害を認める場合には ICSI を，受精障害は認めないが頸管粘液内通過障害を認める場合には AIH を，受精障害も頸管粘液内通過障害も認めない場合にはタイミング療法を選択し，それぞれ治療を開始する[7]．

▶免疫因子（女性側）

女性側では血中の精子不動化抗体の有無を検査する．抗体が陽性の場合は治療方針を決定するため，定量的精子不動化抗体価である SI_{50} 値を測定する．

子宮頸管粘液中にも精子不動化抗体は分泌されるため，PCT 陰性，すなわち子宮頸管粘液内において精子の通過性が障害されることが多く，AIH の適応となる．ところが抗体は子宮～卵管内にも分泌され，また精子の運動障害のほか，受精障害も存在するため，**図 4** に示すように抗体価（SI_{50} 値）が高い症例では AIH による妊娠率

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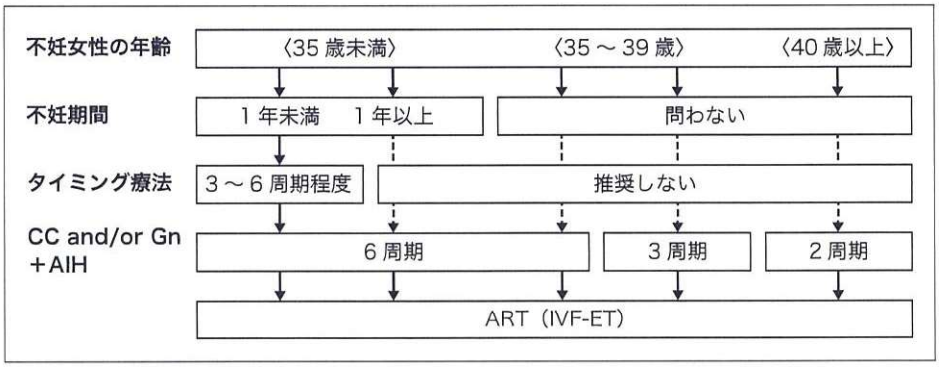


図 5　原因不明不妊の診療指針

は低く，IVF-ET の適応になる．基準として，SI_{50} 値が 10 未満の低抗体価の場合は AIH を，10 以上の高抗体価の場合は IVF-ET を選択する [8]．

▶原因不明

不妊期間が 1 年以上あり，不妊症に対する通常の一次検査を行っても明らかな異常を認めず，原因を特定できない場合を原因不明あるいは機能性不妊と呼ぶ．治療は一般にタイミング療法から開始し，卵巣刺激を併用する AIH，続いて IVF-ET の順にステップアップする．IVF-ET による妊娠率は最も高く，妊娠達成期間も短い．

特に 35 歳以上の高年齢女性の場合や，不妊期間が長い場合には，IVF-ET という選択肢について早期に提示する必要があるとされている [9]．年齢別の診療指針を**図 5** に示す．

一般的な ART のプロトコール

前述の適応に該当し，ART を希望するカップルに対しては，インフォームド・コンセントを十分に行い ART を実施する．

自然の排卵周期では，1 個の卵胞が成長し排卵に至る．したがって，ART の際に自然周期で採卵を計画すると，獲得できる卵子数はせいぜい 1 個である．その後に受精・胚発生の過程がすべて 100％上手くいくことは保証できず，自然排卵周期では胚移植まで至らない確率は決して低率ではない．そこで一般には，卵巣過剰刺激症候群（ovarian hyperstimulation syndrome：OHSS）の発症に注意しながら，ゴナドトロピン（Gn）製剤で卵巣刺激を行い，複数の卵胞発育を目標とする．

▶卵巣刺激法

不妊女性に月経 3 日目頃から Gn 製剤の注射を連日行うと，一般に複数の卵胞が発育する．ただし，採卵前にもし排卵してしまうと，採卵はキャンセルになってしまう．すなわち，Gn 製剤の投与だけでは premature LH surge が発生するために，Gn 製剤をスタートした女性の約 20％は採卵キャンセルに陥る．

その後に開発された GnRH アナログ製剤は，premature LH surge を抑制できる．ART の Gn 療法においても，GnRH アゴニストや GnRH アンタゴニストを併用以来，採卵キャンセルは非常にまれになった．なお，Gn 製剤には自己注射可能な recFSH があり，GnRH アンタゴニストも自己注射可能である．GnRH アゴニストは点鼻投与

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である．以上より，仕事をもつ女性にとってもコンプライアンスは非常に良好である．

▶採卵法

GnとGnRHアナログを併用し，経腟超音波で測定する首席卵胞径が17 mmを超えた段階で，抑制したLH surgeの替わりにhCG 5000 IUを投与し，その35～37時間後に採卵を行う．

採卵は静脈麻酔，あるいは腟円蓋への局所麻酔のいずれかで行う．経腟超音波ガイド下に19G程度の採卵針を用い，腟円蓋からダグラス窩または膀胱子宮窩を経由して左右の卵巣にアプローチし，順次卵胞を穿刺し卵胞液を吸引して卵子を回収する．

採卵室に隣接する培養室で，担当者（通常は胚培養士）が検卵を行い，培養液を満たしたディッシュ内で媒精までインキュベーター内に静置し，卵子を前培養する．

▶受精と胚移植

媒精法にはIVFとICSIがある．採卵当日に採取した精液を調整するか，あらかじめ凍結保存した精子を融解して媒精する．IVFではスイムアップ法などで得た通常5～10万個の運動性良好精子を用いる．一方，ICSIではマイクロマニピュレーターを用い，成熟卵子内に不動化した精子を1個注入する．

媒精の17～20時間後に雌性，雄性の2前核と，第2極体の放出を確認し，受精の有無を判定する．一般に新鮮胚（受精卵）移植は，胚の発育状態や形態観察を行いやすい採卵3日後の8細胞期前後，あるいは5日後の胚盤胞期に実施する．経腹または経腟超音波ガイド下に，1個ないし2個の胚を子宮腔内に移植する．この移植胚の数については，単胎妊娠と比較して母子のリスクが高い多胎妊娠の発生を極力予防するため，日本産科婦人科学会の会告に従い決定している．

▶胚凍結

前述の観点から，新鮮胚移植を行った以外の胚は凍結保存する．あるいは卵巣刺激の結果，OHSS発症のリスクが高いと判断した場合には，新鮮胚移植による妊娠成立はOHSSをさらに重症化することから，全胚凍結（freeze all）とすることがある．

胚の凍結法としては，従来はプログラムフリーザーを用い，耐凍剤を加え徐々に温度を低下させる緩慢凍結法を行っていたが，現在は一気に液体窒素に浸して急速冷却し，氷晶形成なく固化した状態にするガラス化法（vitrification）が主流である．

ARTの最近の話題

若年がん女性の妊孕性温存

これまでは若年者ががんに罹患した場合，手術や抗がん剤，あるいは放射線治療をできる限り早急に進め，生命予後の改善を最優先した．これらの集学的治療により生存率は著しく改善したが，性腺毒性の強い治療を選択した場合には妊孕性の低下や消失などを生じることから，がんサバイバーのQOLの低下が問題視されてきた．この“Oncofertility”と呼ぶ新しい領域では，わが国でも都道府県単位で「がん・生殖医療ネットワーク」が開設され，各地においてがん治療施設と生殖医療施設の連携が密接となった．

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若年者のがんの診断後には，このようなネットワークを通じ患者に妊孕性温存に関する情報提供を行う．希望があれば，すでに臨床の場で定着している受精卵の凍結に加え，未受精卵子や卵巣を凍結する技術も進歩し，実施可能である．その後，すみやかにがん治療に進むというシステムが構築されている．

なお，がん治療が終了し，主治医からの妊娠許可を得て保存した凍結受精卵を融解し，移植により妊娠・出産した例をわれわれも経験している[10]．

反復着床障害

前述のようにARTをめぐる治療技術は進化してきたが，良好な胚を移植し続けても，繰り返し着床が成立しない反復着床障害（repeated implantation failure：RIF）に陥る女性は一定数存在する．原因は胚側か子宮内膜側に求めることになるが，前者には移植前に胚の染色体異常の有無を検査する着床前染色体異数性検査（preimplantation genetic testing for aneuploidy：PGT-A）という手法があり，現在本邦でも日本産科婦人科学会がPGT-Aの有用性について，臨床研究を実施し検証している．

一方，後者の子宮内膜側の検査としては，慢性子宮内膜炎や乳酸菌の不足，あるいは胚受容能検査（endometrial receptivity assay：ERA）という遺伝子検査などが注目されているが，それらの有用性についてはまだ議論の余地がある状況にある．

ARTの今後の課題

本来不妊治療とは，当該夫婦間の遺伝子を継承する子孫を授かるために提供されるべきはずである．したがって，卵子がない，精子がない，子宮がないといういわゆる絶対的不妊のカップルは，かつては不妊治療を断念せざるを得なかった．この自然の摂理を，わが国における現状の不妊治療は踏襲している．

一方で，特別な技術を要することがない卵子提供，精子提供，代理懐胎という第三者に依存する不妊治療は容易に実施できることから，これらを容認する国が存在する．現在わが国の生殖医療の現場ではこれらを容認しておらず，自国でできないのなら高額な治療費を支払ってでも海外流出し，妊娠して帰国したり，代わりに産んでもらった子を連れ帰るカップルが相当数存在する．彼らと同等の苦悩に向き合ったことがない者が，彼らの行動を安易に批判することは決してあるべきではなく，このような状況がいつまで続くのか，いつまで続けなければならないのか，国として一定のルール作成，あるいはサポート体制の構築に真剣に取り組むべきであろう．

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SPECIAL ISSUE ARTICLE

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Pelvic endometriosis and natural killer cell immunity

Atsushi Fukui | Chuxian Mai | Shinichiro Saeki | Mayu Yamamoto | Ryu Takeyama | Toru Kato | Yuji Ukita | Yu Wakimoto | Ayano Yamaya | Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo College of Medicine, Hyogo, Japan

Correspondence
Atsushi Fukui, Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan.
Email: a.fukuipon@mac.com

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Abstract

Endometriosis is a chronic disease that commonly affects women in their reproductive age. It has been reported that the infertility due to endometriosis is largely caused by pelvic adhesion, oocyte damage, and so on. There are several causes of endometriosis including bacterial infections, immunological abnormalities, epigenetics, and aberrant DNA methylation. The natural killer (NK) cells present in the peritoneal fluid express CD16 and CD56. They also express NK cell inhibitory receptors and activating receptors and usually work to eliminate endometrial cells in the retrograde menstruation. However, in women with endometriosis, the changes in these receptors and production of cytokines by NK cells cause the onset and progression of endometriosis. In this review, we have focused on the role of NK cells in pelvic endometriosis and presented the immunological abnormalities in endometriosis including the possibility of future treatment.

KEYWORDS

endometriosis, IFN-γ, implantation failure, NK cell, NKp46, recurrent pregnancy loss

1 | INTRODUCTION

Endometriosis is a common disease that occurs in women of reproductive age. It is difficult to assess the exact prevalence of the disease as not all women suffering from endometriosis visit doctors. However, it has been suggested that 9% of the women of reproductive age suffer from endometriosis. It is also reported that 25% to 50% of the infertile women have endometriosis and 30% to 50% of the women with endometriosis have infertility.[1] Moreover, endometriosis has been related to recurrent pregnancy losses.[2,3] Main symptoms of endometriosis are dysmenorrhea, dyspareunia, pelvic pain, pain in defecation, and infertility. Various kinds of peritoneal lesions and deep endometriotic lesions are observed. Late marriages and delayed pregnancies are worldwide trends, especially in the developed countries.[4] Previously, fertility rates were high with women reporting multiple deliveries. For example, in Japan, total fertility rate in 1949 was 4.32. It has continued to decline since, slipping below 2.00 in 1975, and recorded at 1.42 in 2018 (Vital Statistics by Ministry of Health, Labour and Welfare, Japan). Besides, average age of women at their first delivery in 1950 was 26.4 years, which has continued to increase reaching 30.7 years in 2017 (Declining Birthrate White Paper by Cabinet Office, Japan, 2020). Therefore, contemporary women deliver their first babies at older ages compared with women in the past and the number of babies delivered by each woman is also comparatively low. As a result, contemporary women spend longer periods of time without gestation or lactation. So, the total number of menstruation cycles is higher for contemporary women. Therefore, nowadays, women have greater chances to be exposed to backflow of menstrual blood in peritoneal cavity with every cycle. Usually, it is considered that all women have some backflow of menstrual blood. However, not all of them develop pelvic endometriosis. There are several other factors like bacterial infections,[5] immunological factors,[6,7] epigenetics,[8,9] and mutations[10] that also play key roles in the development of endometriosis. One of the important causes of endometriosis is an immunological change in the immune cells in the peritoneal cavity. Usually, immunological cells such as the

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NK cells, T cells, and macrophages eliminate endometrial cells from backflow of menstrual blood. However, due to certain changes in the characteristics of these immune cells, the endometrial cells are able to attach themselves to the peritoneal cavity. In this review, we have focused on the immunological abnormalities resulting in endometriosis with special emphasis on the role of NK cells in pelvic endometriosis. We have also included the data from our study.

2 | ENDOMETRIOSIS AND INFERTILITY

Women with endometriosis have shown some mechanisms that indicate the relationship between endometriosis and infertility, though these claims remain to be proved. First, endometriosis causes adhesion in the peritoneal cavity around uterus and adnexa resulting in difficulties in oocyte release from the ovary and its passage to the fallopian tube. There are many reports suggesting that women with endometriosis have lower success rates of IVF-ET.[11,12] More importantly, many researchers have reported changes in the functions of immune cells in the peritoneal fluid and cytokine production such as IL-1,[13,14] IL-6,[14,15] IL-8,[14] IL-33,[13] TNF-α,[15-18] IFN-γ,[16,17] and VEGF[19,20] by immune cells as the cause of infertility. Changes in endometrial lymphocytes and endometrial receptivity have also been reported.[21-23] However, as for the oocyte donation cycle, women with moderate to severe endometriosis who received oocytes from women without endometriosis showed normal or better endometrial receptivity and pregnancy rates.[23,24] In contrast, when women with endometriosis became oocyte donors for women without endometriosis, implantation rates were lower and embryo quality was low.[23-25]

3 | ENDOMETRIOSIS AND ENDOMETRIAL RECEPTIVITY

Uterine endometrial receptivity in women with endometriosis is controversial. Abnormal endometrial receptivity for eutopic uterine endometrium in women with endometriosis may be related to the defects of decidualization and the change of endometrial morphology.[22,26,27] Recent studies have reported lower implantation rate in women with endometriosis.[28] They compared the clinical outcome of siblings (without endometriosis) oocyte donation cycle between menopausal women with/without endometriosis. They concluded that in oocyte donation cycles, the recipient's history of endometriosis might have a negative impact on implantation, pregnancy, and live birth rates.[28] In contrast, same observations have been reported regarding the implantation rates, pregnancy rates, and miscarriage rates between women with severe endometriosis and control women using oocyte donation cycle.[23,29,30] Diaz et al reported in their study that healthy oocyte donors were shared between R-ASRM stages III and IV of severe endometriosis women and healthy controls. Half of the donated oocytes were given to severe endometriosis patients, and the others were given to healthy control. They concluded the same implantation rate, pregnancy rate, and miscarriage rate between the two groups.[29] Gene expression of uterine endometrium was also compared between women with/ without endometriosis.[31] The endometrial receptivity analysis (ERA) can identify a personalized window of implantation and is especially used for infertile women with recurrent implantation failure in order to know the appropriate time of embryo transfer. The ERA test has evaluated expression of 238 genes critical for the uterine endometrial receptivity. Researchers found the same gene expression in the ERA test of endometriosis stages compared with controls. They concluded that endometrial receptivity gene signature during the implantation window did not vary among women with endometriosis even considering severity compared with that of healthy women.[31]

4 | ENDOMETRIOSIS AND IMMUNOLOGICAL ABNORMALITY

Thirty to fifty percent of women with endometriosis suffer from infertility.[1] Women with reproductive failure such as recurrent pregnancy loss (RPL) or implantation failure (IF) have also shown some immunological abnormalities. Studies on women with endometriosis have shown decreased NK cell activity especially in the peritoneal fluid and peripheral blood.[32] However, in women with RPL or IF, NK cell activity is usually increased. In these women, higher NK cell cytotoxicity,[33] lower percentage of NK cell inhibitory receptors,[34] higher percentage of NK cell activating receptors,[35] and type 1 shift in NK cells have been reported.[36] In contrast, in women with endometriosis, lower NK cell cytotoxicity, higher percentage of NK cell inhibitory receptor,[37] lower percentage of NK cell activating receptor, and shift away from type 1 to type 2 have been reported.[16] Though the immunological parameters are completely opposite, both result in infertility or reproductive failure.

5 | PHENOTYPES OF PERITONEAL FLUID NK CELLS

Representative dot plots of the endometrial NK cells, peripheral blood NK cells, and peritoneal fluid NK cells using CD16 and CD56 by flow cytometry have been shown in Figure 1. NK cells can be divided into $CD56^{dim}$ cells and $CD56^{bright}$ cells according to their fluorescence intensity. Main population of the peripheral blood NK cells consists of $CD16^{+}/CD56^{dim}$ cytotoxic cells and that of endometrial NK cells is made up of $CD16^{-}/CD56^{bright}$ cytokine-producing NK cells. Interestingly, peritoneal fluid NK cells consist of $CD16^{-}/CD56^{bright}$ cells, similar to that observed in endometrial NK cells.

We have reported NK cell immunological abnormalities in women with the history of RPL and/or IF. In infertile women with IVF failures, the percentage of cytotoxic uterine endometrial $CD16^{+}/CD56^{dim}$ and NK cell cytotoxicity was significantly higher compared

with women who conceived by following IVF cycle.[33] Some studies have also reported no change in the expression of $CD56^+$ NK cells in peritoneal fluid of women suffering from endometriosis.[16,38-41] In contrast, other reported lower expression of $CD56^+$ in peritoneal fluid NK cells.[42,43] Kang et al reported the percentage of CD3-/CD56+ peritoneal fluid NK cells in women with endometriosis was significantly decreased compared with that in control women.[42] These results are in line with lower cytotoxicity of peritoneal fluid NK cells in women with endometriosis.

6 | THE ROLE OF NK CELLS IN ENDOMETRIOSIS

Main function of conventional NK cell is to reject tumor cells or pathogenic microorganisms in the body. As mentioned above, there are some hypotheses for the origin and development of pelvic endometriosis. The oldest one of them is perhaps the Sampson's theory of retrograde menstruation from fallopian tubes to peritoneal cavity.[44] According to this theory, menstrual blood with detached endometrial cells from endometrium flows reverse through the fallopian tubes and endometrial cells attach themselves to the peritoneal cavity, usually at the ovarian fossa. Most of the women have retrograde menstruation, but all of them do not have endometriosis, suggesting that NK cells reject the endometrial cells. It has been reported that expression of a non-classic human leukocyte antigen, HLA-G, which binds to the killer immunoglobulin-like receptor (KIR) 2DL4 (CD158d) on natural killer (NK) cell was observed in eutopic endometrium only during the menstrual phase, and the HLA-G-expressing cells were detected in PF during the menstrual period.[45] Epithelial cells bearing HLA-G may enter the peritoneal cavity during retrograde menstruation, allowing the antigen to react locally with KIR2DL4. In addition, it has been reported that the heterotransplant of human endometrium was accepted 100% in natural killer cell activity-suppressed nude mice.[46] If the function of NK cells is compromised or changed, endometrial cells in the backflow blood may attach to the peritoneal cavity and develop.

7 | NK CELL CYTOTOXICITY AND ENDOMETRIOSIS

Many researchers have reported lower cytotoxicity of NK cells in the peritoneal fluid and peripheral blood.[32] Oosterlynck et al reported reduced NK cell activity using ^{51}Cr release K562 assay in the peritoneal fluid and peripheral blood NK cells in women with endometriosis.[47] They only showed the correlation between the severity of endometriosis and NK cell cytotoxicity in the peripheral blood. Since this report, it has been suggested that reduced NK cell cytotoxicity is involved in the development and progression of endometriosis (Figure 2A). Others have also reported lower

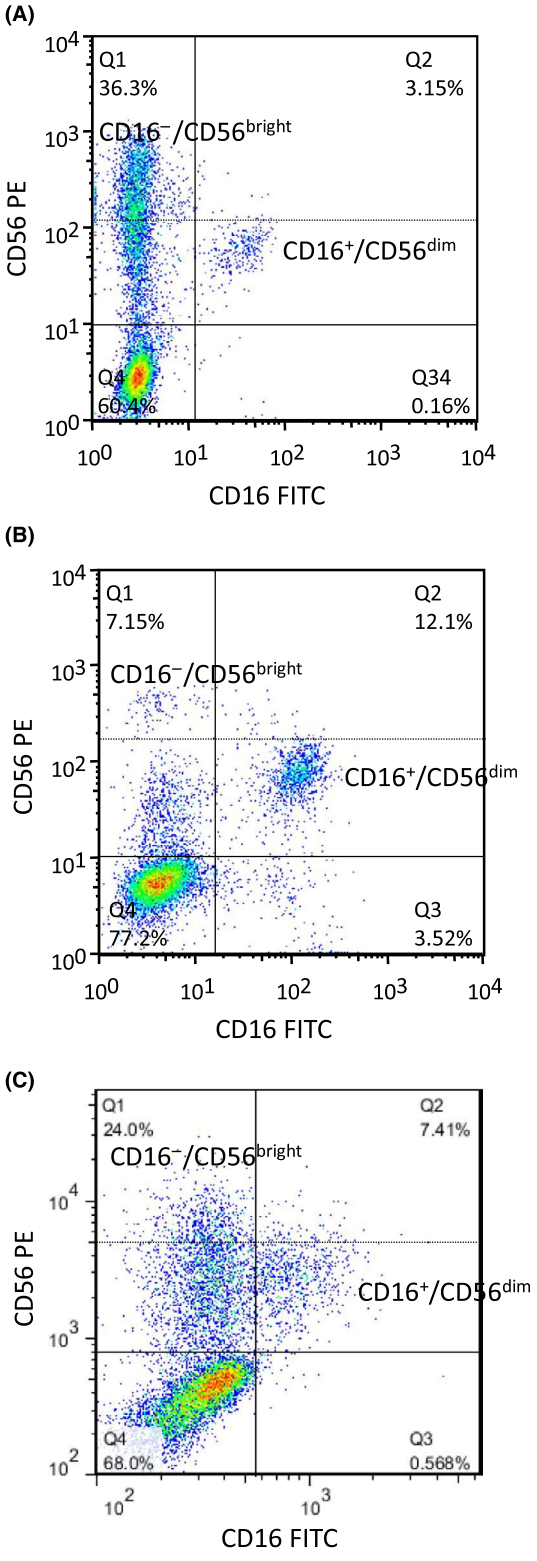


FIGURE 1 Representative dot plots of the endometrial NK cells, peripheral blood NK cells, and peritoneal fluid NK cells using CD16 and CD56 by flow cytometry. A gate is set on the lymphocytes. Lymphocytes are classified into $CD56^+$ and $CD56^-$ cells. $CD56^+$ cells are classified into $CD56^{bright}$ and $CD56^{dim}$ cells. Co-expression of CD56 with CD16 is shown. A: uterine endometrial NK cell, B: peripheral blood NK cell, C: peritoneal fluid NK cell

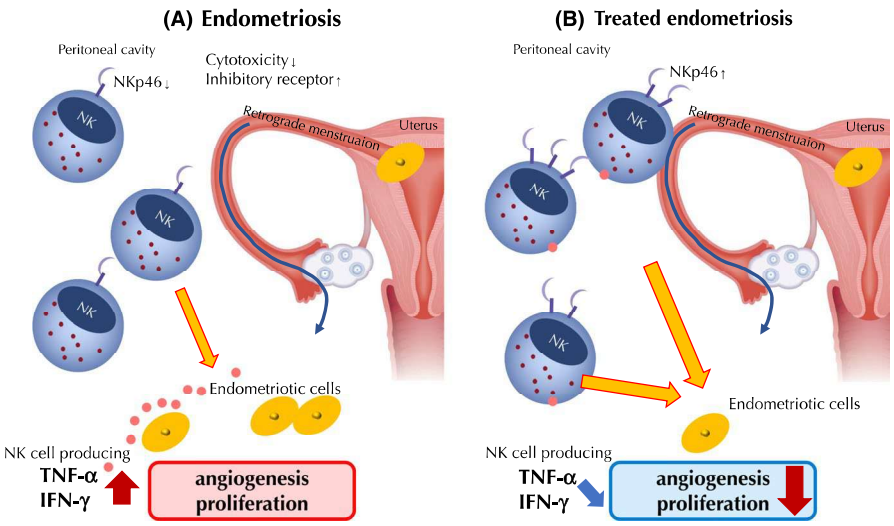


FIGURE 2 Participation of natural killer (NK) cell in peritoneal cavity for women with endometriosis. By the retrograde menstruation, endometrial cells go to peritoneal cavity. Due to lower NK cell cytotoxicity and higher expression of NK cell inhibitory receptors, endometrial cells can attach to the peritoneal cavity. Decreased expression of NKp46 induces abnormal cytokine production, allowing the progression of endometriosis. These NK cell abnormalities may be modified by the administration of some medicine such as OCP or Dienogest

NK cell cytotoxicity in the peritoneal fluid in endometriosis.[38,48] For NK cell activity of the peripheral blood, reports on their cytotoxic function have been controversial. Several authors reported a decrease in the NK cell cytotoxicity using K562 assay using the peripheral blood in endometriosis,[47,49,50] while others did not find any decrease of NK cell cytotoxicity.[38,48] Recently, it is reported that function of platelet on NK cell cytotoxicity is very important to development of endometriosis.[51] They reported platelet-derived transforming growth factor-beta1 (TGF-beta1) suppresses the expression of NKG2D on NK cells, resulting in reduced cytotoxicity in women with endometriosis. The reduced NK cell cytotoxicity was due to reduced IFN-γ production by NK cells, reduced expression of NK cell activating receptors NKG2D and NKp46, and increased expression of NK cell inhibitory receptor CD158a (KIR2DL1).

8 | NK CELL RECEPTORS AND ENDOMETRIOSIS

NK cells express various kinds of activating and inhibitory receptors. NK cell cytotoxicity is decided by the balance of these activating and inhibitory receptors. Namely, if a target cell binds to a NK cell's activating receptor, then the NK cell attacks the target cell. Conversely, if the target cell binds to the NK cell's inhibitory receptor, then the NK cell does not attack the target cell. If the target cell binds to both the activating receptor and the inhibitory receptors on the NK cell, then the response of the NK cell to the target cell will depend on the balance of the expression of the activating and inhibitory receptors on the surface of the NK cell. NK cells preferentially kill target cells with lower-than-normal expression of the major histocompatibility complex (MHC) class I proteins, as fewer inhibitory receptors engage ligands. This concept is known as the *missing-self hypothesis*.[52] In women with endometriosis, abnormal expression of various kinds of activating receptors and inhibitory receptors of the peritoneal fluid NK cells has been reported. For the activating receptors of the NK cells, reduced expression of natural cytotoxicity receptors,[16,53] NKG2D,[53] and CD107a[54] (LAMP-1) was described in women with endometriosis. Additionally, increased expression of NK cell inhibitory receptors such as CD158a (KIR2DL1),[37,55] CD158e1 (KIR3DL1),[55] and CD159a (NKG2A)[56] was reported.

9 | NK CELL INHIBITORY RECEPTORS AND ENDOMETRIOSIS

As mentioned earlier, an increase in the expression of the inhibitory NK cell receptor, KIR2DL1 (CD158a), in the peritoneal fluid is common in endometriosis.[37,57,58] This increase in the NK cell inhibitory receptors may show the suppression of NK cell cytotoxicity to endometrial cells, and this reduced NK cell activity may facilitate the implantation of endometrial cells.[59] They also showed the correlation between the expression of $CD158a^+$ NK cells and the R-ASRM score of pelvic endometriosis.[39] Some percentage of the peritoneal fluid $CD158a^+$ NK cells may have some local cytokine interaction that influences proliferation and migration of endometriotic cells.[60] Wu et al also showed the increase of the expression of CD158a and CD158e1 on the peritoneal fluid NK cells in endometriosis patients. Interestingly, the expression of CD158e1 on the peritoneal fluid NK cells was significantly higher in women with severe endometriosis compared with that in mild endometriosis. They suggested that large amount of retrograde menstruation induced more KIR (CD158a and CD158e1) activation, leading to increased impairment of NK cytotoxicity in the peritoneal cavity. As for the expression of NKG2A and NKG2C, the expression of NKG2A on the peritoneal fluid NK cell was significantly higher compared with that in the control; however, the expression of the activating NKG2C receptor on the peritoneal fluid NK cell was not affected.[56] They concluded that the inhibitory signals provided by the CD94/NKG2A receptor led to the escape of endometrial tissue from the NK cell–mediated immune surveillance, thus favoring the progression of the endometriosis.

10 | NATURAL CYTOTOXICITY RECEPTORS ON NK CELLS AND ENDOMETRIOSIS

Natural cytotoxicity receptor (NCR) that consists of NKp30, NKp44, and NKp46 is expressed exclusively on NK cells. Among them, NKp46 is a unique marker that regulate not only the NK cell cytotoxicity but also production of cytokines from the NK cells. NKp46 is one of the NK cell activating receptors, and it has been reported to recognize viral proteins such as hemagglutinin of the influenza virus or hemagglutinin-neuraminidase of the parainfluenza virus.[61,62] However, endogenic ligands recognized by NKp46 remain to be fully investigated. We reported the abnormal expression of NKp46 in women with reproductive failure such as RPL, IF, hypertensive disorder in pregnancy, and gestational diabetes mellitus. Namely, the expression of NKp46 on the uterine or peripheral blood NK cells was lower in non-pregnant women with RPL or IF compared with control.[63-65] Usually, it has been reported that NK cell cytotoxicity is higher in women with RPL or IF.[33] However, for the expression of NKp46, the expression of activating NKp46 was lower in women with RPL or IF. These results looked strange and to evaluate the expression of NKp46 in other reproductive failures, we performed some research. Our results suggested that the expression of NKp46 was lower in women with preeclampsia[66] and gestational diabetes mellitus.[67] The expression of NKp46 on the peritoneal fluid NK cells was significantly lower in endometriosis patients as compared to that in the healthy individuals (Figure 2A).[16] Furthermore, the expression of $NKp46^{+}/CD56^{dim}$ was significantly higher in women with endometriosis as compared to that in the control subjects, but that of $NKp46^{+}/CD56^{bright}$ was not. Moreover, a negative correlation between the percentages of $NKp46^{+}$ NK cells and the R-ASRM score of endometriosis was found. However, these differences were not seen in the peripheral blood or uterine endometrial NKp46 on NK cells in women with/without endometriosis. Peritoneal fluid is mainly from ovarian exudate, furthermore from developing follicles and corpus luteum. It is reported that in women with endometriosis the volume of peritoneal fluid is slightly increased compared to healthy women without endometriosis.[68] Recently, it is reported that there is a new population of NK cells that reside in the normal, un-inflamed peritoneal cavity.[69] They are similar to immature splenic NK cells and have function to produce IFN-γ, TNF-α, GM-CSF, and so on. Xu et al reported the same condition in peripheral blood from women with endometriosis.[53] They also reported the higher expression of NKp46 on the $CD16^{+}$ peritoneal fluid NK cells and lower expression of NKp30 and NKG2D on the $CD56^{+}$ peritoneal fluid NK cells.[53] Usually, main population of the $CD16^{+}$ NK cells are $CD56^{dim}$ cells, so their findings were consistent with our results. Besides, there was no significant difference between the expression of NKp30 or NKp44 on NK cells in the peripheral blood, endometrium, and peritoneal fluid with/without endometriosis.[16] For the uterine NK cells, the expression of NKp46 on NK cells in the uterine endometrium from women with pelvic endometriosis has not been clarified. We investigated the difference of expression of NKp46 on NK cells in the uterine endometrium with/without endometriosis and found that there were no significant differences in the percentage of $CD16^{+}/CD56^{dim}$ cells and $CD56^{+}/NKp46^{+}$ cells in NK cells with/without endometriosis.[70] Interestingly, in women with RPL, the expression of NKp46 was lower in women coupled with endometriosis than those without endometriosis.[71] We have reported that NKp46 may regulate cytokine production such as at least IFN-γ, TNF-α, IL-4, IL-10, and TGF-β by endometrial NK cells.[72] Thus, the association between NKp46 and reproductive failure may relate to the increased cytotoxic activity and dysregulation of cytokine production following engagement of NKp46 on NK cells.[72]

11 | NK CELL CYTOKINES PRODUCTION FOR THE DEVELOPMENT OF ENDOMETRIOSIS LESION

There are many reports that show the significant changes of peritoneal fluid cytokines in women with endometriosis. Podgaec et al reported higher levels of IFN-γ and IL-10 in women with endometriosis compared with those in normal women.[73] Interestingly, they also reported lower IFN-γ/IL-4 and IFN-γ/IL-10 ratio, that is lower Th1/Th2 ratio in the peritoneal fluid in women with endometriosis. They showed type 2 shift of the peritoneal fluid cytokine concentration in women with endometriosis. These results are very interesting, because they show that women with PRL or infertility have type 1 shift and type 1 cytokines attack their fetuses.

Kang et al reported the increased level of IL-6 in the peritoneal fluid in women with endometriosis.[42] They stated that the increase of IL-6 in peritoneal fluid weakened the cytotoxic activity of NK cells via the regulation of the expression of the Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2).

We evaluated the percentages of NK cells producing IL-4, IL-10, GM-CSF, IFN-γ, and TNF-α in women with/without endometriosis. There were no differences for the percentages of peritoneal fluid NK cells producing IL-4, IL-10, or GM-CSF in these women. However, IFN-γ- and TNF-α-producing NK cells were significantly higher in women with severe (R-ASRM stages III or IV) endometriosis compared with those in the control.[16] Also, a positive correlation between the percentages of IFN-γ- and TNF-α-producing NK cells and the R-ASRM score of endometriosis was found. TNF-α[74] and IFN-γ[64,75] are related to inflammation and angiogenesis. TNF-α is also related proliferation of endometriotic stromal cells in women with endometriosis, and its concentration in PF was increased in women with endometriosis.[76,77] IFN-γ has been also reported to have an essential role in implantation especially in vascular remodeling process. NK cell-derived IFN-γ modifies the expression of genes in the uterine vasculature and stroma, which initiates vessels instability and facilitates pregnancy-induced remodeling of decidual arteries.[78] It is reported the mRNA expression of IFN-γ is significantly higher in endometriotic lesion compared with eutopic endometrium.[79] Therefore, it seemed that angiogenesis of endometriotic cells was promoted by increase of TNF-α- and IFN-γ-producing peritoneal

fluid NK cells. We concluded that proliferation and angiogenesis of the endometriotic cells might be promoted as a result of higher IFN-γ or TNF-α environment in the abdominal cavity (Figure 2A).

12 | MODIFICATION OF NK CELL ABNORMALITIES IN WOMEN WITH ENDOMETRIOSIS

We have previously reported the lower expression of NKp46 on the peritoneal fluid NK cells and its possible correlation with the increase of IFN-γ- and TNF-α-producing NK cells in the peritoneal fluid of endometriosis patients.[16] We tried to investigate the alterations of the expression of NCRs of endometriosis patients by treatment of some medicine such as oral contraceptive pills (OCP) or dienogest.[80] After the administration of OCP or dienogest for at least 3 months, the expression of NKp46 on the peritoneal fluid NK cells increased compared with that in the control. Besides, the percentage of IFN-γ- and TNF-α-producing NK cells was decreased compared with that in the control.

It was suggested that cytokine production of NK cells in the peritoneal fluid in women suffering with endometriosis might be changed by dienogest or OCP because of the change of NCRs (Figure 2B).[80] Recently, we have reported the role of IL-1/IL-33 signaling in the onset and development of endometriosis.[13] In the absence of IL-33 or IL-1R1, endometriotic lesions were reduced. The same results were obtained by the administration of inhibitor against IL-1R associated kinase 4 (IRAK4) orally. Moreover, IRAK4 inhibitor worked in the prevention of endometriosis progression. Our investigation showed that IL-1/IL-33 and associated downstream signaling molecules such as IRAK4 were involved in the pathogenesis of endometriosis and these might provide a novel treatment for endometriosis.

13 | CONCLUSIONS

Endometriosis is a common disease that occurs in reproductive age women and one of the major courses of infertility. It is controversial, but the uterine endometrial receptivity may not vary between with/without endometriosis for the clinical outcome and the gene expression using ERA. The expression of NKp46, one of the activating receptors, on the peritoneal fluid NK cells was significantly lower, and the expression of CD158a, one of the inhibitory receptors, was significantly higher in women with severe endometriosis. Moreover, a positive correlation between the percentage and CD158a on peritoneal fluid NK cells and a negative correlation between the percentage and NKp46 on peritoneal fluid NK cells are found. Also, a positive correlation between the percentages of IFN-γ- and TNF-α-producing NK cells and the R-ASRM score of endometriosis was found. Taken together, abnormal expressions of NK cell receptors and cytokines production by NK cells are involved in the development of pelvic endometriosis.

For the treatment of endometriosis, oral contraceptive pill and dienogest may become useful treatment to normalize the NK cell abnormalities of endometriosis. Further analysis is needed to clarify the roles of NK cells in women with endometriosis, and new forms of treatment such as IRAK4 inhibitor should be developed.

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ORCID

Atsushi Fukui https://orcid.org/0000-0002-9059-7458
Yu Wakimoto https://orcid.org/0000-0001-8989-6998
Hiroaki Shibahara https://orcid.org/0000-0001-7966-3170

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第3章　生殖内分泌分野

10. 抗精子抗体検査

兵庫医科大学産科婦人科学講座　脇本 裕　脇本 剛　柴原浩章

検査の目的とポイント

- 女性側では患者血清を用いて精子不動化試験(sperm immobilization test：SIT)により精子不動化抗体を，男性側では患者精子を用いてイムノビーズテスト(immunobead test：IBT)により抗精子抗体を検出する．
- SITは抗体を検出する定性試験であるのに対して，SI_{50}値の測定は抗体価を測定する定量試験である．
- SI_{50}値を測定することでSIT陽性不妊女性の診療指針が決定できる．

検査対象(適応)

不妊症の原因所在は，女性が約65%，男性が約50%，(重複は約25%)で，原因不明不妊のカップルは約10%強である[1]．抗精子抗体の1つである精子不動化抗体の検出頻度は原因不明不妊女性の5～10%である．また，男性不妊症の原因は，造精機能障害が約80%，精子輸送路の通過障害が約10%，精子機能障害及び原因不明が約10%である[1]．そして，精子機能障害の原因の1つに抗精子抗体がある．

精子抗原には多様性があり，それに対する抗精子抗体も多様である．表1に示すように抗精子抗体の検出方法は多数あるが，これらの方法で検出される抗体すべてが不妊症の原因になるわけではない[2]．抗精子抗体による不妊症の頻度は高いわけではなく，不妊検査の初期スクリーニングとして採用しない施設もある．しかし，近年は高年齢不妊女性が増加しており，特に生殖補助医療(assisted reproductive technology：ART)の適応がある精子不動化抗体の検出は治療期間の短縮・迅速化の観点から，不妊治療を受ける全女性患者を検査対象として，不妊治療開始後早期の実施が望ましい．本稿では，精子不動化抗体を検出する方法である精子不動化試験(sperm immobilization test：SIT)，その抗体価であるSI_{50}値の測定方法，および精子結合抗体を検出する方法である直接イムノビーズテスト(direct-immunobead test：D-

表1　抗精子抗体の検査法

1. 生物学的活性を検出する方法
   1) 精子凝集試験
   2) 精子不動化試験
   3) 受精阻害試験
2. 精子結合抗体を検出する方法
   1) immunobeads テスト
   2) mixed antiglobulin(MAR)test
   3) panning test
3. 精子またはその抽出抗原に反応する抗体の検出法
   1) 間接蛍光抗体法
   2) ELISA 法
   3) radio labeled antiglobulin テスト
   4) fluorescent-activated cell sorter(FACS)
   5) 精子抗原感作血球凝集反応

〔文献2より〕

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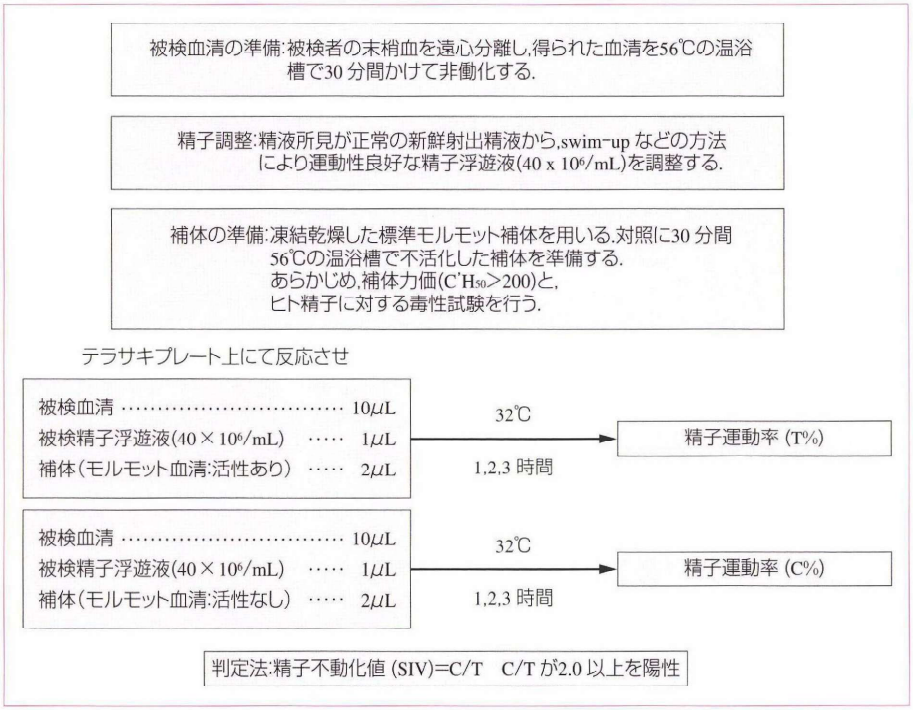


図1 精子不動化試験の方法

IBT)を，図示するとともに解説する．

検査方法

1. SITの測定方法[3)]

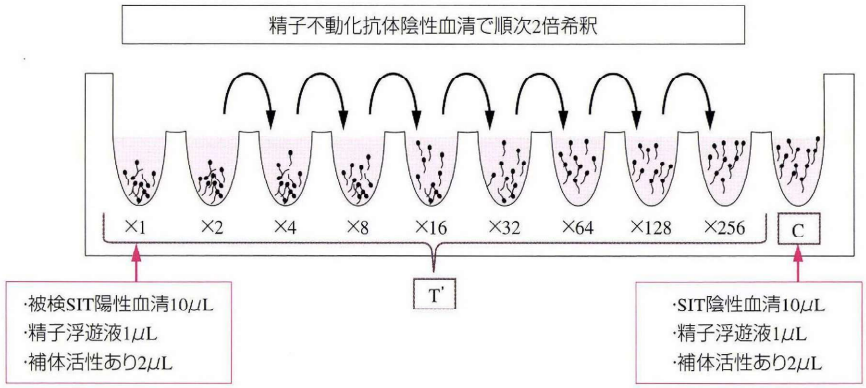
図1に図示したように，非働化した被検血清10 μL，精子濃度40×10⁶/mLに調整した精子浮遊液1 μL，および補体2 μL(対照には不活化補体を用いる)をテラサキプレート上(Greiner Bio-one，Frickenhausen，Germany)の各ウェル内で反応させる．32℃の条件下に1，2，3時間反応後の各ウェル内の精子運動率から，活性を有する補体の存在下での精子運動率をT%，不活化した補体の存在下での精子運動率をC%とし，その比(C/T)を精子不動化値(sperm immobilization value：SIV)値として算出する．

2. SI_{50}値の測定方法[4)~6)]

非働化したSIT陽性の被検血清を，図2に図示しているように非働化した精子不動化抗体陰性の血清で順次2倍希釈する．各希釈血清10 μL，精子濃度40×10⁶/mLに調整した精子浮遊液1 μL，および補体2 μLを，テラサキプレート上の各ウェル内で反応させ，32℃の条件下に1時間後に顕微鏡下に精子運動率を測定する．精子運動性が50%回復する血清の希釈倍率をSI_{50}値と定義する．すなわち，各ウェル内精子運動率から精子不動化活性(Y)をY=(C−T')/C×100を求め，図3のように精子不動化活性が50%と交差する血清希釈倍数がSI_{50}値と定義される．

3. D-IBTの測定方法

図4に記載しているように，ビーズ及び精液を準備する．スライドグラス上で準備した精子懸濁液5 μLと準備した抗IgAビーズをピペットの先端で混和した後，カバースライドを載せ

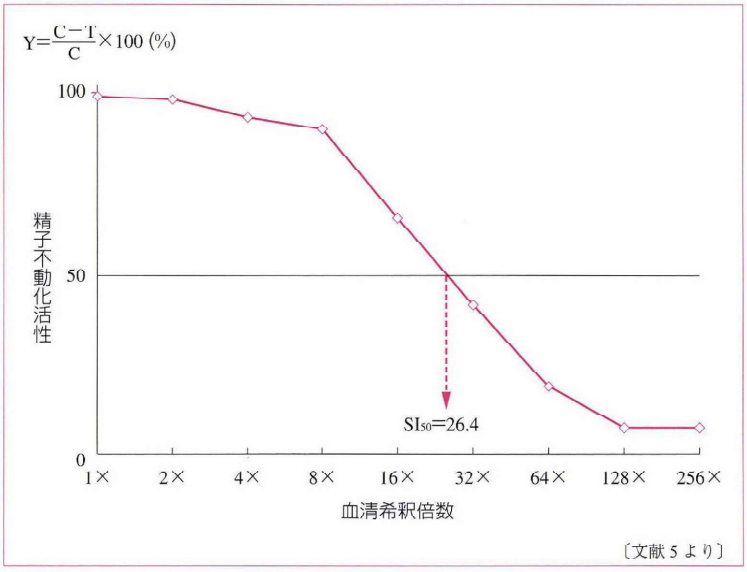


1. SIT陽性の被検患者と希釈及び対照に用いるSIT陰性女性の末梢血を遠心分離した後,血清を30分間56℃の温浴槽で30分間かけて非働化する.
2. 非働化したSIT陽性の被検血清を,非働化した精子不動化抗体陰性の血清で,順次2倍希釈する.
3. 各希釈血清10μL,精子浮遊液1μL,および補体2μL,をテラサキプレートの各ウェル内で反応させ,32℃の条件下に1時間後に顕微鏡下に精子運動率を測定する.

T':被検SIT陽性血清における精子運動率,C: SIT陰性血清における精子運動率

〔文献6より〕

図2 SI_{50}値の測定方法



〔文献5より〕

図3 SI_{50}値の求め方

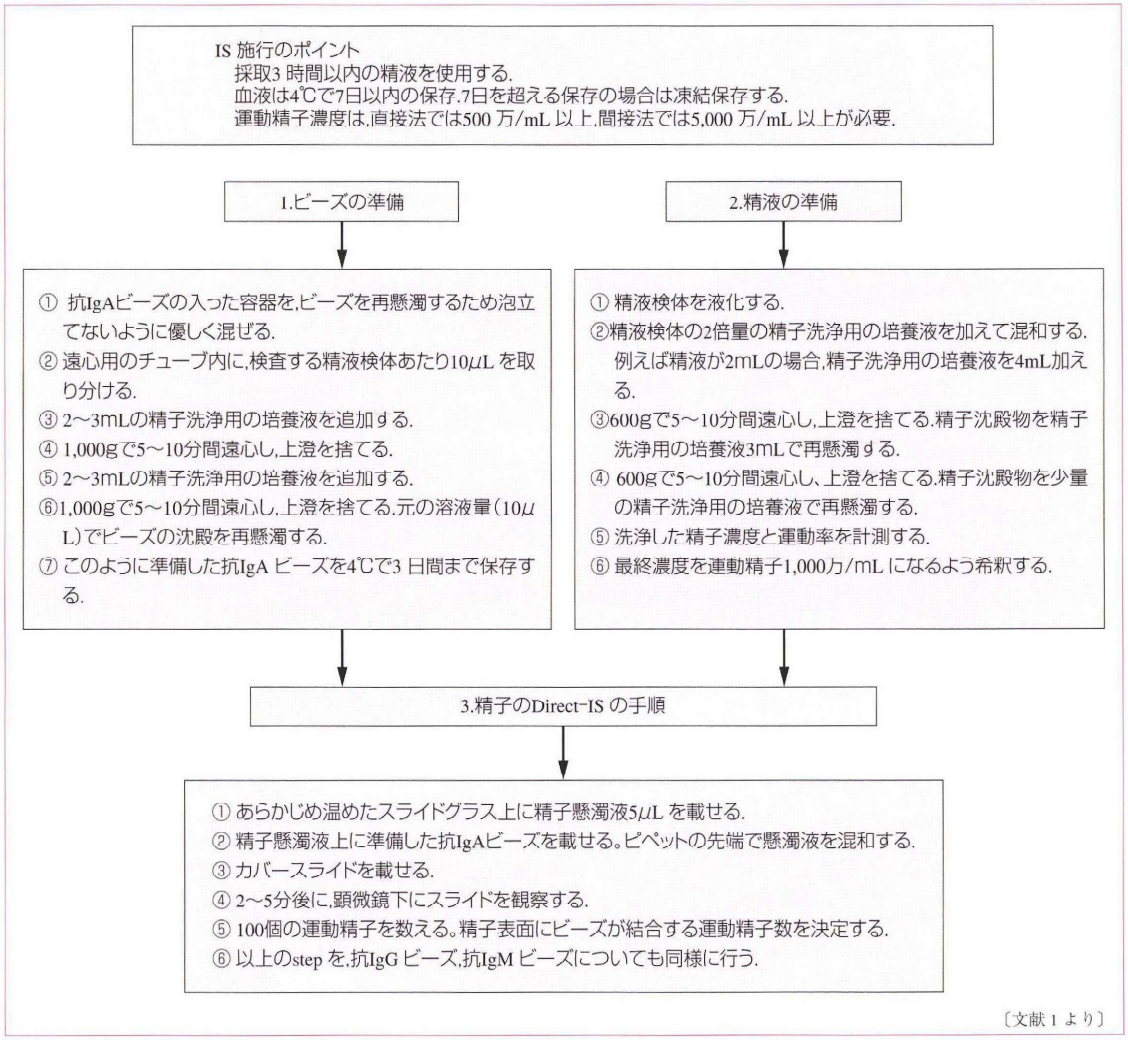


図 4 ImmunoSpheres®(IS)の実施法

る．2～5 分後に，顕微鏡下にスライドを観察し，100 個の運動精子を数え，精子表面にビーズが結合する運動精子数を決定する．抗 IgG ビーズ，抗IgM ビーズについても同様に行う[1]．

検査結果の読み方，評価

1. SIT の評価

SIT は患者血清中の精子不動化抗体とモルモット補体により精子の運動性が低下する現象をもとに，抗体の存在を検出する試験である．すなわち，活性を有する補体の存在下での精子運動率を T%，不活化した補体の存在下での精子運動率を C% とし，その比(C/T)を SIV 値として算出し，2 時間の測定中に SIV 値が 2.0 以上を示した場合を SIT 陽性と判定する[3]．したがって，精子運動率が対照と比較して 1/2 に低下した場合を抗体陽性(SIV=2)と判定する定性試験であると解される．しかし，T が 0 の場合には SIV=∞になる．そこで，定性試験で抗体陽性と判定した場合には抗体量を表現するために，定量試験である SI_{50}値を測定する．

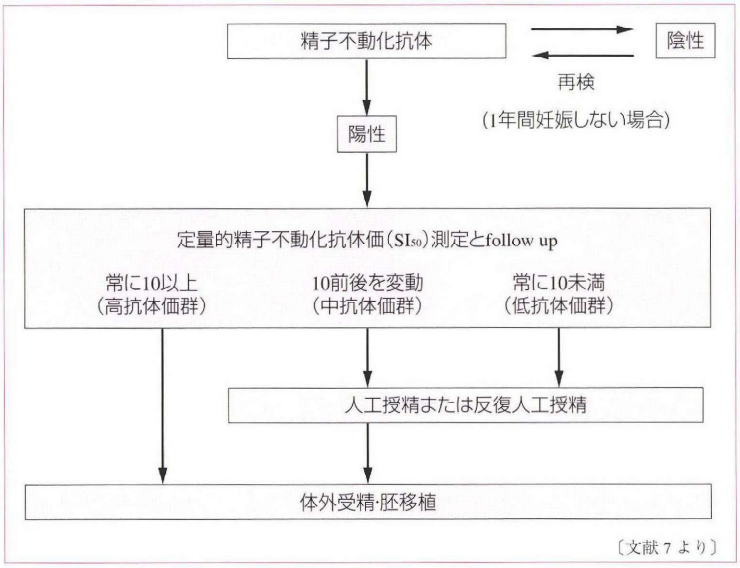


図5　精子不動化抗体陽性不妊女性の診療指針

2. SI_{50}値の評価

図5に精子不動化抗体陽性不妊女性の診療指針を示した[7]．SI_{50}値は自然変動するため定期的に測定する必要がある．そして，常に10以上を推移する高抗体価群の場合は，人工授精(intrauterine insemination：IUI)までの治療では妊娠は困難である．したがって，体外受精・胚移植(*in vitro* fertilization and embryo transfer：IVF・ET)の適応である．10前後を変動する中抗体価群と，常に10未満を推移する低抗体価群の場合は，IUIまたは反復IUIによる治療で妊娠が期待できるが，妊娠成立しない場合にはIVF・ETを選択する．

3. D-IBTの評価法

D-IBTは射出運動精子細胞膜上に付着している抗精子抗体の検出法として，繁用されてきたが[8]，この検査キットは，現在のところ製造中止に伴い入手不能である．そこで，IBTと同じ原理で，高い相関性が報告されている抗精子抗体検出法のイムノスフェア(Immuno-Spheres®：IS)(Bioscreen Inc.)を用いる[9]．基準値について，WHOマニュアル2010年ではイムノビーズを1個でも結合する精子が全体の50%以上存在すれば，抗精子抗体陽性と判定している[10]．ただし，2012年にKoriyamaらは，postcoital test(PCT)の分析結果から，カットオフ値は20%とする方が妥当であると報告している[11]．また，Shibaharaらは，D-IBT陽性不妊男性において，運動精子へのimmunobeads(IBs)結合率が80%以上は80%未満と比較して有意に受精障害を発症することを報告している[12]．

検査に伴う合併症と対処

SITとSI_{50}値の測定は不妊女性患者の血清を，D-IBTは不妊女性患者のパートナーの精子を用いた検査である．採血及び精子の採取が患者に負担となるが，一般的に行われている医療行為である．採血に伴うリスクとして神経損傷や皮下出血などがあるが，頻度は少なく軽症で自然軽快する．

保険適用と留意点

SIT, SI_{50}値の測定, および D-IBT は不妊治療において1次スクリーニング検査には推奨されておらず, これらの検査に保険適用はなく自費診療になる. また, 一般の検査機関で測定されているのは SIT, SI_{50}値の測定であり, D-IBT は新鮮精子を用いるため自施設で検査する必要がある.

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不妊症・不育症患者におけるNK細胞の機能発現異常と機能分担異常

福井淳史* 竹山龍 山本真由 麦楚娴
浮田美里 杉山由希子 浮田祐司 加藤徹
脇本裕 柴原浩章

兵庫医科大学　産科婦人科学

Role of NK Cells in Women with Recurrent Pregnancy Loss and Implantation Failure

Atsushi Fukui*, Ryu Takeyama, Mayu Yamamoto, Chuxian Mai, Misato Ukita, Yukiko Sugiyama, Yuji Ukita, Toru Kato, Yu Wakimoto, Hiroaki Shibahara (*Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, HYOGO 663-8501, JAPAN*)

子宮NK細胞は黄体期から妊娠初期にかけて増加し，妊娠初期には子宮内リンパ球の約70％を占める．それらを適切に制御することは，妊娠の成立・維持において非常に重要なことであると考えられる．一方，子宮，末梢血におけるNK細胞の異常は不妊症や不育症を引き起こすばかりでなく，妊娠高血圧症候群などの周産期合併症の発症にも関与している．

我々は，一貫して不育症や着床不全などの生殖異常におけるNK細胞の関与についての検討を行い，体外受精・胚移植直前周期における子宮内膜NK細胞の状態がその後の妊娠成績に影響を与えること，不育症あるいは着床不全においてNK細胞に発現するNatural Cytotoxicity Receptor（NCR）が変化すること，NK細胞産生サイトカインが変化すること，NCR発現とNK細胞産生サイトカインとの間には関連性があること，NK細胞のサイトカイン制御にNK22細胞が関与する可能性があることなどより妊娠の成立および維持において免疫機構，とりわけNK細胞の機能分担と機能発現の適正化が重要であることを示してきた．本稿ではこれらについて概説する．

Key words：NK細胞，不育症，着床不全，子宮内膜

Main population of midsecretory uterine endometrial lymphocytes and early pregnant decidual lymphocytes is natural killer (NK) cells. So, it is convinced that NK cells have important function for the achievement and maintenance of pregnancy.

We have evaluated the expression of surface markers of NK cells and cytokines production by NK cells. Higher percentage of cytotoxic NK cells both endometrium and peripheral blood causes abortion in IVF-ET program. NKp46 is a unique marker that functions in NK cell cytotoxicity and cytokine production. Interestingly, NKp46^{+} NK cells were lower in women with recurrent miscarriage or implantation failure. We have also shown the abnormal cytokines production in those situations. Moreover, we have shown that the NKp46^{bright}/CD56^{bright} NK cells are cytokines such as IFN-γ producing NK cells. Besides, the percentage of NKp46^{+} NK cells negatively correlated with that of NK22 cells, and the percentage of NK22 cells was negatively correlated with IFN-γ or TNF-α producing NK cells. We believe NK22 cell may be the candidate of regulator of NK cell.

In conclusion, abnormal NK cell may exist in women with reproductive failures.

Key words：NK cell, Recurrent pregnancy loss, Implantation failure, Natural cytotoxicity receptor, endometrium

*Corresponding author：福井淳史　兵庫医科大学　産科婦人科学

はじめに

厚生労働省不育症研究班によれば，不育症の原因は，不育症患者の40％で特定されるに過ぎず，残りの60％は不育症の原因が判然としないリスク因子不明不育症と診断される．リスク因子不明不育症に対する根本的な治療法はなく，その治療には苦慮することが多い．一方，リスク因子不明不育症の中には，Natural Killer(NK)細胞の異常，すなわち免疫学的異常を有するものが含まれていると考えられているものの一定の結論が得られていない．

さて，遺伝学的に非自己である受精卵が子宮内膜に着床し成長して行くためには，その受精卵の受容を可能とする精妙な免疫機能の調整機構が介在し，さらに胎芽の拒絶を阻止しその発育を保証できる機構の存在が必要である．細胞傷害性リンパ球の一種であるNK細胞は，末梢血のみならず子宮内膜にも存在し，子宮内膜における免疫機構の維持に重要な機能を果たしていることが示されている．NK細胞の機能異常は不妊や流産を引き起こすと考えられてきており，子宮内膜，脱落膜，そして末梢血のNK細胞の絶対数の異常および構成比率の異常，あるいは表面抗原発現異常やサイトカイン産生異常についての検討がなされ，妊娠の成立，維持における免疫系，特にNK細胞の関与はほぼ確立された概念と考えてよい．我々は着床機構，妊娠維持機構におけるNK細胞の機能分担と機能発現をより明確にし，流産の原因としての免疫機構の異常を明らかにすることとその正常化を目的として検討を行ってきた．

妊娠の成立と維持におけるNK細胞分布

妊娠成立の場である子宮において，子宮内膜および脱落膜には，子宮NK細胞が存在する．正常の絨毛は組織学的に合胞体栄養膜細胞(syncytiotrophoblast)と細胞性栄養膜細胞(cytotrophoblast)とに分類される．合胞体栄養膜細胞および細胞性栄養膜細胞は，自己・非自己の認識を行う通常のクラスⅠ(HLA-A, HLA-B)およびクラスⅡ(HLA-DP, HLA-DR, HLA-DQ)の主要組織適合抗原(MHC)を発現しない．このため，これらは細胞傷害性T細胞による攻撃から保護されている．しかしHLA(MHCクラスⅠ)を発現しないがために細胞傷害性を持つNK細胞による攻撃を受けることになってしまう．一方，合胞体栄養膜細胞と細胞性栄養膜細胞は非常に多型の少ないクラスⅠのMHCであるHLA-C，HLA-E，HLA-Gを発現している[1]．HLA-Gは細胞表面MHCクラスⅠ分子として働き，母体MHC非依存性のNK細胞の攻撃から，細胞性栄養膜細胞におけるNK細胞傷害感受性を減弱させ細胞性栄養膜細胞を保護している．またHLA-EはHLA-Gとの特異な相互作用により胎盤においてNK活性の制御にかかわっていると考えられている．このように合胞体栄養膜細胞と細胞性栄養膜細胞は母体の免疫機構からの保護作用機構を有しているが，それでも絨毛間腔では合胞体栄養膜細胞と細胞性栄養膜細胞は母体の末梢血NK細胞と直接的に接しており，HLA-Gの発現が末梢血NK細胞による傷害からこれらの細胞を完全に保護しうるというのは難しく，末梢血NK細胞からの作用を無視することはできない．よって妊娠中は子宮NK細胞のみならず末梢血NK細胞の制御も非常に重要であると考えられる．

さてNK細胞はその細胞表面に発現するCD56の発現強度によりCD56^{bright}細胞とCD56^{dim}細胞とに分類することができる．NK細胞の10％を占めるCD56^{bright}細胞は，子宮NK細胞の主構成成分であり，主としてサイトカイン産生に働く．またNK細胞の90％を占めるCD56^{dim}細胞は，末梢血NK細胞の主構成成分であり，主として細胞傷害性に働く．さらにこれらのNK細胞はその表面にNK細胞を活性化したり，抑制したりする種々の受容体を発現している．この受容体はNK細胞の細胞傷害性発現やサイトカイン産生において，さまざまな機能を有する．

NK細胞の妊娠の成立・維持への関与

NK細胞の妊娠の成立・維持への関与を明らかにするため，体外受精・胚移植(IVF-ET)を施行した症例のNK細胞の動態を検討した．胚移植時の末梢血CD16^{+}/CD56^{+}NK細胞は，着床成立群に比して着床不成立群で有意に高値であり，末梢血NK細胞活性は，着床成立群では，胚移植時のNK細胞活性がIVF-ET施行直前周期黄体中期のそれに比して有意に低値であった．一方，着床不成立群では採取時期による差を認めなかった[2]．さらに着床成立群を分娩群と流産群とにわけ検討すると，胚移植時のNK細胞活性は，分娩群に比して流産群で有意に高

値であった[2]．またIVF-ET施行前周期黄体中期子宮内膜CD16^{-}/CD56^{bright}細胞は，分娩群に比して流産群で有意に低値であり，さらにCD16^{+}/CD56^{dim}細胞は，分娩群に比して流産群で有意に高値であった[2]．以上，IVF-ETにおける検討より，末梢血および子宮内膜NK細胞は，着床・妊娠の成立に重要な細胞であることが明らかとなった．さらに，たとえ妊娠が成立したとしてもNK細胞活性やNK細胞サブポピュレーションに異常がある場合，流産に陥ってしまう可能性もあり，NK細胞は妊娠の成立のみならず，妊娠維持にも重要な細胞であることが示された．

NK細胞は活性性受容体と抑制性受容体の2種類で標的細胞を認識することにより，対象となる細胞を攻撃するか否かを決定する（ミッシングセルフ仮説）．つまり，NK細胞は活性性受容体と抑制性受容体とを発現し，抑制性受容体にも活性化受容体にも結合するものがない場合，NK細胞は標的細胞に対して反応しない．また抑制性受容体に結合した場合も同様に反応しない．一方，活性性受容体に結合した場合，NK細胞は標的細胞を攻撃し，さらに活性性受容体，抑制性受容体ともに結合する場合，シグナルのバランスにより標的細胞を攻撃するか否かが決定される．NK細胞の主たる機能は細胞傷害であるが，同時にNK細胞はサイトカイン産生能を有している．これら細胞傷害・サイトカイン産生これら双方に関与すると考えられているのが活性性受容体として知られているNatural cytotoxicity receptor（NCR）である．そこで，NCRの発現意義につき，以下の検討を行った．

NK細胞に発現するNCR発現，NK細胞産生サイトカイン産生と妊娠の成立・維持

NCRには，NKp46，NKp30，NKp44が知られており，このうちNKp46とNKp30は活性化および非活性化NK細胞に発現し，NKp44は活性化NK細胞にのみ発現することが知られている[3]．また細胞傷害性はNK細胞表面に発現するNCRsの密度と相関すると言われている．NKp46とNKp30はインフルエンザウィルスあるいはパラインフルエンザウィルスのヘマグルチニンを認識することが知られている[4,5]が，内因性のNCRsのリガンドはまだ明らかではない．さらにNKp46とNKp30は細胞傷害性のみならずサイトカイン産生とも関連すると考えられている[6]．

当初我々は，NK細胞における活性性受容体であるNCR発現は，不育症（Recurrent pregnancy loss: RPL）患者で高値となると推定した．しかし非妊時末梢血NK細胞NKp46発現（CD56^{+}/NKp46^{+}細胞）はRPLで非RPLに比して有意に低値であった[7]．非妊時子宮内膜におけるNKp46発現も，サイトカイン産生が主であると考えられるCD56^{bright}細胞におけるNKp46発現がRPLで非RPLに比して有意に低値であった．興味深いことに細胞傷害が主であると考えられるCD56^{dim}細胞におけるNKp46発現も，RPLで非RPLに比して有意に低値であった．これらの結果はRPLにおけるNKp46発現低下はNK細胞による細胞傷害性の低下ということよりはNK細胞の産生サイトカインの変化の存在を示唆するものであると思われた．

非妊時末梢血NK細胞，非妊時子宮内膜NK細胞におけるサイトカイン産生

NK細胞はTNF-αやIFN-γといったタイプ1サイトカイン（NK1サイトカイン）やIL-4，IL-5，IL-13といったタイプ2サイトカイン（NK2サイトカイン），およびIL-10，GM-CSFなどのサイトカイン産生能を有している．そこでNK細胞によるサイトカインの産生がどのようにRPLや着床不全に関与するのかを明らかにすることを目的に非妊時末梢血あるいは非妊時黄体中期子宮内膜NK細胞によるサイトカイン産生を検討した．

正常妊娠では正常妊娠ではTh細胞のタイプ2シフト，すなわちタイプ1サイトカイン産生Th細胞とタイプ2サイトカイン産生細胞の比であるTh1/Th2比が低下することが知られている[8,9]．これら妊娠中のTh1/Th2シフトといった母体免疫反応にはNK細胞が関与していると報告されており[10]，さらにはTh1/Th2細胞比やTc1/Tc2細胞比は変化しないで，NK1/NK2細胞比が正常妊娠では低下するとの報告もある[11]．この妊娠中のNK1/NK2比の変化は，タイプ2サイトカイン産生は変化せず，タイプ1サイトカイン産生が低下することによりCD56^{bright}細胞とCD56^{dim}細胞，双方のNK細胞からのサイトカイン産生がタイプ1からタイプ2へとシフトする[12]とされている．このようにこれまで

の報告から正常妊娠においてはT細胞およびNK細胞におけるタイプ2シフトが起こっていることは間違いないと考えられる．我々の検討でもサイトカイン産生が主であるCD 56^{bright}細胞が産生するTNF-αやIFN-γといったタイプ1サイトカインはRPL，着床不全において高値あった[13]．興味深いことにIFN-γのみを産生する細胞が着床不全群でのみ低下しており，IFN-γは着床に重要である事が示唆された[13]．

さらにTh1/Th2比同様にタイプ1サイトカインであるIFN-γ，TNF-αとType2サイトカインであるIL-4の比すなわちNK1/NK2比およびNKr1細胞産生サイトカインであるIL-10との比であるNK1/NKr1比について検討すると，NK1/NK2比，NK1/NKr1比ともにRPLで有意な増加を認めた[13]．この結果は，非妊時においてもRPLにおいて末梢血NK細胞のタイプ1シフト，つまりNK1シフトが起こっていることを示している．しかし，NK細胞はIL-4およびIL-10を極少量しか産生しておらず，これらを分母として比をみた場合，安定した測定結果が得られるとは言い難い．そこで胎盤形成に必要でありNK細胞から安定して産生されるGM-CSFとタイプ1産生サイトカインとの比を測定した．CD56^{bright}細胞におけるTNF-α/GM-CSF比は，RPL，着床不全で有意に増加しており，NK細胞の約50％はTNF-αを，約80％はGM-CSFを産生するので，測定も安定して行うことができることからTNF-α/GM-CSF比は，RPL，着床不全に対する検査法として有用である可能性が示された[13]．

一方，子宮NK細胞によるサイトカイン産生は，CD56^{bright}/TNF-α^{+}細胞，CD56^{bright}/IFN-γ^{+}細胞，CD 56^{bright}/IFN-γ^{+}/TNF-α^{+}細胞，CD56^{bright}/IFN-γ^{-}/TNF-α^{+}細胞ともにRPLで産生細胞数が低下していた[14]．妊娠の成立・維持の場においてTNF-αは血管新生をIFN-γは血管のリモデリングを行っている．よってRPLではTNF-αによる血管新生やIFN-γによる血管のリモデリングの異常を来すことにより流産となっている可能性を示していると思われる．

NCR陽性NK細胞とNK細胞産生サイトカインとの関わり

NK細胞に発現するNCRのうちNKp46はそのサイトカイン産生とも関連が認められる．NKp46はその発現強度によりNKp46^{bright}細胞とNKp46^{dim}細胞とに分類することが可能であり，これらの細胞のIFN-γ産生をみてみるとIFN-γ産生NK細胞のほとんどがNKp46^{bright}細胞であることがわかった[15]．NK細胞の細胞傷害性はNK細胞表面に発現するNKp46の密度と相関すると言われている．細胞傷害性同様にサイトカイン産生にもNKp46発現強度と関連するか否かを末梢血NK細胞および子宮内膜NK細胞をもちいて検討すると，末梢血，子宮内膜ともにNK細胞によるIFN-γ産生能は，有意にNKp46^{bright} NK細胞で高値であり，さらにCD56発現も加味すると，CD56^{bright}/NKp46^{bright}NK細胞において有意にIFN-γ産生が高値であった[15]．すなわちNKp46はNK細胞におけるサイトカイン産生に関与しており，その発現はCD56から見るとサイトカイン産生能が高いとされるCD56^{bright}NK細胞で，NKp46から見るとその発現強度が強いNKp46^{bright}NK細胞でサイトカイン産生能が最も高いことが示された．同様の報告は他家からもなされており[16]，NKp46発現の弱いNK細胞では，発現の強いNK細胞に比して，脾臓，肺，縦隔においてNK細胞によるIFN-γ，IL-13産生が有意に少なかったと報告されている．さらにNK細胞からのサイトカイン産生とNCR陽性NK細胞との相関が，着床不全やRPLではコントロールと逆の相関あるいは相関の欠如を認めており[17]，これは生殖異常でのサイトカイン産生異常を示しているものと思われる．

末梢血NK細胞と子宮内膜NK細胞におけるNCR発現の関連性

末梢血NK細胞を測定することは子宮NK細胞の状態を知ることができるのであろうか？末梢血NK細胞の測定は子宮の中で起こっていることを示してはいないと報告[18]されている一方，末梢血NK細胞と子宮NK細胞とは相関するとも報告[19]されており結論は得られていない．NKp46に着目すると末梢血NK細胞と子宮内膜NK細胞との間で有意な正の

相関を認めた．すなわち末梢血NKp46^{+}NK細胞は子宮内膜NKp46^{+}NK細胞を反映しており，末梢血の測定により子宮の中で起こっていることを推定できる可能性がある[20]．

NK細胞における活性性受容体と抑制性受容体の共発現

先に示したようにNK細胞は活性性受容体と抑制性受容体とを発現する．これらは共発現しており，それらの違いによるNK細胞の機能の違いを検討した．細胞傷害性の少ないNK細胞では，NKp46^{+}NK細胞における活性性受容体との共発現が少なく，さらにNK細胞のタイプ2シフトを示した．またサイトカイン産生が主であるNKp46^{bright}NK細胞は細胞傷害性が少ないCD16^{-}NK細胞との正の相関をみとめ，NKp46発現の意義が解明されつつあると思われる（図1）．

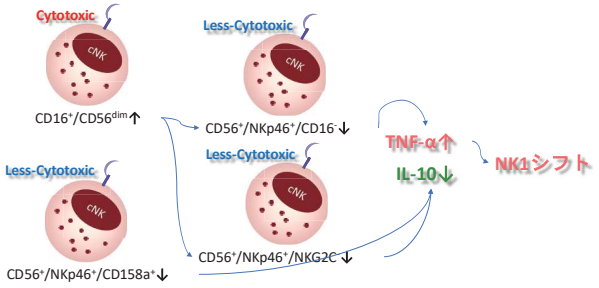


図1．NK細胞における活性性／抑制性受容体共発現とNK細胞によるサイトカイン産生
不育症や着床不全では細胞傷害性のCD16^{+}/CD56^{dim}細胞が増加し，抑制性受容体であるCD158を発現するNKp46陽性細胞が低下する．さらには，細胞傷害性受容体であるCD16や活性性受容体であるNKG2Cを共発現しない，すわなち細胞傷害性が強くないNKp46陽性NK細胞が低下し，タイプ1サイトカインの産生増加，タイプ2サイトカインの産生低下が起こり，NK細胞のタイプ1シフト，すなわちNK 1 シフトが起こるものと推測される．

不育症・着床不全患者NK22細胞におけるNKp46発現

最近，NKp46を発現するIL-22産生性の新しいタイプのNK細胞，NK22細胞の存在が報告された[21,22]．NK22細胞は子宮内膜にも存在することが知られている[23]．我々の検討ではNK22（CD56^{+}/IL-22^{+}）細胞は，不育症患者の子宮内膜で増加しており[24]，さらにNK22細胞内に発現するROR-γtも同様であった[25]．またNK22細胞とタイプ1サイトカインであるIFN-γやTNF-α産生NK細胞とが負の相関を示した[24]．NK22細胞とNK細胞によるIFN-γやTNF-αの低下は不育症患者のみでみられた[26]ことより，不育症患者でのNK22細胞の増加は，不育症患者で起こっているタイプ1シフト（IFN-γやTNF-αの産生増加）を是正する制御性の細胞として働いている可能性が示唆された．NK22細胞は，粘膜防御などにおいて制御的に働くことが知られており[27]，不育症おいてもサイトカイン制御の一端を担っているものと思われる[28]（図2）．

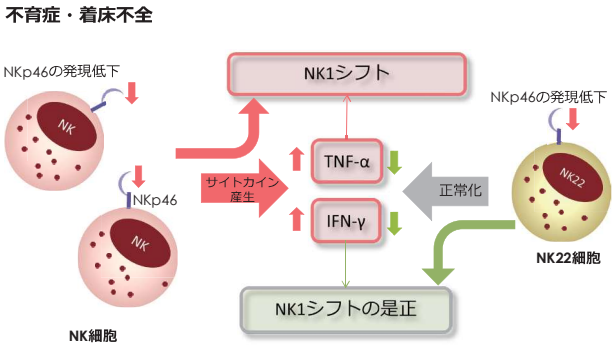


図2．NK22細胞の生殖への関わり
NK22細胞は，不育症や着床不全で生じているNK1シフトに対し，TNF-α産生やIFN-γ産生を低下させてNK1シフトを是正する働きを有しているものと推測される．

不育症患者における妊娠成立後のNKp46発現

不育症・着床不全におけるNKp46発現低下は妊娠成立後，いつまで続くのであろうか？妊娠成立後末梢血を用いて妊娠12週，20週，28週，36週に不育症の既往をもつ妊娠女性と正常コントロールとのNK細胞におけるNKp46発現を検討すると，コントロールでは妊娠期間を通じてNK細胞におけるNKp46発現は低下しなかったのに対し，不育症既往のある女性では妊娠20週まで妊娠前より認められているNKp46低下が持続し，実に妊娠28週になってはじめてNKp46発現がコントロールに追いつくことが示された[14]．

妊娠高血圧症候群患者におけるNKp46発現

妊娠高血圧症候群（Hypertensive disorder in pregnancy: HDP）の発症にもNK細胞が関与することが示唆されている．よってNK細胞におけるNKp46の機能異常は不育症や着床不全のみならずHDP

の発症にも関連する可能性がある．HDPを発症した例と発症しなかった例を比較すると，妊娠12週では末梢血NK細胞のNKp46発現は両群間に差を認めなかったが，本検討のHDP発症週数である妊娠34±6週に対し，NK細胞のNKp46発現は妊娠20週にHDP群で低下し，この低下は妊娠36週まで持続した[29]．またNK細胞におけるNKp46発現とタイプ1サイトカイン(IFN-γ，TNF-α)産生との間には有意な負の相関を認め[29,30]，HDPにおけるNK細胞のNKp46発現低下は，NK細胞によるサイトカイン産生の変化，すなわちHDPにおけるNK細胞のタイプ1シフトを示しているものと思われ，NK細胞NKp46発現測定はHDP発症の予測マーカーとしての有用である可能性が示唆された．

結論と今後の展望

妊娠の成立・維持のためにはNK細胞の適切な機能発現が必要である．つまり末梢血，子宮内膜双方のNK細胞におけるレセプター発現とサイトカイン産生が妊娠の成立・維持のために非常に重要であり，これらの異常発現は着床不全やRPLを引き起こす可能性が示唆された．またこれらのNK細胞異常を有する着床不全やRPL症例に対して免疫グロブリンやイントラリピッドが有効である可能性があり，現在我々もその有効性を検討している．さらにはIVF-ETにおいて胚培養液にサイトカインを添加することにより流産率が低下する可能性も報告されている．今後，NK細胞など免疫細胞の異常を有する生殖異常に対する更なる病態解明とその対応が明らかになっていくものと思われる．

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子宮内免疫

免疫が着床に及ぼす影響
Th1/Th2比，NK細胞

福井 淳史　麦 楚嫻　柴原 浩章

● 妊娠の成立・維持においてNK細胞やT細胞などの免疫担当細胞は，末梢血中や子宮内膜・脱落膜に存在し，子宮における免疫機構の維持に重要な役割を果たしている．
● NK細胞の細胞傷害性の増加，サイトカイン産生の異常は不育症や着床不全を引き起こす．また，精液に曝露されることにより起こるNK細胞機能異常もありうる．
● 不育症や着床不全に対して子宮内免疫異常に着目したさまざまな免疫学的アプローチが行われており，有効な治療法となる可能性があるが，十分なエビデンスは得られていない．

はじめに

遺伝学的に非自己である受精卵が子宮内膜に着床し成長していくためには，その受精卵を受け入れることを可能とする精妙な免疫機能の調整機構が存在し，さらに受精卵を拒絶することを阻止しつつ，その発育を保証できる機構が存在する必要がある．妊娠の成立・維持において，NK細胞やT細胞などの免疫担当細胞は末梢血中や子宮内膜・脱落膜に存在し，子宮での免疫機構の維持に重要な役割を果たしている．これらの細胞の調節異常は良好な受精卵を複数回移植しても妊娠が成立しない着床不全や流産を繰り返してしまう不育症を引き起こすと考えられてきている．これまで末梢血のみならず，子宮内膜，脱落膜中の免疫担当細胞の絶対数の異常および構成比率の異常，あるいは受容体発現やサイトカイン産生異常などについての検討がなされ，妊娠の成立・維持における免疫系の関与，特にT細胞やNK細胞の関与はほぼ確立された概念となっている．

ふくい あつし，まい ちゅうしあん，しばはら ひろあき：兵庫医科大学産科婦人科（〒663-8501　兵庫県西宮市武庫川町1-1）

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着床とは

着床とは，胚盤胞が子宮腔へ到達し，子宮内膜上皮と対位（apposition）・接着（adhesion）することに始まり，間質に浸潤（invasion）し，胚盤胞全体が子宮内膜に被包されるまでの過程をいう．子宮内膜上皮細胞には胚を受容できる時期と受容できない時期とが存在し，胚を受容できる時期は着床ウィンドウ（implantation window）といわれる．着床ウィンドウは排卵後7±2日ほどと考えられ，性ステロイドホルモンにより制御されており，この時期に孵化した胚が子宮内膜上皮に接着する必要がある．

着床期には，子宮内膜に抗原提示細胞である樹状細胞やNK細胞，T細胞などの免疫担当細胞が存在し，着床期から妊娠初期にかけて，その数を増やしていくことが知られている．T細胞は，1型サイトカインを産生するTh1細胞やIL-17を産生するTh17細胞（エフェクターT細胞）と2型サイトカインを産生するTh2細胞や制御性T細胞とのバランスが重要である．また，NK細胞の最大の機能は異物や腫瘍細胞に対する細胞傷害作用であるが，ほかにもらせん動脈のリモデリングに重要な役割を果たしていると考えられている．

T細胞と着床

妊娠中には，1型免疫反応（TNF-αやIFN-γによって誘発される炎症性免疫反応）および2型免疫反応（IL-4，IL-10などによって特徴づけられる抗炎症反応）が起こる．T細胞のうち，1型サイトカインを産生するヘルパーT細胞はTh1細胞と，2型サイトカインを産生するヘルパーT細胞はTh2細胞といわれる．胚の着床と，引き続いて起こる子宮内膜への浸潤のプロセスは，炎症性免疫反応，つまり1型免疫反応によるものであり，その後の胎児の成長と発達は，抗炎症性の2型免疫反応によるものである．妊娠末期の分娩は，子宮内膜への免疫細胞の流入を特徴とする炎症によるものである．

Th1環境が強いと妊娠に有害であり，Th2反応がTh1反応を改善する．1型サイトカインはトロホブラストの浸潤を抑制し，トロホブラストのアポトーシスを促進させる．さらに1型サイトカインは，プロトロンビナーゼの活性化，トロンビンの生成，血栓の形成などによって胎児の成長に悪影響を及ぼすことが知られている．正常妊娠ではTh細胞のタイプ2シフト，すなわち1型サイトカイン産生Th細胞と2型サイトカイン産生細胞の比であるTh1/Th2比が低下する[1, 2]．なお，末梢血のTh1/Th2比はコマーシャルベースで測定可能であり，その結果から治療法が選択されるようになってきた．しかし，Th細胞は子宮内と末梢血ではその挙動が異なることも報告されている[3]ことから，結果の解釈は慎重に行う必要がある．

一方，免疫制御性のT細胞には，制御性T細胞がある．制御性T細胞は$CD4^+/CD25^{bright}/Foxp3^+$に特徴づけられる．制御性細胞は抗原提示細胞によって活性化され，TGF-β，IL-10，CTLA-4などの発現により，Th1細胞，Th2細胞，B細胞，NK細胞などの免疫細胞に対する免疫調節に関与している．制御性T細胞は正常妊娠では増加す

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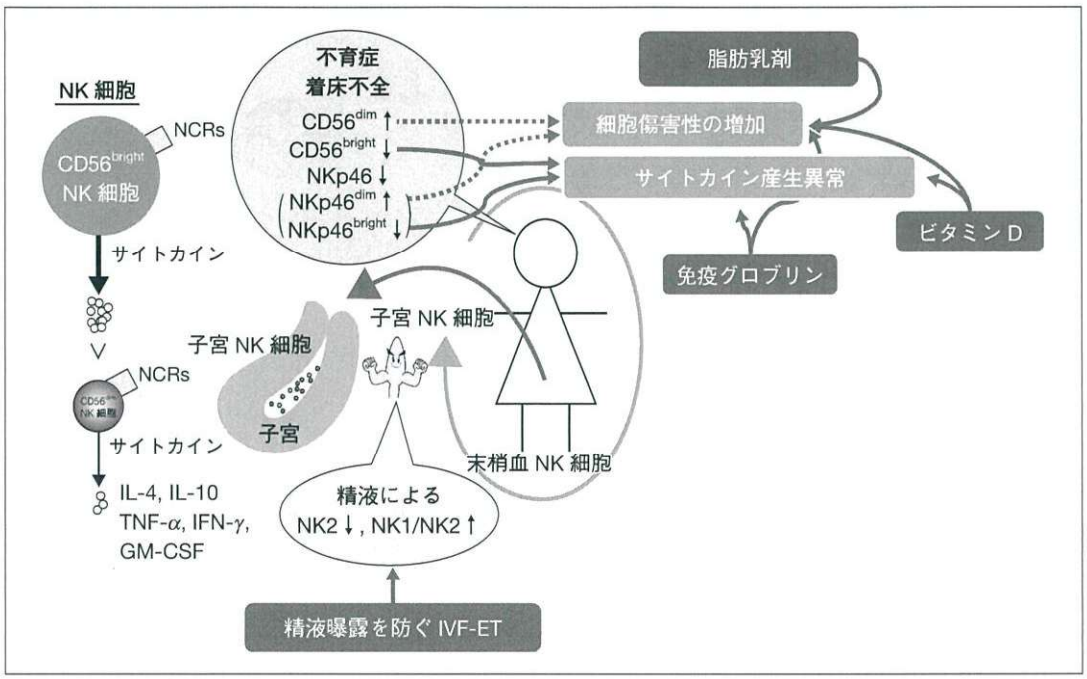


図 1　NK 細胞異常とその治療

ることが知られており，不育症女性では妊娠中絶を受けた女性と比較して末梢血と脱落膜の制御性 T 細胞が減少する．これは，制御性 T 細胞が児に対する母体の免疫寛容に関与している可能性があることを示していると思われる．なお，妊娠中の制御性 T 細胞の増殖は，精子や精漿に曝されることに関連していると報告されている．

最近，Th17 細胞といわれる T 細胞の新たなサブセットが報告された．リスク因子不明不育症女性では，妊娠時末梢血および脱落膜 Th17 細胞，非妊時 Th17 細胞が増加していることが報告されている．

NK 細胞と着床（図 1）

妊娠成立の場である子宮において，子宮内膜および脱落膜には，子宮 NK 細胞が存在する．子宮 NK 細胞は妊娠の成立・維持が行われる子宮に多く存在しており，妊娠に対し何らかの働きをしていると考えるのは想像に難くない．

NK 細胞は，その細胞表面に発現する CD56 の発現強度により，$CD56^{bright}$ 細胞と $CD56^{dim}$ 細胞とに分類することができる．NK 細胞の 10％を占める $CD56^{bright}$ 細胞は，子宮 NK 細胞の主構成成分であり，主としてサイトカイン産生に働く．また，NK 細胞の 90％を占める $CD56^{dim}$ 細胞は，末梢血 NK 細胞の主構成成分であり，主として細胞傷害性に働く．さらに，これらの NK 細胞はその表面に NK 細胞を活性化したり抑制したりする種々のレセプターを発現する．このレセプターが細胞傷害性やサイトカイン産生にお

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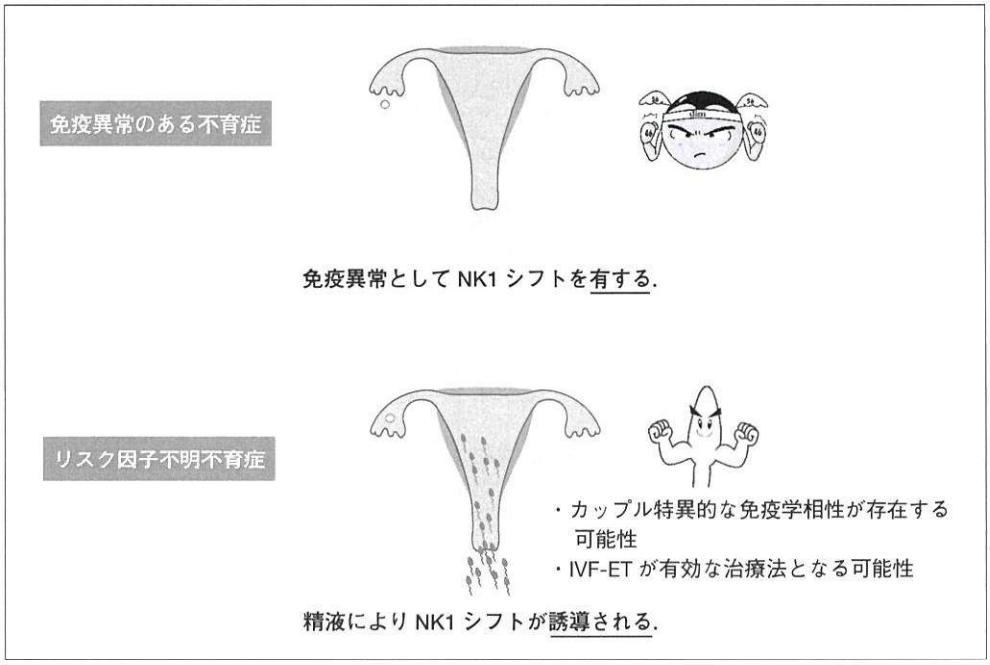


図2　リスク因子不明不育症における新概念：精液曝露によるNK細胞異常の誘導

いて，さまざまな機能を有している．

われわれはNK細胞の妊娠の成立・維持への関与を明らかにするため，当院で体外受精・胚移植（IVF-ET）を施行した症例のNK細胞動態を検討した．胚移植時の末梢血$CD16^{+}/CD56^{+}$細胞は，着床成立群に比して着床不成立群で有意に高値であり，NK細胞活性は，着床成立群では，胚移植時のNK細胞活性がIVF-ET施行直前周期黄体中期のそれに比して有意に低値であった[4]．また，IVF-ET施行前周期黄体中期の子宮内膜$CD16^{-}/CD56^{bright}$細胞は，分娩群に比して流産群で有意に低値であり，$CD16^{+}/CD56^{dim}$細胞は，分娩群に比して流産群で有意に高値であった[4]．以上より，NK細胞は妊娠の成立のみならず，妊娠維持にも重要な細胞であることを示した．

NK細胞は，その細胞表面に発現する活性性レセプターと抑制性レセプターで標的細胞を認識することにより，対象となる細胞を傷害するか否かを決定する．抑制性レセプターにも活性性レセプターにも結合するものがない場合，NK細胞は標的細胞に対して反応しない．また，抑制性レセプターにのみ結合した場合も同様に反応しない．一方，活性性レセプターに結合した場合には，NK細胞は標的細胞を攻撃し，さらに抑制性レセプター，活性性レセプターともに結合する場合では，シグナルのバランスにより標的細胞を攻撃するか否かが決定される．

また，NK細胞はサイトカイン産生能も有しており，細胞傷害・サイトカイン産生双方に関与すると考えられているのが活性性レセプターであるnatural cytotoxicity receptor（NCR）である．NCRの1つであるNKp46は，着床不全患者・不育症患者で低下

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する[5, 6]．NKp46 発現低下は NK 細胞による細胞傷害性の低下ということよりは NK 細胞の産生サイトカインの変化の存在を示唆するものであり，先に述べた Th1/Th2 シフトといった母体免疫反応は NK 細胞にも関与していると報告されている[7]ことから，不育症や着床不全患者における NK 細胞産生サイトカイン異常の有無を検討してみた．すると，TNF-α 産生 NK 細胞や IFN-γ 産生 NK 細胞は，不育症・着床不全患者で有意に高値であった．また，Th1/Th2 比と同様に，1 型サイトカインである IFN-γ，TNF-α と 2 型サイトカインである IL-4 の比すなわち NK1/NK2 比，および NKr1 細胞産生サイトカインである IL-10 との比である NK1/NKr1 比についても検討したところ，NK1/NK2 比，NK1/NKr1 比ともに不育症や着床不全患者で高値であり，これらの患者が NK1 シフトを有することが示された[8, 9]．

さて，自然妊娠の成立時では精液が腟内で射精されるわけだが，この精液への曝露と NK1 シフトとの関連性を明らかにするため，サイトカイン測定時の刺激物質として精液を使用してみると，サイトカインは測定可能であり，またリスク因子不明不育症のなかには精液に曝露することにより子宮内で NK1 シフトを起こすことがあることが示された[10]．これは不育症や着床不全における新概念であり，今後，精液曝露を防ぐことが治療の 1 つになっていくかもしれない（**図 2**）．

着床不全に対する免疫学的治療法

着床不全の原因には胚側と子宮内膜側の問題がある．胚側の問題に関しては，着床前胚スクリーニング（PGT-A）により解明が進むものと思われ，将来的には着床不全患者の治療として PGT-A が選択される可能性もあると思われる．子宮内膜側の問題に対する免疫学的アプローチには以下のようなものがあるが，まだまだデータが不足しており十分なエビデンスが示されてはいないのが現状である．

T 細胞が産生するサイトカインの比である Th1/Th2 比が高値である着床不全に対してはタクロリムスが有効である可能性が報告されている[11]．また，ヒドロキシクロロキンが Th1 サイトカインを抑制することにより Th1/Th2 比が改善する可能性[12]や免疫グロブリン療法が Th1/Th2 比が高値である不育症症例における妊娠成績改善に有効である可能性[13]が報告されている．NK 細胞異常が認められる着床不全に対しては免疫グロブリン療法や脂肪乳剤療法が行われることもあり[14, 15]，これらの治療が有効である症例もある．また，ビタミン D 欠乏症が NK 細胞異常を誘導する可能性およびビタミン D 投与による改善も報告[16, 17]されている．他にも，サイトカイン添加胚培養液（GM-CSF 入りの胚培養液）を使用することにより着床率が改善したとの報告がある[18]．

新型コロナ禍における免疫学的治療法

われわれは，不育症や着床不全の治療に用いられているさまざまな免疫学的治療法の新型コロナ禍における使用について報告した[19]．タクロリムス，ヒドロキシクロロキン，

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免疫グロブリン，脂肪乳剤，ビタミンDはいずれもウイルス感染がない状態では使用可能であるとされている．ただし，脂肪乳剤は重症新型コロナ感染症例における栄養補給に使用されている一方，免疫抑制の可能性があるため使用時には注意が必要であり，またタクロリムスは免疫抑制の可能性があるためウイルス症状が現れたときは投薬中止を検討すべきである．

おわりに

妊娠の成立・維持のためには免疫担当細胞の適切な機能発現が必要であり，それらの破綻は着床不全や不育症を引き起こす．また，それらの異常に対するさまざまなアプローチが試みられているが，まだまだデータが不足しており，今後の検討が必要であると思われる．

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Expression of natural cytotoxicity receptors and cytokine production on endometrial natural killer cells in women with recurrent pregnancy loss or implantation failure, and the expression of natural cytotoxicity receptors on peripheral blood natural killer cells in pregnant women with a history of recurrent pregnancy loss

Atsushi Fukui[1,2], Ayano Funamizu[2], Rie Fukuhara[2] and Hiroaki Shibahara[1]

[1]*Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, and* [2]*Department of Obstetrics and Gynecology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan*

Abstract

Aim: Natural cytotoxicity receptors (NCR) are unique markers that regulate natural killer (NK) cell cytotoxicity and cytokine production. In this study, we investigated the expression of NCR (NKp46, NKp44, and NKp30) and cytokine production in NK cells derived from the uterine endometrium of women with recurrent pregnancy loss (RPL). We also investigated the expression of NCR in peripheral blood NK cells in pregnant women with and without a history of RPL.

Methods: The expression of NCR (NKp46, NKp44, and NKp30) in NK cells ($CD56^{dim}$ and $CD56^{bright}$) in the uterine endometrium was analyzed using 3-color flow cytometry. Cytokine (tumor necrosis factor-α and interferon-γ) production was also analyzed. NK cells from the mid-secretory endometrium of 28 women with RPL, 34 women with implantation failure, and 74 controls were collected and mechanically dispersed using a tissue grinder. The expression of NCR in peripheral blood NK cells from pregnant women with ($n = 17$) and without ($n = 91$) a history of RPL was analyzed.

Results: The percentages of $NKp46^{+}$ NK cells were significantly lower in both women with RPL and pregnant women with a history of RPL. The percentages of tumor necrosis factor-α- and/or interferon-γ-producing uterine endometrial NK cells were significantly lower in women with RPL compared with controls.

Conclusion: The changes in NCR expression and cytokine production, especially decreased NKp46 expression in endometrial NK cells, suggests the presence of abnormal NK cell regulation in women with reproductive failures.

Key words: cytokine, endometrium, NK cell, NKp46, recurrent pregnancy loss.

Introduction

Natural killer (NK) cells have very important roles in human pregnancy. For the achievement and maintenance of pregnancy, appropriate systemic regulation of NK cells is necessary. In general, NK cells recognize their target cells via their activating and inhibitory receptors and NK cell cytotoxicity is decided by the balance of the expression of the activating and inhibitory receptors on their surface. NK cells can attack their target cells without sensitization to eliminate cancer cells or foreign substances.

NK cells express their specific cell surface marker CD56. Moreover, human NK cells can be subdivided into $CD56^{bright}$ and $CD56^{dim}$ subsets by the intensity of

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Correspondence: Dr Atsushi Fukui, Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan. Email: a.fukuipon@mac.com

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fluorescence. NK cells are 5–10% of the peripheral blood lymphocytes and they are mainly $CD56^{dim}$ cells. They have higher cytotoxicity. On the other hand, NK cells are 70–90% of uterine lymphocytes and they are $CD56^{bright}$ cells. Uterine NK cells present mainly in the uterine endometrium and the decidua. The abnormal regulation of uterine NK cells (through abnormalities in the number and ratio of NK cells, expression of NK cell surface receptors, and cytokine production by NK cells) has been associated with reproductive failures, such as recurrent pregnancy loss (RPL), implantation failure, or pre-eclampsia. It is becoming clear that the appropriate regulation of NK cells is very important for reproductive success.

Natural cytotoxicity receptors (NCR), which contain NKp30, NKp40, and NKp46, are one of the NK-cell-activating receptors and expressed solely on NK cells. Intriguingly, NKp30 and NKp46 receptors are expressed on the surface of both activated and non-activated NK cells, while the NKp44 receptor is exclusively expressed on the surface of activated NK cells only. More interestingly, the functions of NKp30 and NKp46 are not only cytotoxic activity of NK cells, but also cytokines production. The cytokine repertoire of peripheral blood NK cells includes mainly type 1 cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α (NK1 cell). However, NK cells are capable with stimulation to produce type 2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13[1,2] (NK2 cell). Besides, additional types of NK cells can produce transforming growth factor-β[3] or IL-10 (NKr1 cell).[4,5] It has been proposed that cytokines produced by NK cells enable decidualization, control trophoblast invasion, and promote angiogenesis at the implantation site.[6,7] Previous studies suggest that appropriate systemic regulation of peripheral blood NK cells is indicative of reproductive success[8,9] and that implantation-related immune abnormalities, such as abnormal expression of NK cell surface markers and abnormal cytokines production by NK cells, cause reproductive failure.[10–15] We have previously reported decreased expression of NKp46 in peripheral blood NK cells in women with RPL or implantation failure.[16]

In this study, in order to determine the further participation of NKp46 for reproduction, we conducted a flow cytometric analysis of uterine endometrial NK cell expression of NCR, as well as cytokine production, by uterine endometrial NK cells in women with RPL. Moreover, we evaluated the expression of NCR in peripheral blood NK cells in pregnant women with a history of RPL.

Methods

Study subjects

Cases for this prospective study were recruited at the Department of Obstetrics and Gynecology, Hirosaki University Hospital from 1 May 2007 to 31 March 2012. All study subjects signed informed consent forms prior to entering the study, in accordance with local Institutional Review Board protocol.

Uterine endometrial NK cells were collected from the mid-secretory endometrium of 28 women with RPL, 34 women with previous implantation failure, and 74 controls. Peripheral blood NK cells were collected at 12, 20, 28, and 36 gestational weeks (GW) from pregnant women with and without a history of RPL (17 and 91 women, respectively). Women with anatomical, endocrine, infectious, or autoimmune disorders, including antiphospholipid syndrome and genetic etiologies of RPL, were excluded from the study. Endometrial samples were drawn from study groups prior to treatment. Patient characteristics are shown in Tables 1 and 2. There were no significant differences in age or number of prior deliveries among the RPL, implantation failure, and control groups (Table 1), nor between pregnant women with and without a history of RPL (Table 2). The numbers of pregnancies ($P < 0.01$) and miscarriages ($P < 0.01$) were significantly different among the RPL, implantation failure, and control groups (Table 1) and between pregnant women with and without a history of RPL (Table 2). There was no significant difference in newborn birthweight in pregnant women with and without a history of RPL (Table 2).

Table 1 Age, obstetric, and infertility histories of women with RPL, implantation failure, and controls

	RPL ($n = 28$)	Implantation failure ($n = 34$)	Controls ($n = 74$)	P
Age	33.8 ± 4.3	35.3 ± 3.8	33.3 ± 4.9	NS
No. of pregnancies	2.9 ± 1.5	0.3 ± 0.6	0.5 ± 0.7	<0.01
No. of deliveries	0.2 ± 0.5	0.0 ± 0.2	0.2 ± 0.5	NS
No. of miscarriages	2.6 ± 1.5	0.0 ± −.4	0.2 ± 0.4	<0.01

NS, not significant; RPL, recurrent pregnancy loss.

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Table 2 Age, obstetric, and infertility histories of pregnant women with and without a history of RPL

	Pregnant women without a history of RPL ($n = 91$)	Pregnant women with a history of RPL ($n = 17$)	P
Age (years)	32.3 ± 4.6	34.1 ± 4.1	NS
No. of pregnancies	1.2 ± 1.1	3.8 ± 1.9	<0.01
No. of deliveries	0.8 ± 0.8	0.6 ± 0.8	NS
No. of miscarriages	0.2 ± 0.4	3.2 ± 1.4	<0.01
Birthweight (g) of newborn	3043 ± 395	3256 ± 455	NS

NS, not significant; RPL, recurrent pregnancy loss.

Preparation of peripheral blood and endometrial leukocytes

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using HISTOPAQUE-1077 (Sigma-Aldrich). Endometrial tissue was obtained at the mid-secretory phase of the menstrual cycle using the Endo-suction endometrial sampler. Samples were first minced using scissors and were placed into sterile Roswell Park Memorial Institute medium (RPMI) 1640 medium (Gibco) and supplemented with 10% Fetal calf serum (FCS) (Gibco), 2% L-glutamine, 1% penicillin, and 1% streptomycin (Gibco). After macroscopic exclusion of blood, samples were then mechanically disrupted with a Pyrex glass Broeck tissue grinder (Fisher Scientific Co.) and gentle magnetic-activated cell sorting (Miltenyi Biotec).

Flow cytometric analysis of peripheral blood and endometrial NK cells

The following monoclonal antibodies (mAb) were used to analyze the surface antigens of peripheral blood and uterine (endometrial) leukocytes: anti-CD56 fluorescein isothiocyanate (FITC) (Beckton Dickinson), anti-CD335 (NKp46) phycoerythrin (PE) (Beckman Coulter), anti-CD336 (NKp44) PE (Beckman Coulter), and anti-CD337 (NKp30) PE (Beckman Coulter). For uterine lymphocytes, anti-CD45 phycoerythrincyanin 5.1 (PC5) (Beckman Coulter) was used to detect and exclude other cells. Labeling of peripheral blood and endometrial leukocytes with this panel of mAb was performed using standard techniques. Briefly, 0.5×10^6 cells were incubated with mAb for 20 min at room temperature, and the cells were washed twice in phosphate-buffered saline (PBS). Immunofluorescence and 3-color flow cytometric analysis were performed using a FACSCanto flow cytometer (Becton Dickinson). BD FACSDiva software (Becton Dickinson) was used for full-list-mode data storage, recovery, and analysis. Gates were set on the lymphocyte region (for peripheral blood analysis) and the anti-CD45 PC5 positive events (for endometrial analysis) using characteristic forward and side scatter parameters. For each sample, 10^4 peripheral blood lymphocytes were evaluated. The percentage of $CD56^+$ cells or subsets of $CD56^+$ cells expressing each NCR was determined by flow cytometry. The percentage of NCR^+ cells /$CD56^+$ or subsets of $CD56^+$ cells was calculated from this data. We distinguished $CD56^{dim}$ and $CD56^{bright}$ NK cells according to their fluorescence.

Intracellular cytokine expression by NK cells

Endometrial samples were washed twice in PBS. These cells were stimulated with phorbol 12-myristate 13-acetate (25 ng/mL) (Sigma-Aldrich) and ionomycin (1 mmol/L; Sigma-Aldrich) in the presence of brefeldin A (10 mg/mL; Sigma-Aldrich) for 4 h at 37°C in a 5% CO2 humidified incubator, followed by washing. The cells were stained for 20 min at room temperature with anti-CD56 PC5, then washed and fixed with Cytofix/Cytoperm (Pharmingen). After permeabilization to allow anti-cytokine antibodies to enter the cells, cells were stained for 30 min with anti-TNF-α FITC and anti-IFN-γ PE mAb (both from Becton Dickinson). Appropriate isotype controls were used for each antibody. Cells were washed twice with 1× Perm Wash solution (Pharmingen) and resuspended in 0.5 mL of PBS for subsequent flow cytometric analysis. Immunofluorescence and 3-color flow cytometric analysis were performed. For each sample, 3×10^4 peripheral blood lymphocytes were evaluated.

Statistical analysis

All data are presented as the mean ± standard deviation, with the exception of the cytokine analysis, which is presented as the median and interquartile range (IQR). The data were analyzed using SPSS 22 (IBM). Age distribution of study groups and controls were analyzed by one-way ANOVA. Expression of NCR in peripheral blood and endometrial NK cell subsets were analyzed by Fisher's exact probability test of frequency distribution. For the

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cytokines analysis, the laboratory data are presented as the median and IQR. Statistical differences among the study and control groups were analyzed using a non-parametric test. The Kruskal–Wallis analysis of ranks for multiple comparisons was applied to analyze differences between study groups and controls. The Mann–Whitney *U*-test was applied to compare differences between two groups. Differences were considered significant at $P < 0.05$.

Results

NCR expression is decreased in RPL endometrial NK cells

Representative dot plots of NCR expression (NKp46, NKp44, NKp30) in $CD56^+$ NK cells and NK cell subsets are shown in Figure 1. Flow cytometric analysis of NCR expression in uterine endometrial NK cells of women with RPL, women with implantation failure, and controls are shown in Figure 2 and Table 3.

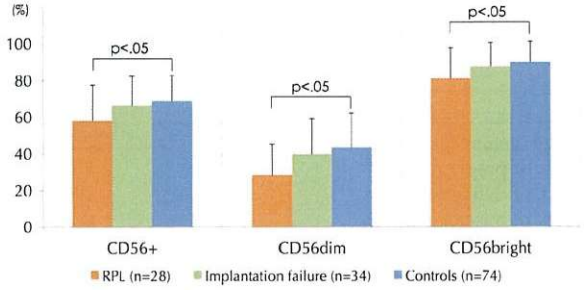


Figure 2 The expression of NKp46 on uterine endometrial natural killer (NK) cells. The ordinate indicates the percentage of NKp46-positive cells in each subset of NK cells. (•) Recurrent pregnancy loss ($n = 28$). (•) Implantation failure ($n = 34$). (•) Controls ($n = 74$).

For the expression of NKp46, the percentage of $CD56^+/NKp46^+$ cells in women with RPL (58.0 ± 19.6%) was significantly lower than that in controls (68.9 ± 14.1%) ($P < 0.05$) (Fig. 2). For NK cell subsets, the percentage of $CD56^{bright}/NKp46^+$ cells in

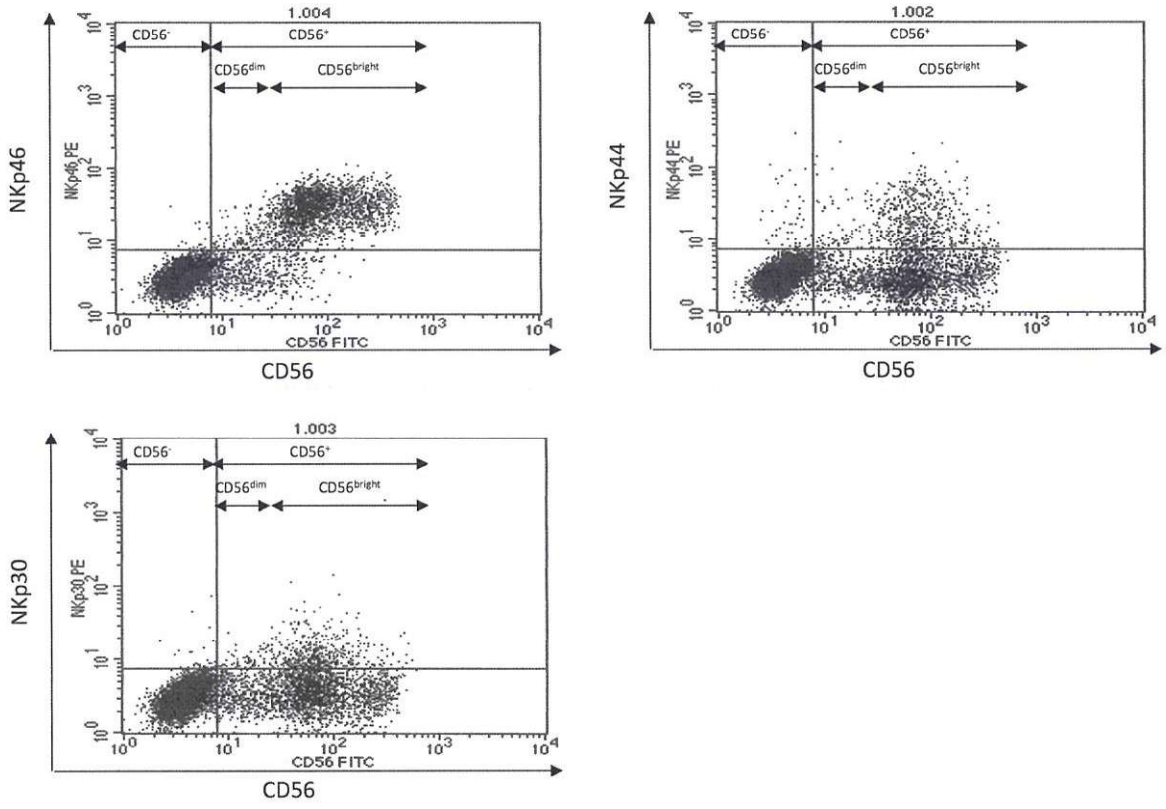


Figure 1 Representative dot plots of the expression of natural cytotoxicity receptors (NCR) on uterine endometrial natural killer cells. A gate is set on the lymphocytes. Lymphocytes are classified into $CD56^+$ and $CD56^-$ cells. $CD56^+$ cells are classified into $CD56^{bright}$ and $CD56^{dim}$ cells. Co-expression of CD56 with NCR is evaluated. FITC, fluorescein isothiocyanate.

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Table 3 Expression of NCR on uterine endometrial NK cells

	RPL	Implantation failure	Controls	*P*
$CD56^+/NKp46^+$	58.0 ± 19.6	66.5 ± 16.0	68.9 ± 14.0	<0.05
$CD56^{bright}/NKp46^+$	80.9 ± 16.8	87.5 ± 13.0	89.8 ± 11.5	<0.05
$CD56^{dim}/NKp46^+$	28.4 ± 16.8	39.6 ± 19.5	43.5 ± 18.6	<0.05
$CD56^+/NKp44^+$	6.6 ± 8.1	7.5 ± 4.8	9.1 ± 7.0	NS
$CD56^{bright}/NKp44^+$	9.2 ± 11.9	8.8 ± 7.1	10.0 ± 9.6	NS
$CD56^{dim}/NKp44^+$	3.3 ± 3.3	6.0 ± 3.9	6.6 ± 3.9	NS
$CD56^+/NKp30^+$	18.7 ± 19.4	18.7 ± 10.7	17.8 ± 12.8	NS
$CD56^{bright}/NKp30^+$	31.2 ± 26.7	37.1 ± 31.9	35.8 ± 48.7	NS
$CD56^{dim}/NKp30^+$	11.8 ± 19.2	13.3 ± 9.1	11.7 ± 8.4	NS

NCR, natural cytotoxicity receptors; NK, natural killer; NS, not significant; RPL, recurrent pregnancy loss.

women with RPL (80.9 ± 16.8%) was significantly lower than that in controls (89.8 ± 11.6%) ($P < 0.05$) and the percentage of $CD56^{dim}/NKp46^+$ cells in women with RPL (28.4 ± 16.8%) was significantly lower than that in controls (43.5 ± 18.6%) ($P < 0.05$) (Fig. 2). There was no significant difference in expression of NKp46 on $CD56^{bright}$ NK cells, nor in expression of NKp44 and NKp30 on NK cells ($CD56^+$, $CD56^{dim}$, $CD56^{bright}$) between the three groups (Table 3).

IFN-γ or TNF-α production is deceased in uterine endometrial NK cells

The percentages of IFN-γ- or TNF-α-producing $CD56^+$, $CD56^{bright}$, and $CD56^{dim}$ NK cells in women with RPL, women with implantation failures, and controls are listed in Table 4. The percentages of $CD56^+/TNF\text{-}\alpha^+$, $CD56^{bright}/TNF\text{-}\alpha^+$, and $CD56^{dim}/TNF\text{-}\alpha^+$ cells were significantly different among the three groups (all $P < 0.05$). Furthermore, the percentages of $CD56^+/IFN\text{-}\gamma^+/TNF\text{-}\alpha^+$, $CD56^+/IFN\text{-}\gamma^-/TNF\text{-}\alpha^+$, and $CD56^{dim}/IFN\text{-}\gamma^-/TNF\text{-}\alpha^+$ cells were all significantly different among the three groups (all $P < 0.05$). In women with RPL, the percentages of TNF-α-producing $CD56^+$ ($P < 0.05$), $CD56^{bright}$ ($P < 0.01$), and $CD56^{dim}$ ($P < 0.05$) NK cells were significantly lower compared with controls, as was the percentage of $CD56^{bright}/IFN\text{-}\gamma^+$ cells ($P < 0.05$). Furthermore, the percentages of TNF-α-only-producing NK cells ($IFN\text{-}\gamma^-/TNF\text{-}\alpha^+$ cells)

Table 4 Cytokines-producing NK cells in women with RPL, implantation failure, and controls

	RPL ($n = 20$)		Implantation Failure ($n = 34$)		Controls ($n = 74$)		*P**
	Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	
$CD56^+/IFN\text{-}\gamma^+$	19.5	(8.5–47.2)	28.9	(11.6–50.8)	39.0	(26.4–51.6)	NS
$CD56^+/TNF\text{-}\alpha^+$	20.6‡	(10.3–34.9)	28.4	(24.7–55.1)	41.3	(28.5–53.1)	<0.05
$CD56^+/IFN\text{-}\gamma^+/TNF\text{-}\alpha^-$	6.6	(2.9–15.5)	8.1	(3.6–12.7)	9.5	(4.7–15.2)	NS
$CD56^+/IFN\text{-}\gamma^+/TNF\text{-}\alpha^+$	14.2‡	(6.2–23.8)	23.0	(7.6–32.9)	26.3	(20.4–37.5)	<0.05
$CD56^+/IFN\text{-}\gamma^-/TNF\text{-}\alpha^+$	4.2§	(2.2–5.9)	6.4	(2.5–12.4)	6.5	(5.0–14.0)	<0.05
$CD56^{bright}/IFN\text{-}\gamma^+$	21.2‡	(7.0–33.4)	31.3	(11.8–49.2)	41.5	(23.5–54.3)	NS
$CD56^{bright}/TNF\text{-}\alpha^+$	20.9§	(11.3–34.1)	28.8	(20.2–49.2)	38.5	(27.8–58.8)	<0.05
$CD56^{bright}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^-$	7.6	(1.6–7.6)	8.0	(3.2–12.9)	11.9	(4.7–20.7)	NS
$CD56^{bright}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^+$	14.0‡	(5.6–26.8)	24.2	(7.7–33.5)	26.8	(18.8–36.3)	NS
$CD56^{bright}/IFN\text{-}\gamma^-/TNF\text{-}\alpha^+$	4.6‡	(0.8–7.5)	7.4	(3.0–10.1)	7.6	(4.4–16.7)	NS
$CD56^{dim}/IFN\text{-}\gamma^+$	19.0	(10.0–52.2)	37.4	(17.2–45.1)	35.6	(20.8–47.0)	NS
$CD56^{dim}/TNF\text{-}\alpha^+$	21.0‡	(10.9–39.1)	32.0	(19.6–52.6)	42.0	(26.0–43.7)	<0.05
$CD56^{dim}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^-$	5.8	(2.8–11.8)	5.5	(3.4–13.8)	7.9	(4.5–12.0)	NS
$CD56^{dim}/IFN\text{-}\gamma^+/TNF\text{-}\alpha^+$	14.1	(5.4–22.6)	27.0	(11.7–31.3)	25.6	(13.4–37.0)	NS
$CD56^{dim}/IFN\text{-}\gamma^-/TNF\text{-}\alpha^+$	4.4‡	(3.0–6.7)	6.7	(2.7–15.7)	8.0	(4.8–14.4)	<0.05

†Difference between groups was analyzed by Kruskal–Wallis analysis of ranks. ‡$P < 0.05$: Difference between RPL and controls was analyzed by Mann–Whitney *U*-test. §$P < 0.01$: Difference between RPL and controls was analyzed by Mann–Whitney *U*-test. IFN, interferon; NK, natural killer; NS, not significant; RPL, recurrent pregnancy loss; TNF, tumor necrosis factor.

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in CD56^{+} NK cells ($P < 0.01$) and its subset (CD56^{bright} and CD56^{dim}, both $P < 0.05$) were significantly lower. The percentages of IFN-γ- and TNF-α-producing NK cells (IFN-γ^{+}/TNF-α^{+} cells) in CD56^{+} NK cells ($P < 0.05$) and the CD56^{bright} subset ($P < 0.05$) were also significantly lower (Fig. 3). There were no differences in the percentage of IFN-γ- or TNF-α-producing NK cells between women with implantation failure and controls.

NCR expression is decreased in peripheral blood NK cells in pregnant women with a history of RPL

The percentages of cells expressing NCR in pregnant women with and without a history of RPL are shown in Table 5. There were no significant differences in the percentages of NKp44 and NKp30 between pregnant women with and without a history of RPL at each gestational age. However, the percentage of CD56^{bright}/NKp46^{+} NK cells was significantly lower in pregnant women with a history of RPL compared with pregnant women without RPL at both 12 and 20 GW. This difference disappeared at 28 GW. There was no significant difference in the percentage of CD56^{dim}/NKp46^{+} NK cells in pregnant women with and without a history of RPL (Fig. 4).

Discussion

Uterine endometrial NK cells have a unique receptor repertoire – they express NKp46, but display little to no expression of NKp30 and NKp44, in both the proliferative and secretory phases.[17] Nevertheless, our data showed that, in the mid-secretory phase, uterine endometrial NK cells express not only NKp46 but also NKp30 and NKp44.[18,19] Our study showed that 60–70% of uterine endometrial NK cells are NKp46^{+} NK cells, about 20% are NKp30^{+}, and about 10% are

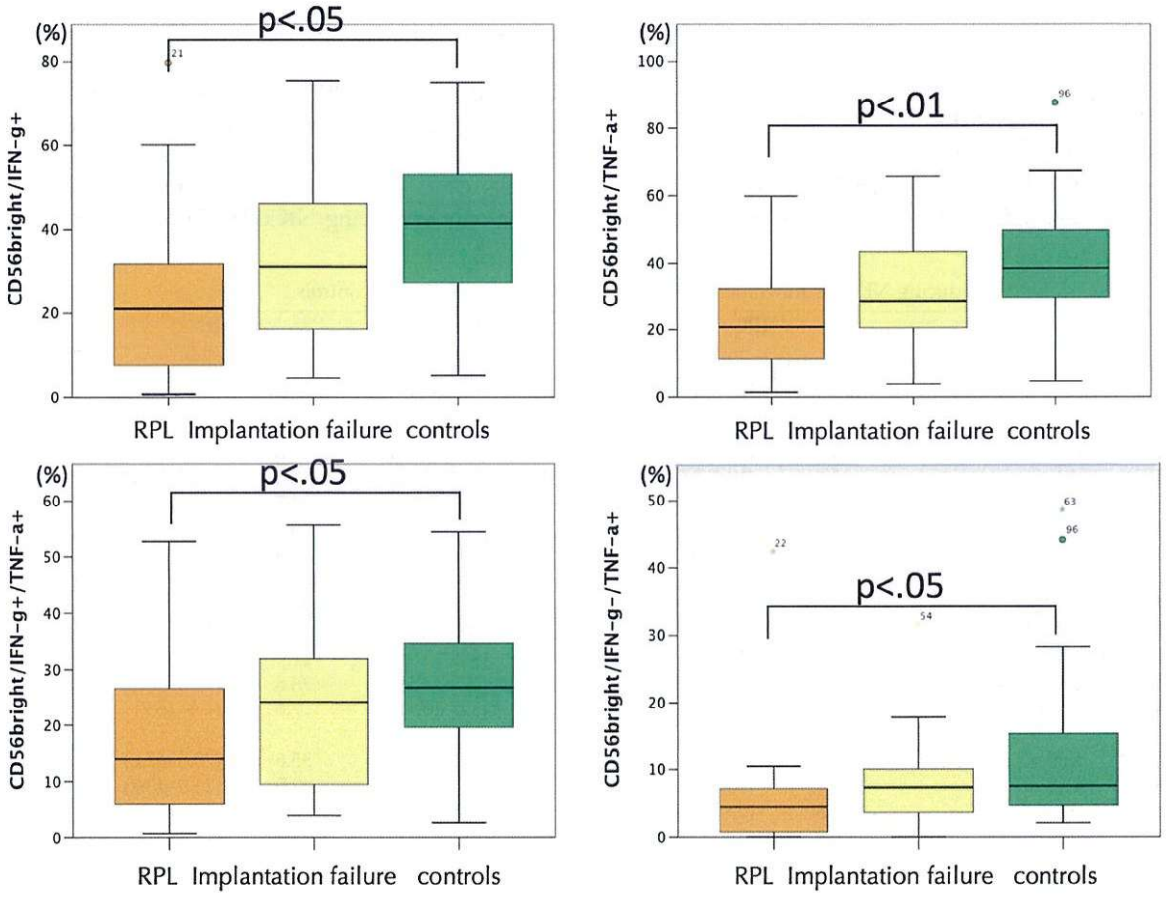


Figure 3 Cytokines production by uterine endometrial natural killer (NK) cells. The ordinate indicates the percentage of cytokine-producing CD56^{bright} NK cells. IFN, interferon; RPL, recurrent pregnancy loss; TNF, tumor necrosis factor.

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Table 5 Expression of NCR on peripheral blood NK cells in pregnant women

		12 weeks	20 weeks	28 weeks	36 weeks
RPL†	$CD56^{bright}/NKp46^{+}$	61.5 ± 32.6*	65.3 ± 56.6*	85.2 ± 9.5	91.6 ± 5.6
	$CD56^{dim}/NKp46^{+}$	28.5 ± 25.6	36.6 ± 36.2	37.2 ± 8.2	39.5 ± 9.8
Controls‡	$CD56^{bright}/NKp46^{+}$	89.5 ± 10.2	91.6 ± 21.1	89.3 ± 10.1	93.4 ± 8.5
	$CD56^{dim}/NKp46^{+}$	46.4 ± 20.2	53.8 ± 19.2	47.0 ± 16.4	50.6 ± 17.9
RPL†	$CD56^{bright}/NKp44^{+}$	5.8 ± 6.2	5.3 ± 3.8	5.3 ± 7.5	0.4 ± 0.6
	$CD56^{dim}/NKp44^{+}$	1.0 ± 0.7	1.6 ± 1.9	3.5 ± 5.7	0.4 ± 0.3
Controls‡	$CD56^{bright}/NKp44^{+}$	13.0 ± 19.0	8.8 ± 8.7	7.0 ± 8.4	6.2 ± 6.4
	$CD56^{dim}/NKp44^{+}$	2.4 ± 5.4	1.8 ± 3.1	2.4 ± 3.1	1.1 ± 2.6
RPL†	$CD56^{bright}/NKp30^{+}$	22.4 ± 13.5	30.1 ± 23.3	31.2 ± 18.4	16.5 ± 2.0
	$CD56^{dim}/NKp30^{+}$	12.9 ± 7.4	14.1 ± 13.0	17.4 ± 12.0	12.0 ± 3.9
Controls‡	$CD56^{bright}/NKp30^{+}$	38.4 ± 24.6	40.3 ± 25.2	36.8 ± 18.2	33.2 ± 16.7
	$CD56^{dim}/NKp30^{+}$	20.9 ± 18.9	26.4 ± 21.0	21.1 ± 14.3	18.5 ± 8.0

*$P < 0.001$: Difference between pregnant women with and without a history of RPL was analyzed by Fisher's exact probability test. †Pregnant women with a history of RPL. ‡Pregnant women without a history of RPL. NCR, natural cytotoxicity receptors; NK, natural killer; RPL, recurrent pregnancy loss.

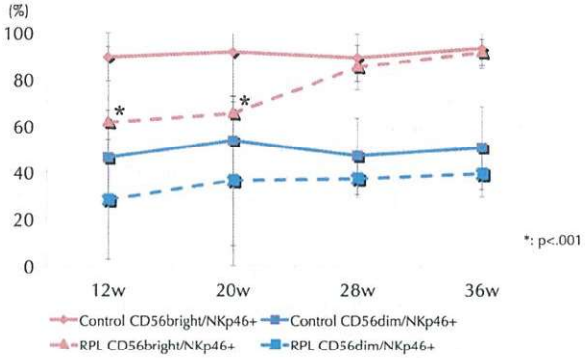


Figure 4 The expression of NKp46 on peripheral blood natural killer (NK) cells in pregnant women with and without a history of recurrent pregnancy loss in each gestational week. The ordinate indicates the percentage of NKp46-positive NK cells in each subset of NK cells. The abscissa indicates gestational week. *$P < 0.001$. (•) Control $CD56^{bright}/NKp46^{+}$. (•) Recurrent pregnancy loss (RPL) $CD56^{bright}/NKp46^{+}$. (•) Control $CD56^{dim}/NKp46^{+}$. (•) RPL $CD56^{dim}/NKp46^{+}$.

$NKp44^{+}$ NK cells. It has been reported that NKp46 is a ubiquitous marker of NK cells[20] and uterine NK cells express NKp46, NKp44, and NKp30.[21–23] From our study, the expression of NCR on endometrial NK cells might fluctuate in response to the patient's condition.

In this study, the expression of NKp46 in uterine endometrial NK cells was significantly lower in non-pregnant women with RPL compared with non-pregnant control women. Previously, we have reported lower expression of NKp46 in peripheral blood NK cells in women with RPL.[16] NKp46 is an NCR, and acts as an activating receptor of NK cells. NKp30 and NKp46 perform functions in the cytotoxic activity and cytokine production of NK cells. Recently, we have shown a functional role for NKp46 in cytokine production by NK cells in an *in vitro* study on the peripheral blood and uterine endometrium of women with reproductive failure, such as RPL.[24] Interestingly, the expression of NKp46 was associated with cytokine-production in both $CD56^{bright}$ and $CD56^{dim}$ NK cells. Moreover, the intensity of NKp46 was associated with cytokine production by NK cells, especially IFN-γ production. That is, $NKp46^{bright}$ NK cells produced more cytokines than $NKp46^{dim}$ NK cells. Besides, among the NK cell subtypes, $CD56^{bright}/NKp46^{bright}$ NK cells produce the most IFN-γ.[24] A previous study examined the relation between the expression of NCR and cytokines production for decidual NK cells,[21] reporting that $NKp46^{+}$ decidual NK cells did not have function for cytokine secretion, but that $NKp44^{+}$ and $NKp30^{+}$ decidual NK cells did. However, other researchers have reported a relation between low NKp46 expression and decreased production of cytokines, such as IFN-γ and IL-13 in allergic responses.[25] Previously, we have also reported the relation between the expression of NKp46 on NK cells and cytokines production by NK cell.[26] We are convinced that NKp46 on NK cell is one of the key regulators of NK cell cytokines production and dysregulation of NKp46 may cause reproductive failure.

In this study, uterine endometrial NK cells from women with RPL had not only lower expression of NKp46, but also lower production of IFN-γ and TNF-α. We have previously demonstrated the importance of IFN-γ for embryo implantation. Briefly, the ratio of IFN-γ-only ($CD56^{+}/IFN\text{-}\gamma^{+}/TNF\text{-}\alpha^{-}$)-producing peripheral blood NK cells was significantly lower in women

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with previous implantation failures compared with controls.[27] We have speculated that IFN-γ-only-producing NK cells are essential for implantation and the decrease of this type of cell causes implantation failure. In fact, the importance of NK-cell-derived IFN-γ for embryo implantation via decidual vascular remodeling has been reported.[7] Uterine endometrial NK cell-derived IFN-γ modifies the expression of genes in the uterine vasculature and stroma, which initiates vessel instability and facilitates pregnancy-induced remodeling of decidual arteries.[7]

In addition, the percentage of TNF-α-producing $CD56^{bright}$ NK cells was significantly lower in women with RPL compared with non-pregnant control women. For successful pregnancy, it is reported that maternally derived TNF-α is essential for trophoblast cell growth[28] and apoptosis,[29] cell differentiation and angiogenesis.[28] On the other hand, exposure of large amounts of TNF-α to pregnant mice causes abortion.[30] For the systemic TNF-α, it is reported that there are no associations between systemic TNF-α level and reproductive outcomes, such as implantation failure and recurrent miscarriage.[31–33] Taken together, uterine NK cell-derived TNF-α and IFN-γ may be important for cell differentiation, angiogenesis, and vascular remodeling. Successful function of TNF-α and IFN-γ in pregnancy, therefore, depends on the appropriate regulation and concentration of TNF-α and IFN-γ produced by NK cells.

We have also evaluated the expression of NKp46 in the peripheral blood of pregnant women at various gestational ages. Intriguingly, pregnant women with a history of RPL had lower expression of $CD56^{bright}$/$NKp46^{+}$ NK cells at 12 and 20 GW compared to pregnant women without a history of RPL. As mentioned above, we have reported lower expression of NKp46 in peripheral-blood NK cells in non-pregnant women with RPL.[16] Taken together, lower expression of NKp46 occurs in both peripheral blood and uterine endometrium NK cells, and that abnormal expression in women with RPL continues during early pregnancy even though their pregnancy is ongoing. Therefore, women with a history of RPL may experience dysfunctional cytokine production by NK cells.

In conclusion, we have demonstrated the importance of NKp46 in reproduction. We have reported decreased expression of NKp46 in peripheral blood in women with previous reproductive failures, such as RPL and implantation failure,[16,26,27,34,35] in peritoneal fluid in women with pelvic endometriosis,[36] and in peripheral blood in pregnant women with gestational diabetes mellitus.[37] These studies suggest that regulation of NKp46 expression in various types of NK cells may be one of the key factors in reproductive failure. Additionally, analysis of NKp46 expression may be a useful tool to investigate and diagnose reproductive failures, such as RPL and implantation failure.

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Disclosure

None declared.

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妊孕性温存のためのランダムスタート法の有用性の検討

Usefulness of the Random Start Ovarian Stimulation Method for Fertility Preservation

浮田 美里　浮田 祐司　表　摩耶　杉山由希子　藤井 雄太
加藤　徹　脇本　裕　福井 淳史　柴原 浩章

MISATO UKITA, YUJI UKITA, MAYA OMOTE, YUKIKO SUGIYAMA, YUTA FUJII, TORU KATO, YU WAKIMOTO, ATSUSHI FUKUI, HIROAKI SHIBAHARA

Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawacho, Nishinomiya, Hyogo 663-8501, Japan

要旨:がん治療前の卵子･胚凍結では，原疾患の治療を早急に開始しなければならない場合が多い。このような場合に，月経周期のうち卵胞期後期や黄体期のいずれの時期から卵巣刺激を開始しても成熟卵や胚盤胞を得られることが近年報告され，ランダムスタート法と呼ばれている。我々も妊孕性温存を要する若年がん女性に対してランダムスタート法を導入し，従来のGnRHアナログ製剤を用いる卵巣刺激法（従来法）と比較検討した。対象はランダムスタート法を行った5人の若年がん女性で，40歳未満の患者62人（採卵回数76回）を対照とした。年齢，AMH値，総ゴナドトロピン（Gn）量，採卵数，M Ⅱ卵数，受精卵数，凍結胚盤胞数をそれぞれ比較検討した。ランダムスタート法VS従来法とすると，年齢（34.8 ± 2.2歳 vs. 34.4 ± 3.3歳），AMH値（3.4 ± 1.1 ng/ml vs. 3.2 ± 1.8 ng/ml），総Gn量（2,475 ＋ 450 IU vs. 2,449 ＋ 762 IU），採卵数（17.2 ± 5.5個 vs. 14.5 ± 6.4個），M Ⅱ卵数（13.8 ± 3.4個 vs. 11.1 ± 5.1個），受精卵数（9.6 ± 1.9個 vs. 9.1 ± 4.4個），凍結胚盤胞数（6.2 ± 2.6個 vs. 5.1 ± 2.8個）と全てにおいて有意差を認めなかった。またランダムスタート法では，初診時から採卵までの日数は平均12.2 ± 1日と，全例において速やかにがん治療を開始できた。以上より，ランダムスタート法は妊孕性温存症例において有用であると考える。

キーワード：ランダムスタート法，妊孕性温存，ART

ABSTRACT: In ovum and embryo freezing before cancer treatment, treatment of the primary disease often must begin promptly. For such cases, it has been reported that mature eggs and blastocysts can be obtained even when ovarian stimulation is initiated in the later stages of the follicular or luteal phases of the menstrual cycle; this is known as a random start ovarian stimulation. We adopted a random start ovarian stimulation protocol for young women with cancer who required fertility preservation, and compared this method with conventional ovarian stimulation using a GnRH analogue. The study subjects were five young women with cancer who underwent random start ovarian stimulation. Seventy-six cycles of 62 infertile patients under the age of 40 were selected as controls. Data for the random start method vs. conventional ovarian stimulation were not significantly different: age (34.8 ± 2.2 vs. 34.4 ± 3.3 years old), AMH value (3.4± 1.1 vs. 3.2 ± 1.8 ng/ml), total Gn amount (2,475 ± 450 vs. 2,449 ± 762 IU), number of eggs (17.2 ± 5.5 vs. 14.5 ± 6.4), number of M II eggs (13.8 ± 3.4 vs.11.1 ± 5.1), number of fertilized eggs (9.6 ± 1.9 vs. 9.1 ± 4.4), and number of frozen blastocysts (6.2 ± 2.6 vs. 5.1 ± 2.8). In random start ovarian stimulation, the average number of days from initial diagnosis to OPU was 12.2 ± 1. Cancer treatment could begin promptly. These findings suggested that random start ovarian stimulation is useful for fertility preservation.

Key words: random start ovarian stimulation, fertility preservation, ART

緒　言

若年がん患者の罹患率は，近年増加傾向を示す一方で，集学的治療や早期診断の進歩に伴いその治療成績は向上し，生存率が増加している。そこで，若年がん患者のQOL向上のため，がん治療前の妊孕性温存が注目されている。2004年に世界で初めて，凍結保存した卵巣組織から妊娠，出産した症例が報告され[1]，2006年に米

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兵庫医科大学病院　産科婦人科学講座：
〒663-8501　兵庫県西宮市武庫川町1-1
TEL: 0798-45-6482　FAX: 0798-45-6936
e-mail: coco.misato.0309@gmail.com

国のWoodruffは，がん患者における妊孕性温存として，OncologyとFertilityを合わせたOncofertility（がん・生殖医療）という概念を提唱し，新たな医療の一領域として認識され，広く知られるようになった[2]。

若年がん患者の妊孕性温存法には，女性では胚（受精卵），未受精卵子，および卵巣組織の凍結保存が[3, 4]，男性では精子の凍結保存が行われている[5]。胚や未受精卵子の凍結保存では，調節卵巣刺激法により少しでも多くの卵子採取（採卵）を目指すことになるが，一方でがん治療を早急に開始しなければならない場合が多い。一般に，ARTで従来から行う卵巣刺激法は月経発来後から開始するため，月経周期に合わせる必要があることから，採卵までに2～5週間必要である。原疾患の治療をできるかぎり早期に開始するためには，これらの月経発来を待つことが困難である場合がある。

このような場合に，月経周期のうち卵胞期後期や黄体期のいずれの時期から卵巣刺激を開始しても成熟卵や胚盤胞を得られる方法が報告され，ランダムスタート法（random start controlled ovarian stimulation）と呼ばれている[6]。以降，本法はがん治療を早急に開始しなければならない，若年がん女性に対する妊孕性温存のための卵巣刺激に有用であると報告されている[6–9]。我々もランダムスタート法を導入し，より早期に妊孕性温存に成功した5症例を経験したため，従来のGnRHアナログ製剤を用いる卵巣刺激法（従来法）と比較検討した。

方　法

対象は2016年7月から2017年8月までに，兵庫医科大学病院生殖医療センターにおいてランダムスタート法を行った5人の若年がん女性（Random start；RS群）である。同時期に同センターで採卵を行った不妊患者101人（採卵回数142回）のうち，40歳未満の患者62人（採卵回数76回）（Conventional；C群）を対照とした。

年齢，AMH値，総Gn量，採卵数，MⅡ卵数，受精卵数，凍結胚盤胞数をそれぞれ比較検討した。統計処理はStudent's t-testを用い，$p<0.05$を有意差ありとした。なお本研究は，当院倫理委員会の承認を得ている。

結　果

RS群の5症例について表1に示す。原疾患は乳癌が2人，急性骨髄性白血病が1人，子宮体癌が1人，肛門管癌が1人であった。平均年齢は34.8 ± 2.2歳，AMH値は3.4 ± 1.1 ng/ml，総ゴナドトロピン（Gn）量は2,475 ± 450 IU，採卵数は17.2 ± 5.5個，MⅡ卵数は13.8 ± 3.4個，受精卵数は9.6 ± 1.9個，凍結胚盤胞数は6.2 ± 2.6個であった。また初診時から採卵までの日数は，平均12.2 ± 1日であった。5例の卵巣刺激開始時期は，卵胞期初期からが2例，卵胞期後期からが1例，黄体期からが1例，無月経期からが1例であった。

RS群とC群の比較を表2に示す。年齢はRS群34.8 ± 2.2歳，C群34.4 ± 3.3歳（P=0.848），AMH値はRS群3.4 ± 1.1 ng/ml，C群3.2 ± 1.8 ng/ml（P=0.886），総Gn量はRS群2,475 ± 450 IU，C群2,449 ± 762 IUであった（P=0.951）。採卵数はRS群17.2 ± 5.5個，C群14.5 ± 6.4個（P=0.483），MⅡ卵数はRS群13.8 ± 3.4個，C群11.1 ± 5.1個（P=0.349），受精卵数はRS群9.6 ± 1.9個，C群9.1 ± 4.4個（P=0.833），凍結胚盤胞数はRS群6.2 ± 2.8個，C群5.1 ± 2.8個であった（P=0.537）。全項目において有意差を認めなかった。

考　察

ARTで用いられてきた従来の卵巣刺激法には，一般にGnRH agonistを用いるlong法・short法と，GnRH antagonist法がある[10]。いずれも月経開始3日前後から卵巣刺激を開始するのが一般的である。その理由は従来，卵胞発育は前月経周期の黄体期後期に前胞状卵胞のリクルートがあり，その後卵胞期中期に1個の卵胞が選択されると，そのほかの卵胞は閉鎖に向かうと考えられていたためである[11]。しかし最近では，同一月経周期のなかで卵胞のリクルートは多くの波があることが示されている[12, 13]。したがって，卵胞期後期や黄体期に認める前胞状卵胞は閉鎖に向かうものではなく，卵胞発育の初期の段階であると考えられている。そのため，どの月経周期でも卵巣刺激を行えば卵胞が発育し，採卵は可能になるという原理による発想がランダムスタート法である。

しかし，月経周期と無関係であることから新鮮胚移植は計画できないため，全胚凍結が必要となる。一般の不妊治療では，子宮内環境不良やOHSSが危惧される症例以外では採卵周期での新鮮胚移植が一般的であり，ランダムスタート法は不向きであるかもしれない。一方で妊孕性温存法の場合は，がん治療終了後の胚移植を前提とするため，全胚凍結が必要であるランダムスタート法の選択が可能となる。

ランダムスタート法は，卵巣刺激の開始時期により，卵胞期初期からの刺激開始，卵胞期後期からの刺激開始，黄体期からの刺激開始に分けられる。卵胞期初期および黄体期から刺激を開始する場合には，早急にGn投与を開始する。卵胞期後期から刺激を開始する場合には，早急にGn投与を開始し，首席卵胞以外の卵胞を発育させる，または首席卵胞を排卵させてからGn投与を開始するこ

表1　ランダムスタート法による治療成績

症例	原疾患	年齢（歳）	AMH値（ng/ml）	平均ゴナドトロピン投与量（IU）	採卵数（個）	MⅡ卵数（個）	媒精法	凍結胚数（個）	初診から採卵までの日数（日）
①	白血病	31	5.2	2,700	31	20	IVF	6	11
②	肛門管癌	35	4.52	2,025	17	14	IVF	8	10
③	子宮体癌	33	2.83	1,800	9	8	ICSI	2	12
④	乳癌	39	1.2	3,150	13	11	IVF	4	14
⑤	乳癌	36	3.27	2,700	16	16	IVF	11	14
平均		34.8 ± 2.2	3.4 ± 1.1	2,520 ± 486	17.2 ± 5.5	13.8 ± 3.4		6.2 ± 2.6	12.2 ± 1

表2　ランダムスタート法（RS群）と対照群（従来法；C群）による治療成績の比較

	RS群（n=5）	C群（n=101）	P値
年齢（歳）	34.8 ± 2.2	34.4 ± 3.3	0.848
AMH値（ng/ml）	3.4 ± 1.1	3.2 ± 1.8	0.886
総Gn量（IU）	2,475 ± 450	2,449 ± 762	0.951
採卵数（個）	17.2 ± 5.5	14.5 ± 6.4	0.483
MⅡ卵数（個）	13.8 ± 3.4	11.1 ± 5.1	0.394
受精卵数（個）	9.6 ± 1.9	9.1 ± 4.4	0.833
胚盤胞獲得数（個）	6.2 ± 2.6	5.1 ± 2.8	0.537

とが推奨されている[6]。今回の5症例に関しては，卵胞期初期からの刺激開始が2例（表1：症例②・④），卵胞期後期からの刺激開始が1例（表1：症例⑤），黄体期からの刺激開始が1例（表1：症例①），無月経期からの刺激開始が1例（表1：症例③）であった。卵胞期後期からの刺激開始以外では，早急に卵巣刺激を開始した。卵胞期後期からの刺激開始の症例ではhCG 5,000単位を投与し，首席卵胞を排卵させてから卵巣刺激を開始した。

従来法，卵胞期後期からのランダムスタート法，黄体期からのランダムスタート法，それぞれの獲得卵子数，成熟卵子数，受精卵数には有意差がないが，ランダムスタート法の方が有意に総Gn量は多かったことがこれまでに報告されている[6, 7, 14]。今回の検討では，総Gn量においても両群に有意差を認めなかった。これは患者の年齢を限定することで，両群のAMH値を同等にして比較検討できたためと推察している。今回の我々の検討では，対象患者数が少なく，卵胞期初期，卵胞期後期，黄体期それぞれの時期から刺激を開始した場合においての比較検討はできなかったが，これまでの報告では，従来法，卵胞期初期，卵胞期後期，黄体期開始群の各々の成熟卵子回収数，受精率に，有意差は認めなかったと報告されている[7]。

妊孕性温存の症例においては原疾患の治療を早急に開始しなければならない。今回の5症例では初診から採卵までの平均日数が12日であり，最大に日数を要した症例においても14日であった。従来法であれば月経開始を待たなければならないため，少なくとも2週間以上の日数を要することを考えると，明らかに早期に採卵可能であった。

妊孕性温存だけではなく，通常のARTで卵巣刺激への反応が低下している症例に対して，Filippoらはランダムスタート法を応用したDuoStim法を初めて報告した[15]。これは同一周期に2回採卵するという方法で，従来法による卵胞期初期からの刺激により，1回目の採卵を行い，引き続き同一周期の黄体期から再度卵巣刺激を行い，2回目の採卵を行う。彼らは卵胞期初期と黄体期の獲得卵子数，成熟卵子数，胚盤胞数に有意差を認めなかったと報告している。妊孕性温存症例に対して，がん治療開始までに許容される日数次第では，DuoStim法を積極的に選択してもよいのではないかと考える。

ランダムスタート法は初めて報告されて以来，まだ経験が少ない。そのため，妊孕性を温存した女性に対して胚移植を行い，従来法と比べて妊娠，分娩率に影響がないかは今後の課題である。一方，がんに罹患していない不妊女性に対し，黄体期から卵巣刺激を開始するランダ

ムスタート法を行い，従来法と変わらない周産期転帰であったとする報告もある[16]。したがって，がん女性に限らず，可及的速やかに卵巣刺激を開始しなければならない高年齢の女性などに対しては，ランダムスタート法による卵巣刺激法は積極的に考慮してよいものと考える。

結　論

今回我々は，ランダムスタート法により早期の妊孕性温存に成功した5症例を経験した。採卵数，獲得卵子数，成熟卵子数，受精卵数，総ゴナドトロピン量の全てにおいて従来法と比較し，非劣性であることを確認した。また，初診時から採卵までの日数も短縮でき，がん治療をより早く開始できた。以上より，ランダムスタート法は妊孕性温存症例において有用であると考える。

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ORIGINAL ARTICLE

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Fertility preservation and pregnancy outcomes in adolescent and young adult male patients with cancer

Yuji Ukita[1] | Yu Wakimoto[1] | Yukiko Sugiyama[1] | Yuta Fujii[1] | Atsushi Fukui[1] | Akiko Hasegawa[1] | Nobuyuki Kondoh[2] | Shingo Yamamoto[2] | Hiroaki Shibahara[1]

[1]Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan

[2]Department of Urology, Hyogo College of Medicine, Nishinomiya, Japan

Correspondence
Yuji Ukita, Department of Obstetrics and Gynecology, Hyogo College of Medicine, Ishinomiya, Japan.
Email: y.ukita0926@gmail.com

Abstract

Purpose: Sperm cryopreservation is the gold standard for maintaining fertility in male survivors of cancer. In order to help increase the future success of fertility preservation in these patients, the present state of sperm cryopreservation was examined at the current institution and its challenges were discussed.

Methods: Between January, 2004 and February, 2017, 31 male patients with cancer were introduced to the center for fertility preservation. The ages and semen characteristics of these patients were examined and compared between those whose sperm were cryopreserved before (the pretreatment group) and after (the post-treatment group) cancer treatment.

Results: The mean sperm concentration of the pretreatment group was significantly higher than that of the post-treatment group. Normozoospermia was found in eight and three patients in the pretreatment and the post-treatment groups, respectively, albeit this difference was not significant. In contrast, the prevalence of azoospermia was higher in the post-treatment group (five patients) than in the pretreatment group (one patient).

Conclusion: As many patients possibly suffer from infertility following chemotherapy, it is necessary to provide fertility preservation opportunities to young male patients with cancer prior to the commencement of cancer treatment.

KEYWORDS
male fertility, oncology, sperm cryopreservation

1 | INTRODUCTION

Human sperm cryopreservation first was achieved in 1953, when cryopreserved human sperm was stabilized successfully and artificial insemination with this sperm led to pregnancy.[1]

Subsequently, the first cases of delivery following in vitro fertilization and intracytoplasmic sperm injection (ICSI) were reported in 1983 and 1994, respectively.[2,3] Traditionally, the cryopreservation of sperm for use in methods such as these has been performed in order to increase the fertility of existing couples that wish to conceive. Testicular sperm extraction (TESE) also has been performed to collect sperm from patients with azoospermia.

Due to rapid developments in medicine, many patients with cancer are now able to survive over longer periods. However, the

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negative effects of various cancer treatments, such as chemotherapy, radiation therapy, and surgery, on future pregnancies often have been reported, especially when the patient is an adolescent or young adult (AYA) man. Chemotherapy first disrupts spermatotype type B and then disrupts sperm cell type A. If type A is impaired, it becomes an irreversible insemination dysfunction.[4] After cytotoxic therapies, germ cells frequently appear to be absent as a result of the killing of spermatogenic stem cells, loss of the support of somatic cells, or a combination of both.[5] In addition, there is sexual dysfunction as a fertility disorder by surgery. In other words, it is due to anatomical changes in the male reproductive organs and pathways, innervation to the reproductive organs, and changes in the endocrine environment. In order to maintain the possibility of fertility in these patients, sperm cryopreservation (the creation of a sperm bank) is the current gold standard.

The authors recently reported on the status of fertility preservation in AYA women with cancer, which involves the cryopreservation of the oocytes, embryos, or ovarian tissue.[6] Although the performance of fertility preservation in male AYA patients with cancer also has been occurring here by means of sperm cryopreservation since 2004, there has been no report on this. Therefore, the aim was to examine the present state of sperm cryopreservation at the current center in terms of patient age and semen characteristics and provide herein some discussion of its challenges. It was planned to study the changes in the semen samples before and after the cancer treatment, as the authors believe that this will help to enable more successful fertility preservation in male AYA survivors of cancer, which will be attempted increasingly in the future.

2 | MATERIALS AND METHODS

This study was approved by the University Ethics Committee, Hyogo College of Medicine, Nishinomiya, Japan, prior to its commencement.

2.1 | Semen sample collection

The semen samples were collected before the cancer treatment in 18 patients (the pretreatment group) and after the cancer treatment in 13 patients (the post-treatment group). After the cancer treatment, four patients visited us within 6 months, nine patients over 3 years. The amount of semen that was collected was confirmed first, and after it was confirmed that it was in a liquefied state, the semen concentration and the exercise rate were evaluated with a Makler calculation board. The semen test was evaluated at least twice. These samples were cryopreserved for future use with the liquid nitrogen vapor method. Briefly, the semen was dispensed into a serum tube with a cryopreservation solution (KITAZATO Bio Pharma, Shizuoka, Japan) and stored in liquid nitrogen.

2.2 | Data analysis

The patient's age and the semen's characteristics at cryopreservation were compared between the pre- and post-treatment groups. The semen's characteristics also were compared within the pretreatment group between those with testicular tumors and those with other cancer types. These statistical analyses were retrospective, with the Student's *t* test and the chi-square test being used, with $P < .05$ being regarded as a significant difference. The course of pregnancy and delivery after the cancer treatment also was reviewed as extensively as possible in the relevant cases.

3 | RESULTS

3.1 | Patient characteristics

Between January, 2004 and February, 2017, 31 male patients with cancer were introduced to the current center for fertility preservation. Their ages at the first visit ranged from 14 to 49 years. In terms of the age distribution within this range, two (3.4%) patients were aged under 20 years, 11 (37.4%) were aged from 20 to 29, 11 (37.4%) from 30 to 39, and seven (20.9%) from 40 to 49 years. The types of cancer among these patients were as follows: testicular cancer in 15 patients (48.6%), with eight patients with testicular cancer in the pretreatment group and six in the post-treatment group; malignant lymphoma in five (16.1%); leukemia in three (9.7%); myelodysplastic syndrome in one (3.2%); germ cell tumor in one (3.2%); pancreatic cancer in one (3.2%); rectal cancer in one (3.2%); colon cancer in one (3.2%); hepatoma in one (3.2%); and maxillary rhabdomyosarcoma in one (3.2%). Three patients died of their original disease after sperm cryopreservation.

3.2 | Comparisons of age and semen characteristics

The average age did not differ significantly between the pretreatment group (33.1 ± 6.2 y) and the post-treatment group (31.5 ± 7.8 y) ($P = .482$). The semen volume also did not differ significantly between groups (pretreatment group, 2.8 ± .7 mL; post-treatment group, 2.8 ± .8 mL) ($P = .896$), nor did the sperm motility between groups (pretreatment group, 39.0 ± 17.1%; post-treatment group, 33.2 ± 29.0%) ($P = .551$). However, the sperm concentration was significantly higher in the pre- than in the post-treatment group (pretreatment group, 60.2 ± 50.6 × 10 mL; post-treatment group, 5.3 ± 6.3 × 10 mL) ($P = .031$). These results are summarized in Table 1.

Other semen characteristics at cryopreservation also were compared between the pre- and post-treatment groups. Normozoospermia was found in eight and three pre- and post-treatment group patients, respectively, which did not represent a significant between-group difference ($P = .275$). The prevalence of oligozoospermia and/or asthenozoospermia also did not differ significantly between groups (pretreatment group, nine patients; post-treatment group, five patients) ($P = .717$). However, the prevalence of azoospermia was lower in the pretreatment group (one patient) than in the post-treatment group (five patients) ($P = .059$). All the patients with azoospermia after cancer treatment had cancers other than testicular tumors. These results are summarized in Table 1.

TABLE 1 Comparison of the semen characteristics at cryopreservation before and after the cancer treatment of 31 patients

Characteristic	Before cancer treatment (n = 18)	After cancer treatment (n = 13)	*P*-value
Age (y)	33.1 ± 6.2	31.5 ± 7.8	.482
Semen volume (mL)	2.8 ± 0.7	2.8 ± 0.8	.896
Sperm motility (%)	39.0 ± 17.1	33.2 ± 29.0	.551
Sperm concentration (10^6/mL)	60.2 ± 50.6	5.3 ± 6.3	.031*
Normozoospermia (%)	8 (44.4)	3 (23.0)	.275
Oligozoospermia and/or asthenozoospermia (%)	9 (50.0)	5 (38.4)	.717
Azoospermia (%)	1** (5.5)	5 (38.4)	.059

*$P < .05$.
**The sperm could be cryopreserved by onco-testicular sperm extraction.

Lastly, no significant difference was found between the patients with a testicular tumor or other cancer within the pretreatment group with regard to any of the characteristics examined (normozoospermia: two patients with a testicular tumor and six other patients with cancer, $P = .170$; oligozoospermia and/or asthenozoospermia: five patients with a testicular tumor and four other patients with cancer, $P = .637$; and azoospermia: one patient with a testicular tumor and five other patients with cancer, $P = .444$). These results are summarized in Table 2.

3.3 | Pregnancy and delivery outcomes after the cancer treatment

Among the 31 patients that were included in this study, there were three cases where infertility treatment resulted in a successful pregnancy and delivery. The first case was a 28 year old man who had a germ cell tumor. His wife delivered a female baby weighing 2698 g following ICSI by using fresh sperm 4 years after chemotherapy. The second case was a 37 year old man who had testicular cancer. His wife conceived twice after ICSI by using frozen-thawed sperm. Although the first pregnancy ended in an abortion at 9 weeks' gestation, the second pregnancy resulted in the delivery of a female baby weighing 2948 g. The third case was a 46 year old man with a hepatoma. His wife delivered a male baby weighing 2948 g following ICSI by using frozen-thawed sperm. These results are summarized in Table 3.

TABLE 2 Comparison of the semen diagnosis at cryopreservation in the testicular tumor to the other cancer before the cancer treatment of 18 patients

Characteristic	Testicular (n = 8)	The others (n = 10)	*P*-value
Age (y)	32.0 ± 2.7	32.4 ± 8.1	.700
Semen volume (mL)	2.8 ± .9	2.7 ± 0.6	.813
Sperm motility (%)	34.5 ± 20.8	42.5 ± 11.9	.422
Sperm concentration (10^6/mL)	26.1 ± 23.1	87.4 ± 73.2	.166
Normozoospermia (%)	2 (25.0)	6 (60.0)	.170
Oligozoospermia and/or asthenozoospermia (%)	5 (62.5)	4 (40.0)	.637
Azoospermia (%)	1 (12.5)	0 (.0)	.444

TABLE 3 Pregnancy and delivery outcomes after cancer treatment

Case	Age (y)	Type	ICSI	Outcome
1	28	GCT	Fresh sperm	Delivery (♀, 2698 g)
2	37	Testis	Frozen sperm	Miscarriage (9 wk)
			Frozen sperm	Delivery (♀, 2948 g)
3	46	Hepatoma	Frozen sperm	Delivery (♂, 2948 g)

♀, female; ♂, male; GCT, germ cell tumor; ICSI, intracytoplasmic sperm injection.

4 | DISCUSSION

In recent years, cancer treatment outcomes for young persons have improved significantly. As a result, their quality of life after cancer treatment has been attracting attention. For both sexes, fertility preservation greatly influences the post-treatment quality of life, especially for young persons of reproductive age. This highlights the importance of using fertility preservation measures prior to cancer treatments, such as chemotherapy or radiotherapy. As previously mentioned, sperm cryopreservation is a well-established method and is the current gold standard for fertility preservation, with reports of a successful delivery in 50% of patients with cancer following ICSI with cryopreserved sperm.[7] As such, it should be the first fertility preservation choice for men of reproductive age. Indeed, the American Society of Clinical Oncology (ASCO) strongly recommends the cryopreservation of sperm prior to cancer treatment for men of reproductive age.[8]

In order to facilitate improvements in fertility preservation for young male patients with cancer, this study aimed to examine the

present state of sperm cryopreservation at the current institution. It was found that although there was no significant difference in the semen volume or sperm motility between the pre- and post-treatment groups, the sperm concentration was significantly higher in the former. These findings are similar to data from other studies in terms of sperm concentration, but not with respect to sperm motility.[9] However, it should be noted that as the sperm motility already might be impaired in patients with a testicular tumor prior to the cancer treatment, the inclusion of data from these patients could have affected this study's results. Indeed, it was found that when the patients with a testicular tumor were excluded, the sperm motility tended to be higher in the pre- than in the post-treatment group.

Other semen characteristics that are known to be affected by cancer treatment also were examined, with a key finding being that azoospermia was more prevalent in the post- than in the pretreatment group. This is unsurprising as it is known that both chemotherapy and radiotherapy, which are often used to treat testicular tumors following high-grade radical orchiectomy, can cause irreversible azoospermia.[10] Specifically, chemotherapy often impairs spermatogenic function and causes azoospermia within 50-60 days. If low-testicular toxicity drugs are selected, however, the spermatogenic function can be recovered within 3 months.[11] In contrast, recovery can be delayed or, in the worst cases, prevented altogether if high-testicular toxicity drugs are selected.[12] With regard to radiotherapy, it is known that testicular irradiation of <.8 Gy leads to oligozoospermia, irradiation of .8-2.0 Gy leads to azoospermia, and irradiation of >2.0 Gy leads to irreversible azoospermia.[13] Considering this study's finding that the semen characteristics tend to deteriorate further after cancer treatment, it is suggested that fertility preservation by sperm cryopreservation needs to be carried out prior to treatment. This, in addition to continued medical progress, could enable the harmful impacts of testicular tumor treatment on fertility to be minimized even further. However, oligozoospermia and asthenozoospermia often are found in patients with a testicular tumor even before the cancer treatments are started.[10,14,15] Indeed, it has been reported that 10%-15% of patients with testicular cancer suffer from azoospermia and that >50% suffer from oligozoospermia prior to any cancer treatment.[14,15] This study found that 11.2% of the patients had azoospermia and 62.5% had oligozoospermia. In particular, one patient with a testicular tumor was found to have azoospermia prior to his cancer treatment. In such cases where sperm cryopreservation cannot be performed by using ejaculated semen, a radical orchiectomy with simultaneous onco-TESE could be an option for fertility preservation.[16]

Sperm cryopreservation, or the creation of a "sperm bank," is the only clinically available method of fertility preservation for men. This potentially could impede sperm collection in some preteen boys who have never masturbated. Although this study included one 14 year old boy, fortunately he could collect sperm by masturbation. Also, of the two patients under 20 years, the same was true for another 18 year old man. If a patient never has experienced masturbation, however, sperm can be collected by vibrator stimulation or by electrical stimulation of the penis.[10,17] The preservation of fertility in prepubertal boys also presents a major challenge as there is no mature sperm in their gonads. However, fertility preservation methods for preteen boys whose testes have not begun spermatogenesis have yet to be established. The ASCO expresses at present that the only option for prepubertal boys is testicular freezing, though this method is still at the experimental stage. Specifically, although the key aim of this method is sperm induction by autologous transplantation after the thawing and in vitro culture of frozen testicular tissues, this has yet to be achieved.[18] Therefore, further research on improving the techniques for the cryopreservation of testicular tissues or immature spermatozoa is required.

Regarding the viability of frozen sperm, it is thought that fertility remains sufficient even after cryopreservation. There are many reports that ICSI outcomes are the same, regardless of whether fresh or frozen sperm are used.[3,19–21] In this study, the pregnancies in total were established from three patients who underwent infertility treatment. Although one pregnancy ended in an abortion at 9 weeks' gestation, three reached delivery. Among these three successful delivery cases, one involved a patient with a testicular tumor who was referred to the center for sperm cryopreservation prior to cancer treatment. However, the findings from his semen examination 4 years after the cancer treatment were normal and his wife conceived by ICSI using freshly ejaculated, rather than frozen, sperm.

As aforementioned, this indicates that fertility might be sufficiently maintained and that the seminal findings can return to normal after treatment in some patients with testicular cancer. In other cases, however, such as where alkylating agents are used for the treatment of Hodgkin's disease, testicular toxicity occurs. Therefore, as previously stated, sperm cryopreservation before cancer treatment is important, especially when drugs that are known to result in testicular toxicity are used. In addition, there might be a significantly increased risk of chromosomal abnormalities in the sperm of chemotherapy patients during and immediately after treatment. It is suggested that doctors should provide patients with advance notice that semen examination or counseling could be required after cancer treatment.

With regard to the usage patterns of cryopreserved sperm, frozen sperm was thawed and used three times in this study. The usage rate was 9.7%, which is similar to the rates of 2.7%-11.0% in both domestic and foreign reports.[22–24] In these reports, the low usage rate of frozen sperm appears to have been related to patients remaining single throughout and after malignant tumor treatment.[25] At the authors' center, only 10 (32.3%) of the patients were married. Thus, the low usage rate here can be assumed to be due to the same reasons as in other reports. In this study, the number of target patients was small and it was impossible to examine the difference by type of cancer. In the future, such a study as this should be done on more target patients. Each patient visited us during the post-treatment period. It was not possible to compare the difference between before and after the cancer treatment in the same patient.

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DISCLOSURE

Conflict of interest: The authors declare no conflict of interest. *Human rights statement and informed consent:* The protocol for the research project was approved by the Hyogo College of Medicine Ethics Committee, Nishinomiya, Japan. All the procedures were followed in accordance with the ethical standards of the responsible committees on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all the patients to be included in the study. *Animal studies:* This article does not contain any studies with animal participants that have been performed by any of the authors.

ORCID

Yuji Ukita http://orcid.org/0000-0001-9963-4705

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臨床経験

当センターにおける不妊患者の周産期予後の検討

松岡理恵* 浮田祐司* 浮田美里* 脇本 裕* 坂根理矢*
原田佳世子* 福井淳史* 田中宏幸* 柴原浩章*

ART後の妊娠は，母児ともにハイリスクであると報告されている。そこで，当センターにおいて不妊治療後に妊娠し，分娩に至った妊婦の周産期予後について後方視的に検討した。最近の4年間に当センターで妊娠・分娩に至った単胎妊娠190例を対象とし，その対象を〈ART後妊娠群(以下，ART群)〉，〈ART以外の不妊治療妊娠群(以下，非ART群)〉の2群に分類し，周産期予後について比較した。ART群は，今回の全分娩数の40.5%であった。今回の検討において，ART群の母体年齢は非ART群と比較して有意に高く，帝王切開率もART群で有意に高かった。また妊娠高血圧症候群(HDP)率はART群(26.53%)で有意に高く，切迫早産，妊娠糖尿病(GDM)，前置胎盤は有意差を認めなかったものの，高い傾向にあった。従来からの報告どおり，ART妊娠はハイリスク妊娠と位置づけ，より慎重な周産期管理を行うべきである。

はじめに

現在，周産期医療において，高年齢妊娠，合併症妊娠，帝王切開分娩などのいわゆるハイリスク妊娠が増加しているが，その一因に不妊治療後の妊娠が関与していることが指摘されている。最近では生殖補助医療(assisted reproductive technology；ART)により出生する児は増加しており，この傾向は今後も続くものと考えられる。ART妊娠では母体の高年齢化や基礎疾患の合併などの増加が指摘されている。そこで当センターにおける不妊患者の周産期予後を，ART群と非ART群に分け，検討した。

1．方　法

2013年1月～2016年12月の4年間に当センターを受診した510人のうち，妊娠成立(妊娠判定後，胎嚢を確認)した166人を対象とした。妊娠は延べ190例であり，その対象を，〔ART後妊娠群(以下，ART群)〕〔ART以外の不妊治療妊娠群(以下，非ART群)〕の2群に分類し，両群の周産期予後を比較検討した。なお，統計処理はχ^2検定，student's t-testを用いた。統計学的有意水準は$p<0.05$とした。なお本研究は施設内倫理委員会の承認を得ている。

2．結　果

対象となる190例の妊娠のうち，分娩に至ったのは121例(63.7%)で，そのうちA群が49例(40.5%)，B群が72例(59.5%)であった(図1)。

流産率はART群で38.75%，非ART群で34.54%であり，有意差を認めなかった。帝王切開率はART群で59.2%，非ART群で33.3%であり，ART群において有意に高頻度であった

* M. Matsuoka, Y. Ukita, M. Ukita, Y. Wakimoto, R. Sakane, K. Harada, A. Fukui, H. Tanaka, H. Shibahara　兵庫医科大学病院生殖医療センター

(p＝0.006)。切迫早産率はART群で32.6%，非ART群で18.05%であり，有意差を認めなかった。妊娠高血圧症候群(hypertensive disorders of pregnancy；HDP)率はART群で26.53%，非ART群で19.72%であり，ART群で有意に高頻度であった(p＝0.023)。妊娠糖尿病(gestational diabetes mellitus；GDM)率はART群で12.24%，非ART群で4.16%であり，有意差を認めなかった。前置胎盤率はART群で6.1%，非ART群で1.3%であり，有意差を認めなかった。分娩週数はART群で37w6d±2.1d，非ART群で38w5d±1.3dで，有意差を認めなかった。分娩時年齢はART群で36.8±3.5歳，非ART群で34.9±3.2歳で，ART群において有意に高齢であった(p＝0.015)。出生体重はART群で2,765±561 g，非ART群で2,848±385 gで，有意差を認めなかった(表1)。

以上より，帝王切開率，HDP率，分娩時年齢は，ART群で有意に高かった。流産，切迫早産，GDM，前置胎盤は両群で有意差を認めなかったものの，ART群で有病率が高い傾向にあった。

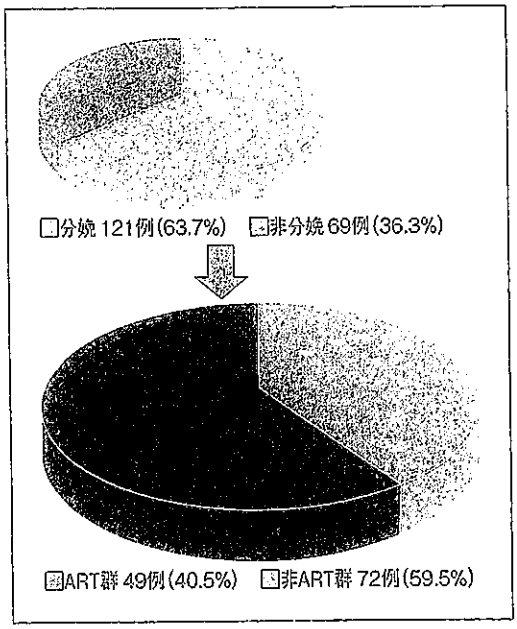


図1　分娩に至った症例における不妊治療でのART依存度

3．考　察

ARTによる妊娠分娩症例について，帝王切開率，早産，HDP，前置胎盤や多胎妊娠などの増加がこれまでに報告[1)~3)]されており，今回のわれわれの検討もこれらを支持するものであり，帝王切開率，HDP，分娩時年齢がART群で有意に高かった。

分娩時年齢はART群で有意に高かったが，加齢に伴い不妊治療を受ける患者が増加し，そのなかでもARTが不妊治療の最終的な選択肢になることが多いためだと考えられる。

帝王切開率に関しては，初産においては，母

表1　不妊患者の周産期予後の比較(ART群vs非ART群)

	ART群	非ART群	p
分娩時年齢(歳)	36.8±3.5	34.9±3.2	0.015
流産(%)	38.75	34.54	0.647
帝王切開(%)	59.2	33.3	0.006
切迫早産(%)	32.6	18.05	0.083
HDP(%)	26.53	9.72	0.023
GDM(%)	12.24	4.16	0.156
前置胎盤(%)	6.1	1.3	0.302
分娩週数	37w6d±2.1d	38w5d±1.3d	0.09
出生体重(g)	2,765±561	2,848±385	0.471

体年齢の上昇に伴って帝王切開率が上昇するという報告がある。40歳以上の初産においては，5人に1人がART妊娠であると報告されている[4]。20歳代後半の初産群は8.7%であるのに対し，40歳以上の初産群では23.4%と有意に増加するとの報告[5]もあり，ART群では高年齢初産の割合が高く，それにより帝王切開率が上昇したと考えられる。また，高年齢妊婦では，子宮筋腫核出術や帝王切開，流産による子宮内掻爬といった子宮手術の既往が多いため，前置胎盤や癒着胎盤の発生のリスク因子が多いことが考えられる。加えて，不妊症の原因として子宮内膜の形成不全や欠損が一部の症例に存在すること，胚移植時の子宮内操作の影響なども考えられる[2]。また，当センターにおいては，母体が高年齢であるという年齢因子だけでは帝王切開の適応とはしていないが，高帝王切開率の背景には，高年齢を理由に妊婦自身が帝王切開を強く希望することや，医療者側の意識などの要因も挙げられている[6]。

切迫早産については，ART群と非ART群で有意差は出なかった。2009年に単一胚移植を原則とする日本産科婦人科学会の会告[7]が発表され，その後多胎妊娠率は低下傾向にあり，多胎妊娠による周産期合併症が低下した影響と考えられる。

HDPについては，ART群で有意に有病率が高く，非ART群でも高い傾向にあった。HDPの一時的な原因は胎盤の形成不全にあり，二次的に母体の全身の内皮細胞障害が起こるため発症する[8]。高年齢婦人では潜在的に高脂血症や動脈硬化の予備軍となり，血管の弾力性が減弱している可能性がある。この場合，妊娠前より内皮細胞障害があるために，HDPを発症しやすいと考えられる。ART群では妊婦が高年齢である割合が高く，そのため有病率が有意に高かったことが推測される。

GDMについては，2011年1月～2016年12月の当院でのデータ[9]では合併率3.4%であり，それと比較するとART群では有意に高く($p=0.004$)，非ART群でも高い傾向にあった。血糖の調節にはインスリン分泌も重要であるが，インスリン抵抗性も関与しており，両者のバランスによって血糖値が正常範囲に保たれる。インスリン抵抗性が増大しても，インスリン分泌がそれに見合って増加すれば正常血糖値が保たれるが，インスリン抵抗性の増大に対して相対的にインスリン分泌が不足すると，高血糖をきたすことになる[10]。インスリン抵抗性を増大させる因子の1つとして加齢があり，ART群で有意に合併率が高くなったと考えている。

妊婦の高齢化に伴い基礎疾患の合併も増加するため，ART群で高い傾向となったが，当センターは周産期センターであるため，非ART群においても，通常よりも高年齢であるハイリスクの患者が多く，そのため両群で有意差が出なかったと考えられる。

ART群では，前置胎盤の合併を6.1%に認めた。このことも帝王切開率の上昇に寄与しているといえる。胎盤の位置異常を引き起こす要因には，高年齢(35歳以上)，5回以上の妊娠歴，3回以上の子宮内掻爬の既往などが報告されている[11]。ART群では有意に高齢であるため，前置胎盤の合併率が高くなったと考えられる。

流産，切迫早産，GDM，前置胎盤，分娩週数，出生体重など有意差を認めなかった項目に関しては，当院は総合周産期母子医療センターであり，非ART群であってもハイリスクな症例が多く含まれていることが原因である可能性がある。

おわりに

今回の結論は既報のデータを支持する結果である。ART妊娠はハイリスク群と位置づけ，より慎重な周産期管理を行うべきであり，われわれもそれを認識して患者に説明する必要があるといえる。非ART妊娠についても，不妊治療後の妊婦も一般妊婦と比較すると，同様に慎重な周産期管理が必要とされる。

また，高齢初産婦の割合も高く，吸引・鉗子分娩率の上昇，微弱陣痛や回旋異常が原因の帝王切開率や癒着胎盤の上昇も報告[12]されてお

り，分娩時も慎重に管理する必要があるといえる。

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〔パネルディスカッション1－3／子宮内膜症合併不妊～次の一手は～〕

子宮内膜症性嚢胞合併不妊症に対するエタノール固定術

1）兵庫医科大学産科婦人科学講座
2）弘前大学大学院医学研究科産科婦人科学講座

福井　淳史[1)]，山谷　文乃[2)]，横田　　恵[2)]，福原　理恵[2)]，柴原　浩章[1)]

はじめに

子宮内膜症性嚢胞の手術療法は妊孕性の向上につながるとの報告が多く，これに対する手術療法は嚢胞核出術が標準的治療となっている．一方，嚢胞核出術により正常卵巣組織の一部が摘出される可能性や卵巣刺激に対する反応が減弱する可能性も指摘されている．そもそも内膜症性嚢胞は月経血の逆流により腹腔内に流入した子宮内膜組織とともに卵巣皮質が嵌入することにより生じる偽嚢胞であると考えられており〔1〕，嚢胞壁には多くの卵が存在する．すなわち安易な卵巣嚢胞の核出は，卵の喪失を意味しており〔2, 3〕，内膜症性嚢胞への対応は慎重にする必要がある．実際，欧州ヒト生殖医学会では，3 cm 以上の内膜症性嚢胞に対する ART 前の核出術は，妊孕能を向上させるとのエビデンスはなく，3 cm 以上の内膜症性嚢胞に対する ART 前の核出術は，内膜症関連の疼痛や卵胞へのアクセス性を向上させるためにのみ行うとしている〔4〕．われわれは，不妊症症例の子宮内膜症性嚢胞に対して卵巣機能を温存することを目的に子宮内膜症性嚢胞に対して施行した腹腔鏡補助下経腟的エタノール固定術を施行している．本稿ではわれわれの行っている腹腔鏡補助下経腟的エタノール固定術の方法を詳述するとともにエタノール固定術の有用性を文献的に考察する．

エタノール固定術とは

エタノール固定術とは，嚢胞内にエタノールを注入することにより，嚢胞の上皮細胞を脱水・凝固固定させることにより分泌を抑制する方法である．卵巣内膜症性性嚢胞に対するエタノール固定術は，経腟的，経腹的など種々のアプローチが可能である．再発卵巣内膜症性嚢胞に対して行われる場合には経腟的に行われていることが多いものと思われる．現在でも体外受精・胚移植の採卵に先だって，あるいは採卵時などに行っている施設があるものと思われる〔5, 6〕．エタノール固定法についても種々の報告がなされており，0分から15分間エタノール固定を行った後に内腔を洗浄する方法やエタノールを嚢胞内に充填したままにする方法などが報告されているが，次に示すエタノール漏出による癒着形成やエタノールの長時間暴露による卵への影響を考慮したうえで，われわれはエタノール固定術を施行している．

エタノール固定術の問題点

Okagaki らは，経腟的エタノール固定術後の腹腔鏡手術所見から経腟的エタノール固定術の問題点を報告している．彼らは，エタノールの卵巣外漏出によりエタノール固定術後の高度かつ非定型的な癒着が起こる可能性があること，エタノールの卵巣皮質への影響がある可能性があること，エタノール固定では組織学的検索ができないことから卵巣内膜症性嚢胞を有する不妊婦人に対して施行すべきではないと報告している〔7〕．

われわれの施行している腹腔鏡補助下経腟的エタノール固定術および子宮内膜症病巣除去術

先に述べたようにエタノール固定術にはいくつかの問題点がある．確かに不妊婦人への腹腔鏡手術は子宮内膜症などの症状を改善させるこ

とだけではなく，妊孕能を高めることが求められる．このため，卵巣内膜症性嚢胞を小さくすることだけを目的に安易にエタノール固定術が行われることは正しい選択ではないと思われる．われわれは，エタノール固定の欠点をなくし，卵巣機能を可能な限り温存させる方法を試みている．われわれの術式を以下に示す．術式は経腟超音波ガイド下経腟的卵巣内膜症性嚢胞エタノール固定術と腹腔内操作に分かれる．手術は全身麻酔下に施行し，経腟的操作に先立って腹腔鏡のスコープおよび鉗子操作用のトロッカーを設置する．この後エタノール固定による卵巣からのエタノール漏出予防のために腹腔内に生理食塩水を1,000 ml～2,000 ml注入する（図１）．これにより万が一エタノール固定中にエタノールが漏出したとしても希釈されるため，術後の癒着は防止できると思われる．また卵巣内膜症性嚢胞の発生メカニズムを考慮した場合，腹腔内操作をすることにより卵巣内膜症性嚢胞と腹膜との癒着が剝離されることにより嚢胞が破綻し，内容液が漏出してしまい固定が困難となるため，生理食塩水を注入する以外の腹腔内操作はすべてエタノール固定術後に行う．また卵巣内膜症性嚢胞への穿刺は経腹的に行うことも可能であるが，同様の理由で腹腔内からの操作による嚢胞破綻を防ぐため行わない．続いて経腟超音波断層法下に卵巣内膜症性嚢胞を穿刺し，十分に内容を吸引・洗浄する．この時内容液は非常に粘稠であることもあるので，その際は温生食を適宜注入するなどして少しずつ内容液を吸引し，内腔を洗浄することが肝要である．また穿刺は細径のものでは吸引が困難であることがあるため，われわれは16Gの穿刺針を用いている．洗浄液が十分に綺麗になるまで生理食塩水で５～10回ほど洗浄したのちに嚢胞容積の80％量の純エタノールを用いて固定する（図２）．固定時間は種々の報告があるが，われわれは卵巣皮質に存在する卵への影響を最小限にするために７分間としている〔8〕．固定終了後は，再度嚢胞内を十分に洗浄した後に子宮マニピュレータを挿入し腹腔内操作へ移る（図３）．

続いて腹腔内に注入した生理食塩水を吸引し，腹腔内を観察し，通常の子宮内膜症病巣除去術と同様に腹腔内の癒着を剝離する．このとき卵巣周囲と腹膜との間の癒着を剝離すると，病変が発生したと思われる癒着部分が開放され

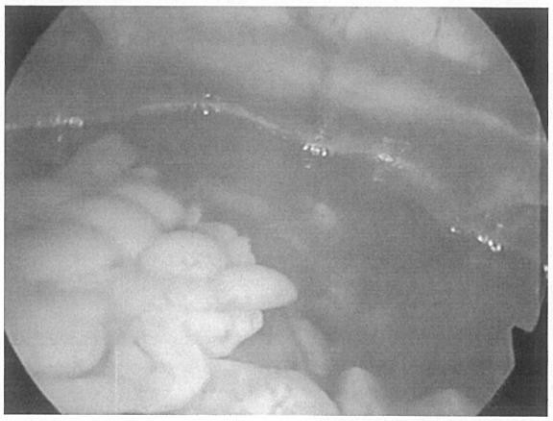


図１　腹腔内への生理食塩水注入

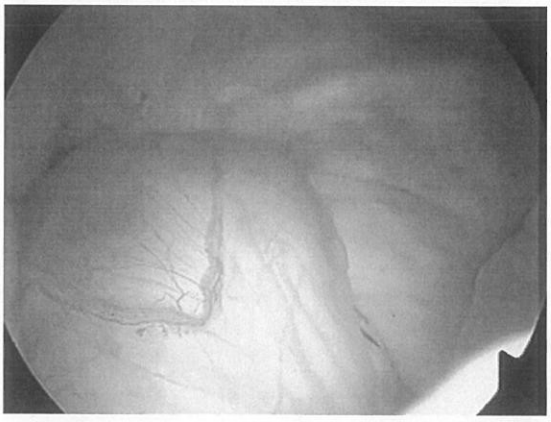


図２　内膜症性嚢胞の吸引・洗浄・固定

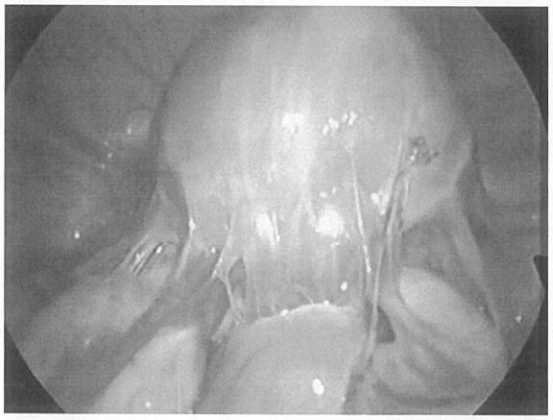


図３　子宮マニピュレータを挿入し剝離操作を開始する

る（図４）．この部位から鈍的に内腔を開放するとスムースに嚢胞内腔が開放される．これは内膜症性嚢胞の多くが卵巣皮質の嵌入による偽嚢胞であることを示しており，また開放された嚢胞壁がもともと卵巣皮質であるということを示していると思われる．開放された嚢胞壁は飜転し，充実部分の有無など嚢胞壁を十分に観察する．また一部を生検することにより嚢胞壁の組織学的検索が可能となる（図５）．卵巣内膜症性嚢胞以外への操作以外の腹腔内操作は通常の子宮内膜症性嚢胞除去術と同様であり，腹腔内の癒着剝離や，病変の焼灼，病巣除去，腹腔内洗浄などを行い手術を終了する．なお，抗生剤投与は他の腹腔鏡手術と同様に術中投与のみとしている．

エタノール固定術の有用性

エタノール固定はその再発率の高さが問題となる可能性がある．最近の報告〔9〕によると，

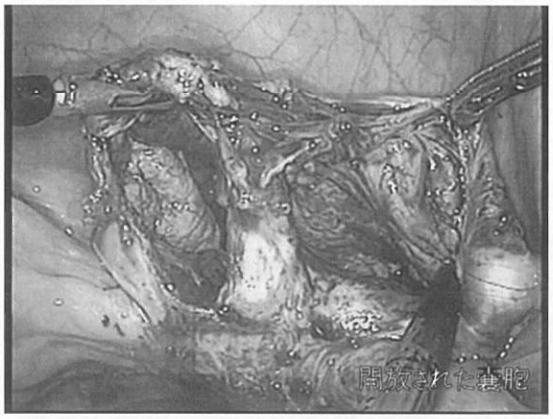


図４　開放された内膜症性嚢胞

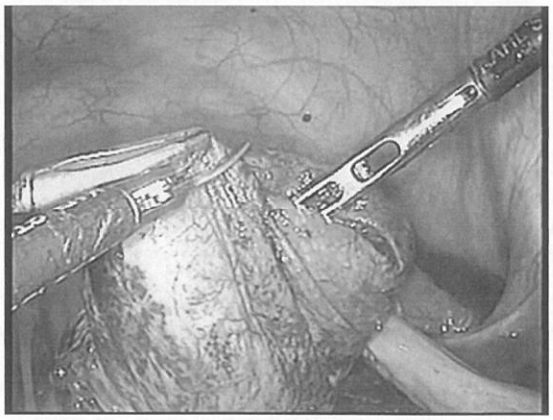


図５　内膜症性嚢胞を飜転して生検を行う

エタノール固定による再発率は０～62.5%であるとされており，嚢胞内にアルコールを残したままにした場合で０～13.3%，エタノール固定後に嚢胞を洗浄した場合で０～62.5%であるとされている．さらに固定時間が10分未満の場合の再発率は62.5%，10分以上の場合は9.1%であったとする報告もあり〔10〕，再発率だけからみると嚢胞内にエタノールを充填したままにしたほうがよいように思われるが，われわれの検討〔8〕では，長時間の固定により卵が傷害されたり，万が一漏出が起こった場合，高度癒着を引き起こしたりする可能性がある〔7〕ため注意が必要である．われわれの検討では，再発率は内膜症性嚢胞核出術に比してエタノール固定術では有意に再発率が高値であった（未発表データ）．しかし，不妊症症例を対象とした術後の妊娠率には，術後の自然妊娠においても体外受精・胚移植による妊娠においても，内膜症性嚢胞核出術群，エタノール固定術群との間には差を認めていない（未発表データ）．さらに，体外受精・胚移植を施行した患者では採卵数には差を認めないものの，調節性過排卵刺激に使用したFSH製剤の量はエタノール固定術群で有意に少なく，エタノール固定術は内膜症性嚢胞核出術に比して低侵襲であろうといえる１つの根拠であると思われる（未発表データ）．他家の報告をみても，エタノール固定術後に施行した体外受精・胚移植による妊娠率は嚢胞核出術と差がないことが報告されており〔9〕，少なくともエタノール固定術により嚢胞核出術に比して妊孕能が低下することはないようである．

腹腔鏡補助下経腟的エタノール固定術は，エタノール固定術によって起こりうる合併症を軽減させつつもエタノール固定術による卵巣に対する低侵襲性を担保することができ，さらには腹腔内の子宮内膜症病変に対する処置も可能となるため，妊孕性の温存，完遂度など内膜症性嚢胞核出術に匹敵する治療法になり得ると考えられた．

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Case Reports and Images in Surgery

Research Article

Status of ovarian tissue cryopreservation for adolescent and young adult patients with cancer in Hyogo College of Medicine College Hospital

Wakimoto Y*, Ukita Y, Fuji Y, Wakimoto G, Fukui A and Shibahara H

Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Abstract

Objective: Cancer treatment for adolescent and young adult (AYA) patients with cancer can have lasting effects on their reproductive health by causing premature menopause. Fertility can be preserved by cryopreservation of oocytes, embryos, or ovarian tissue. Recently, many cases of pregnancy and delivery after ovarian tissue warming and transplantation have been reported. The number of ovarian tissue cryopreservation cases has been increasing in our hospital. Herein, we evaluated the current status of ovarian tissue cryopreservation among AYA patients.

Method: Ovarian tissue cryopreservation was performed in nine cases at our hospital between February 2017 and March 2018. In 6 of the 9 patients, oocyte pick-up without controlled ovarian stimulation was performed for both ovaries during the removal of one ovary by laparoscopic surgery. We retrospectively collected data from the patients' medical records.

Result: The patients ranged in age from 11 to 43 years, and all the patients were nulligravida. Among these patients, 3 had breast cancer; 2, leukemia; 1, Ewing sarcoma; 1, vulvar sarcoma; 1, mediastinal tumour; and 1, suprasellar germ cell tumour. Five of the 9 patients underwent ovarian tissue cryopreservation after chemotherapy. The mean anti-Müllerian hormone (AMH) level was 1.54 ng/ml. Twenty-six oocytes were collected from 6 patients. Ten oocytes matured in vitro could be cryopreserved.

Conclusion: So far, there have been no cases of pregnancy by ovarian tissue warming and transplantation. Further accumulation of cases and consideration is needed to yield any findings on the usefulness of this technique.

Introduction

Cancer treatment in adolescent and young adult (AYA) patients with cancer can have lasting effects on their reproductive health by causing premature menopause [1,2]. Fertility can be preserved by cryopreservation of oocytes, embryos, or ovarian tissue [1,3].

At our institution, we have been performing fertility preservation by cryopreservation for AYA patients with cancer since 2013 [3]. Recently, many cases of pregnancy and delivery after ovarian tissue warming and transplantation have been reported [1,4]. The number of ovarian tissue cryopreservation cases in our hospital has been increasing.

Here, we evaluated the status of ovarian tissue cryopreservation in AYA patients with cancer in Hyogo College of Medicine College Hospital.

Materials and methods

Ovarian tissue cryopreservation was performed in 9 cases at our hospital between February 2017 and August 2017. Vitrification of the ovarian tissue was performed using the cryotissue method (Cryotissue Kit; *Kitazato* BioPharma Co., Ltd., Shizuoka, Japan). In 6 of 9 patients, oocyte pick-up (OPU) without controlled ovarian stimulation was performed during removal of one ovary under laparoscopic surgery. The contents of small follicles on the ovarian surface are aspirated under laparoscopic surgery by using a suction needle (Hakko Medical Co. Ltd., Tokyo, Japan) equipped with a syringe to retrieve oocytes from one ovary. The contents of the small follicle on the ovarian surface are manually aspirated from another removed ovary by using a 23-G needle equipped with a syringe. Immature oocytes were then incubated in *Sage IVM* medium (Cooper Surgical, Trumbull, CT, *USA*) and matured in vitro to the MII stage. Mature oocytes obtained from IVM were cryopreserved using the Cryotop method (Kitazato BioPharma Co., Ltd., Fuji, Shizuoka, Japan).

We retrospectively collected data from medical records and analysed age, gravidity or parity, marriage, primary disease, previous treatment, anti-Müllerian hormone (AMH) levels, OPU, the number of oocytes collected, the number of cryopreserved oocytes, hospitalization period, the number of days from first visit to operation, and the number of fertility preservation cases by cryopreservation of oocytes, embryos, or ovarian tissue.

Result

Figure 1 shows the comparison between the number of cycles and fertility preservation techniques by cryopreservation of oocytes, embryos, or ovarian tissue. Fertility preservation was performed in

Correspondence to: Wakimoto Y, Department of Obstetrics and Gynecology, Hyogo College of Medicine, Mukogawa-cho, Nishinomiya, Hyogo, Japan, Tel: +817984564 81; Fax: +81798464163; E-mail: yuuwakimoto@gmail.com

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7 cycles in 2018 (January to March), 20 cycles in 2017, 18 cycles in 2016, 15 cycles in 2015, three cases in 2014, and one case in 2013. The first ovarian tissue cryopreservation under medical indication for an unmarried woman with breast cancer was performed at our centre in February 2017. The distributions of fertility preservation cases with oocytes, embryos, and ovarian tissue cryopreservation were as follows respectively: 1, 0, and 0 in 2013; 3, 0, and 0 in 2014; 13, 2, and 0 in 2015; 9, 8, and 0 in 2016; 6, 6, and 7 in 2017; and 3, 2, and 2 in 2018 (January to March). In 2016, split cryopreservation, which includes both embryo and oocyte freezing in an OPU cycle, was performed in 1 case.

Table 1 shows 9 cases of ovarian tissue cryopreservation. The patients' ages ranged from 11 to 43 years, and all patients were nulligravida. Among these patients, 3 had breast cancer; 2 had leukemia; 1 had Ewing sarcoma; 1 had vulvar sarcoma; 1 had mediastinal tumour; and 1 had suprasellar germ cell tumour. Five of the 9 patients had their ovarian tissue cryopreserved after chemotherapy. The mean AMH level was 1.54 ng/ml. Twenty-six oocytes were collected from 6 patients. Ten oocytes matured in vitro could be cryopreserved. The mean length of hospitalization was 5.11 days. The mean interval from first visit to operation was 16.3 days.

Discussion

The world's first live birth after cryopreserved ovarian tissue transplantation was reported in a woman with stage IV Hodgkin lymphoma in Belgium in 2004 [5] . According to this report, in 1997, ovarian tissue was taken and cryopreserved before cancer treatment. Following this, the patient had premature ovarian insufficiency. In 2003, transplantation of thawed ovarian tissue was performed using laparoscopy. The first pregnancy after this procedure was reported in 2004; since then, the pregnancy and live-birth rates have continued to increase steadily and have been exponentially increasing in the past 2 years [1]. Although the number of transplantations performed worldwide is unknown, the reported number of live births by ovarian tissue cryopreservation and transplantation is >130 cases as of June 2017, with pregnancy and live-birth rates being approximately 30% and 25%, respectively [1].

At our hospital, ovarian tissue cryopreservation was performed in 9 cases. A center for reproductive medicine was established in 2013 at our hospital. A regional oncofertility network of patients, oncologists, and fertility specialists in Gifu prefecture (GPOFS) was established for the first time in Japan in February 2013 [6]. This oncofertility network is a medical institution cooperation system to provide information related to oncofertility counselling for AYA patients with cancer [7] . In Hyogo prefecture, the Hyogo oncofertility network (Hyogo-OFN) was established in collaboration with the Hyogo Society of Obstetrics and Gynaecology and Hyogo Prefecture Regional Cooperation Conference of Cancer Management in January 2016 [3]. Therefore, the number of young women with cancer who received fertility preservation increases every year in our hospital. The number of fertility preservation cases by means of ovarian tissue cryopreservation has been increasing since 2017. So far, at our hospital, none of the cases has requested the use of ovarian tissue warming and transplantation.

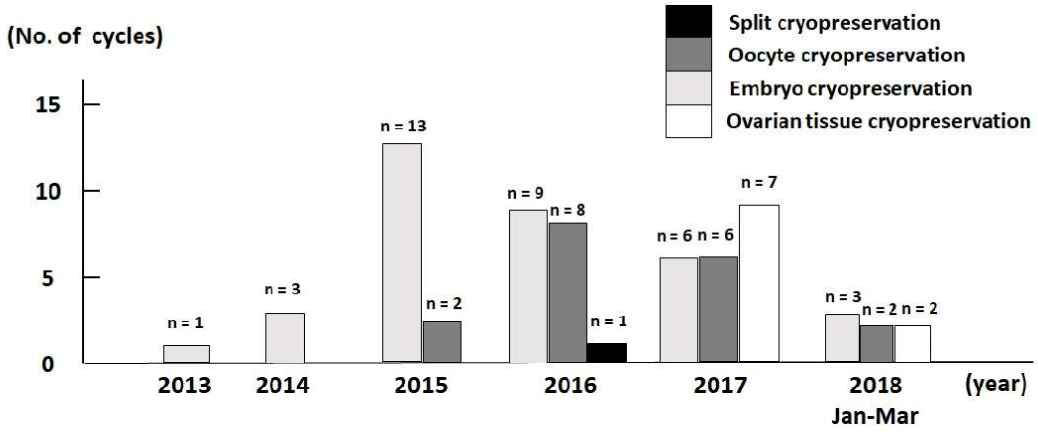


Figure 1. Comparison of the number of cycles for fertility preservation between the 4 groups from 2013 to 2018 (January to March).

Table 1. 9 cases of ovarian tissue cryopreservation

CASE	AGE yr	G/P	Marital status	AMH ng/ml	Primary disease	Previous treatment	Treatment plan	OPU	Freeze	Hospitalization period (days)	From first visit to operation (days)
1	11	G0	unmarried	2.0	Mediastinal tumor	Chemo	Chemo+HSCT	5	MII 1	5	4
2	15	G0	unmarried	1.12	Vulvar cancer	Chemo+ope	Chemo	3	0	5	11
3	14	G0	unmarried	0.23	AML recurrence	Chemo	Chemo+HSCT	1	0	5	4
4	15	G0	unmarried	0.13	ALL recurrence	Chemo	Chemo+HSCT	1	0	5	8
5	16	G0	unmarried	0.03	Ewing sarcoma	Chemo	Ope+chemo	Not done		5	22
6	42	G0	unmarried	0.44	Breast cancer	No	Ope+chemo	Not done		6	27
7	33	G0	unmarried	2.16	Breast cancer (Lynch syn.)	No	Ope+chemo	Not done		5	48
8	43	G0	unmarried	1.63	Breast cancer	No	Ope+chemo	9	MII 5	5	20
9	14	G0	unmarried	6.11	Suprasellar germ cell tumor	No	chemo	7	MII 4	5	3

HSCT: Hematopoietic stem cell transplantation, OPU: oocyte pick-up
AML: acute myelogenous leukemia, ALL: acute myeloid leukemia

Currently, the fertility of AYA women with cancer can be preserved through oocyte, embryo, or ovarian tissue cryopreservation [1]. Embryo cryopreservation requires sperm from the husband or partner. In the case of mature oocyte and ovarian cryopreservation, sperm is not needed. However, both embryo cryopreservation and mature oocyte cryopreservation might delay cancer treatment because of controlled ovarian *stimulation* (COS). Conversely, ovarian cryopreservation avoids COS, which is needed in embryo or oocyte cryopreservation. As a result, cancer treatment can be initiated immediately and can be indicated for pubertal girls before starting their *menstrual period.* Therefore, ovarian cryopreservation could be the only option for fertility preservation in prepubertal patients [1]. In addition, this method allows the storage of a large number of follicles. However, when metastasis of the tumour cells in the ovary is involved, that is, minimal residual disease (MRD) [8], transplantation of thawed ovarian tissue is associated with the risk of recurrence. Especially patients with leukemia are at high risk for transfer of malignant cells with the transplanted tissue [9]. In our hospital, we have performed fertility preservation through ovarian tissue cryopreservation for 2 AYA women with leukemia. After the transplantation of cryopreserved ovarian tissue, these AYA women may be at increased risk of cancer relapse because of ovarian tissue contamination by leukemic cells. As alternative approaches to auto-transplantation, in vitro maturation of primordial follicles or their transplantation in an artificial ovary is assumed to be necessary [10-13]. However, they are yet to be applicable in humans and cannot be offered to patients [9].

In November 2017, the first delivery in a leukemia survivor after transplantation of cryopreserved ovarian tissue was reported [9]. This case report suggests that transplantation safety should be increased through comprehensive tissue evaluation for malignant contamination when transplantation is considered in leukemia survivors. Moreover, tissue collection is recommended during the safest time possible, preferably during complete remission [9]. In our hospital, ovarian tissue samples were obtained from 2 AYA women with leukemia during complete remission before hematopoietic stem cell transplantation (HSCT), and cryopreservation of the ovary was performed. In addition, as the use of frozen oocytes matured in vitro should avoid this risk, we performed cryopreservation of in vitro matured oocytes in addition to ovarian tissue cryopreservation [14]. Six of the 9 patients were treated with OPU during ovarian tissue cryopreservation. Twenty-six oocytes were collected, and 10 MII oocytes were frozen.

In cases 1–5, the AMH level was low despite that the patients were in their teens. However, the AMH level immediately after the end of cancer therapy was reported to be inappropriate for the evaluation of the ovarian reserve [15]. At 11 years after a successful allogeneic bone marrow transplantation (BMT), a patient became pregnant and delivered a healthy infant in full term [16].

In conclusion, so far, at our hospital, there have been no cases of pregnancy by ovarian tissue warming and transplantation. As the success rate of births in women transplanted with frozen-thawed ovarian tissue is low worldwide [1], further accumulation of cases and consideration is needed to yield findings on the usefulness of this technique. However, we believe that fertility preservation of ovarian tissue through cryopreservation will help AYA patients fight cancer by providing hope that they can conceive in the future.

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Interleukin-1/-33 Signaling Pathways as Therapeutic Targets for Endometriosis

Toru Kato[1], *Koubun Yasuda*[2]*, *Kazufumi Matsushita*[2], *Ken J. Ishii*[3], *Seiichi Hirota*[4], *Tomohiro Yoshimoto*[2] *and Hiroaki Shibahara*[1]

[1] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan, [2] Department of Immunology, Hyogo College of Medicine, Nishinomiya, Japan, [3] Division of Vaccine Science, Department of Microbiology and Immunology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan, [4] Department of Surgical Pathology, Hyogo College of Medicine, Nishinomiya, Japan

Endometriosis is an estrogen-dependent disease with symptoms of dysmenorrhea, chronic pain, and infertility that affects 6–10% of women of reproductive age. Medical or surgical therapy, such as administration of an anti-gonadotropin or ovarian cystectomy, provide effective pain relief. However, neither therapy can be used for patients wishing to become pregnant. Despite the high morbidity, the pathogenesis of endometriosis has not been well-elucidated. Several inflammatory cytokines are reported to participate in the onset of endometriosis. Here, we examined the role of interleukin (IL)-1/IL-33 signaling in the development of endometriosis using a mouse model of endometriosis. Endometriotic lesion volume was significantly reduced in *Il33*$^{-/-}$ and *Il1r1*$^{-/-}$ mice, and almost completely suppressed in *Myd88*$^{-/-}$ mice. Mice intraperitoneally administered with an antibody against IL-1 receptor 1 (IL-1R1) or IL-33 developed limited endometriotic lesions. Oral administration of an inhibitor against IL-1R-associated kinase 4 (IRAK4), a downstream signal molecule of MyD88, also suppressed lesion formation. Furthermore, even after the development of cystic lesions the IRAK4 inhibitor prevented the enlargement of lesions. These treatments all significantly reduced cellular proliferation, shown by decreased Ki-67 expression. These results reveal that IL-1/IL-1R1, IL-33/IL-33R and associated downstream signaling molecules are involved in the pathogenesis of endometriosis, and may provide novel therapeutic targets for endometriosis.

Keywords: MyD88, IRAK4, ST2, IL-1R1, estrogen

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Diana Boraschi,
Istituto di Biochimica delle Proteine (IBP), Italy

Reviewed by:
Paola Italiani,
Italian National Research Council (CNR), Italy
Detlef Neumann,
Hannover Medical School, Germany

***Correspondence:**
Koubun Yasuda
koubun@hyo-med.ac.jp

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INTRODUCTION

Endometriosis is defined as endometrial tissue that occurs outside the uterine cavity, and an estrogen-dependent disease with predominant symptoms of dysmenorrhea, dyspareunia, chronic pain, and infertility. Endometriosis affects 6–10% of women of reproductive age (1) and up to 50% of women with infertility (2), highlighting its negative impact upon female reproductive health. It is also associated with a moderate increase in ovarian cancer risk (3). The pathogenesis of endometriosis appears to be multifactorial, including ectopic endometrial tissue, altered immunity, imbalanced cell proliferation and apoptosis, aberrant endocrine signaling, and genetic factors. The endometrial transplantation theory by Sampson is one possible cause of endometriosis, involving reflux of menstrual blood into the abdominal cavity via the oviduct (4). However, because the reflux

of menstrual blood is observed in most women, endometriosis cannot be explained by reflux alone. In addition, inflammatory cytokines, such as tumor necrosis factor α (5, 6), interleukin (IL)-1β (7, 8), and IL-33 (9), and immune cells, such as macrophages (10) and natural killer cells (11, 12), were reported to be involved in the pathogenesis of endometriosis. However, limited studies have investigated the mechanism of their actions in endometriosis.

IL-33 is a member of the IL-1 family cytokines that stimulates various cell types, such as Th2 cells, mast cells and group 2 innate lymphoid cells (ILC2s), and it has pleiotropic functions (13–15). Various types of cells such as epithelial cells and endothelial cells express IL-33 in the nucleus and release it in response to cellular stress (16, 17). In general, IL-1 family member cytokines, including IL-1β and IL-18, are synthesized as precursor proteins in the cytoplasm, and become active after processing in the inflammasome. In contrast, the full-length form of IL-33 is active, and processing by proteases enhances its activity, whereas cleavage by caspases abrogates its function (18). IL-33 is important for innate-type mucosal immunity in the lungs and gut, and airway inflammation, as well as peripheral antigen-specific responses by induction of Th2 cytokines (19–22).

Interleukin-1 is the most important proinflammatory cytokine and is mainly produced by monocytes/macrophages (23). Both IL-1α and IL-1β use a heterodimeric receptor complex consists of IL-1 receptor 1 (IL-1R1) and IL-1R accessory protein (IL-1RAcP) for the induction of inflammatory processes (24). The IL-1R uses the signal transduction adaptor protein MyD88, which recruits a serine/threonine kinase IL-1R-associated kinase 4 (IRAK4), resulting activation of transcription factor NF-κB and mitogen-activated protein kinases (25, 26). IL-33 uses a receptor complex of ST2 (IL-1R-like 1) and IL-1RAcP for its receptor, and also uses MyD88/IRAK4 signaling pathway. ST2 has two forms, a full-length membrane-bound form and a short secreted form (soluble ST2) consisting of an extracellular domain. The membrane-bound form acts as a receptor for IL-33 and the secreted form neutralizes IL-33 as a decoy receptor.

Currently, almost all drugs for the treatment of endometriosis are suppressive, not curative. Medical therapy with non-steroidal anti-inflammatory drugs (NSAIDs), oral contraceptives or anti-gonadotropin provides effective pain relief (27), but does not resolve the endometriosis. Surgical therapy is more effective for pain reduction and endometriosis-associated symptoms (28). However, after medical or surgical treatment, there is a high recurrence frequency of endometriosis; 21.5% at 2 years and 40–50% at 5 years (29). Furthermore, either therapy is limited to patients who do not wish to become pregnant (30). The limitations of currently available options prompted us to seek novel therapies for endometriosis. Here, we used an experimental mouse model to determine the molecular mechanisms underlying endometriosis and examine novel drug targets for endometriosis. We investigated the role of IL-33, IL-1R1, and MyD88 in the development of endometriosis in mice, and demonstrated the potential of inhibitors for IL-1/IL-33 and their signaling pathway as therapeutic targets for endometriosis.

Abbreviations: Ab, Antibody; IL-1R1, IL-1 receptor 1; IL-1Ra, IL-1R antagonist; ILC2, Group 2 innate lymphoid cell; NK, Natural killer; PBL, Peripheral blood leukocyte; Q-PCR, Quantitative PCR.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with recommendations of the Regulations for Animal Experimentation in Hyogo College of Medicine, Hyogo College of Medicine Animal Experiment Committee. The protocols were approved by the Hyogo College of Medicine Animal Experiment Committee (No. 14-028, 16-087).

Mice

Wild type (WT) BALB/c and C57BL/6 mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). BALB/c-background *Tlr4*$^{-/-}$ mice were obtained from Oriental BioService Inc. (Kyoto, Japan). C57BL/6-background *Il1r1*$^{-/-}$ mice were purchased from The Jackson Laboratory. BALB/c-background *Myd88*$^{-/-}$, *Il18*$^{-/-}$, *Il33*$^{-/-}$, and *Il1rl1*$^{-/-}$ mice were generated as described (20, 25, 31). Mice were maintained under specific pathogen-free conditions at the animal facilities of the Hyogo College of Medicine.

Mouse ST2-Human Fc Fusion Protein (mST2Fc)

The mST2Fc was made by Mitsubishi Tanabe Pharma. The sequence of mouse ST2 ectodomain (amino acids 1–332) fused to the N-terminus of the Fc region of human IgG1 was inserted into the mammalian expression vector pEE12.4 (Lonza Biologics, Slough, UK). The expressed mST2Fc was purified by Protein A chromatography, followed by hydroxyapatite chromatography. The fusion protein was concentrated and buffer-exchanged to PBS (pH 7.4). Protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Mouse Endometriosis Model

The experimental endometriosis model was made as described previously with minor modification (32, 33). Six-week-old female mice were ovariectomized under anesthesia with isoflurane, and treated with estradiol subcutaneously (0.5 μg/week/mouse, Fuji Pharma, Tokyo, Japan). Two weeks later, mice were randomly divided into two groups, donor and recipient. Donor group mice were euthanized by cervical dislocation, then uteri were removed, minced, and suspended in PBS containing ampicillin (1 mg/ml). Uteri samples were transplanted (40 mg/recipient) into the peritoneal cavity of recipient mice under isoflurane-induced anesthesia, using a 1 ml syringe (without a needle) into a small median abdominal incision. The control group received 400 μl of ampicillin/PBS. Unless otherwise indicated, the donor and recipient mice were of the same strain. Two weeks after transplantation, mice were euthanized by cervical dislocation and lesion size in the abdominal cavity was evaluated (**Supplementary Figure 1**). There are several lesions with various sizes in each mouse. The lesion volume was calculated using the following formula: $V = 4/3\pi A^2 a$; A: long radius, a; short radius.

The sum of each lesion volume was taken as the total volume. Lesion sections were photographed with an iPhone (scale: mm). There are several lesions with different appearances (white, red or brown). The reason for these differences is unknown.

In some experiments, mice were administered 100 ng recombinant human IL-33 (rhIL-33, HOKUDO, Sapporo, Japan) (20) intraperitoneally (ip) on day 1, 3, 5, 8, 10, and 12 after transplantation of uterine samples.

For neutralization of human IL-33, mice were administered anti-human IL-33 antibody (10 mg/kg ip, A10-1C04, provided from Mitsubishi Tanabe Pharma, Yokohama, Japan, Patent No. WO2015099175) once a week. Normal human IgG1 (Eureka therapeutics, CA) was used as a control. For neutralization of murine IL-33, mice were administered mST2Fc (20 mg/kg iv) once in 3 days. Human IgG1-Fc (Bio X cell, NH) was used as a control. For IL-1 neutralization, mice were administered (iv) 250, 125, and 125 μg anti-IL-1R1 antibody (35F5, #553693, BD Biosciences, CA) at day −1, day 4, and day 8, respectively. Anti-horseradish peroxidase rat IgG1 (HRPN, BP0088, BioXcell) was used as a control.

For blocking IRAK4 signaling, an IRAK4 inhibitor (AS2444697; Sigma-Aldrich Japan, Tokyo, Japan) was solubilized at 10 mg/ml with DMSO and diluted with water, then orally administered twice a day at 5 mg/kg for 5 days after transplantation, or 10 mg/kg for 14 days from 2 weeks after transplantation.

Flow Cytometry

The uteruses from untreated WT and ST2 deficient mice (*Il1rl1*$^{-/-}$) were minced and digested with complete medium (RPMI 1640 supplemented with 10% fetal bovine serum, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) containing 400 U/ml collagenase (032-22364, Wako, Osaka, Japan) and 10 μg/ml DNase I (11284932001, Roche, Mannheim, Germany) for 60 min at 37°C. Cell suspensions were filtered using a cell strainer. Uterus cells were stained with antibodies for CD45 (I3/2.3, PerCP-Cy5.5, 147706, BioLegend, CA), EpCAM1 (G8.8, APC, 118213, BioLegend), ST2 (DJ8, Biotin, 101001B, MD BioSciences, MN), and MAdCAM1 (MECA-367, AlexaFluor 488, 120708, BioLegend) followed by streptavidin (Brilliantviolet 421, 405225, BioLegend). Data were acquired using SP6800 flow cytometer (SONY, Tokyo, Japan) and analyzed using FlowJo software (Tree Star Inc., OR).

Histological Analysis

For histological analysis, lesion specimens were fixed with 4% (w/v) paraformaldehyde (PFA) and embedded in paraffin. Specimens were sectioned at 5-μm thickness and deparaffinized sections were stained with hematoxylin and eosin.

For immunohistochemical staining, deparaffinized sections were microwave-heated in citrate buffer (pH 6.0) for antigen retrieval, then blocked with 1% BSA, stained with rabbit anti-mouse Ki-67 polyclonal antibody (ab16667, Abcam, Tokyo, Japan) or anti-mouse ERβ (Santa Cruz, TX) and detected by Envision+ Dual link (K4063, DAKO, CA). After development with 3-amino-9-ethylcarbazole, specimens were stained with hematoxylin. Sections were evaluated by light microscopy (AX80, Olympus Lifescience, Tokyo, Japan). For analysis of proliferating cells, sections were observed by microscopy to calculate Ki-67 positive nuclei in epithelial cells. The Ki-67 positive epithelial cells were counted in three fields of view per slide and averaged. To detect apoptotic cells, sections of cystic lesions were stained using TUNEL with an Apoptosis *in situ* Detection Kit (FUJIFILM Wako Pure Chemical, Osaka, Japan), as per manufacturer's instruction. For analysis of apoptotic cells, sections were observed by light microscopy to calculate the TUNEL positive nuclei in epithelial cells. The TUNEL positive epithelial cells were counted in three fields of view per slide and averaged.

Cytokine Measurements

After uterine transplantation, sera were collected at indicated time points. Peritoneal lavage fluids were collected by washing the peritoneal cavity with 400 μl PBS, then centrifuged at 20,000 g for 10 min. Supernatants were analyzed for IL-1β (DY401, R&D systems, MN), IL-1α (433403, BioLegend) and IL-33 (88–7333, eBioscience, CA) levels by ELISA kits, as per manufacturer's instructions.

Gene Expression Analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Netherlands), and cDNA synthesized using ReverTra Ace (TOYOBO, Osaka, Japan). Gene expression levels were quantified with TaqMan Gene Expression Assays (Applied Biosystems, Tokyo, Japan). Results are shown as relative expression standardized against the expression of β-actin. Specific primers and probes used for quantitative RT-PCR were *Il33* (Mm00505403_m1) and *actin-b* (4352933E) (Applied Biosystems).

Statistical Analysis

Data are expressed as the mean ± S.D. Statistical significance was determined by the two-tailed Student's *t*-test, one-way analysis of variance (ANOVA) with Tukey's multiple range test, or two-way ANOVA with the Holm-Sidak multiple range test using Prism 6 (MDF, Tokyo, Japan). *P*-values <0.05 were considered significant.

RESULTS

Administration of IL-33 Exacerbates Endometriosis

A previous report indicated that IL-33 levels were elevated in ascites from severe endometriosis patients (9). Therefore, we first examined the effect of IL-33 on lesions in our mouse endometriosis model. Mice were ovariectomized and treated with estrogen as described in methods, then 2 weeks later, uterine samples were transplanted into the peritoneal cavity. After transplantation, mice were administered rhIL-33 intraperitoneally three times a week and the growth of endometriosis-like lesions were

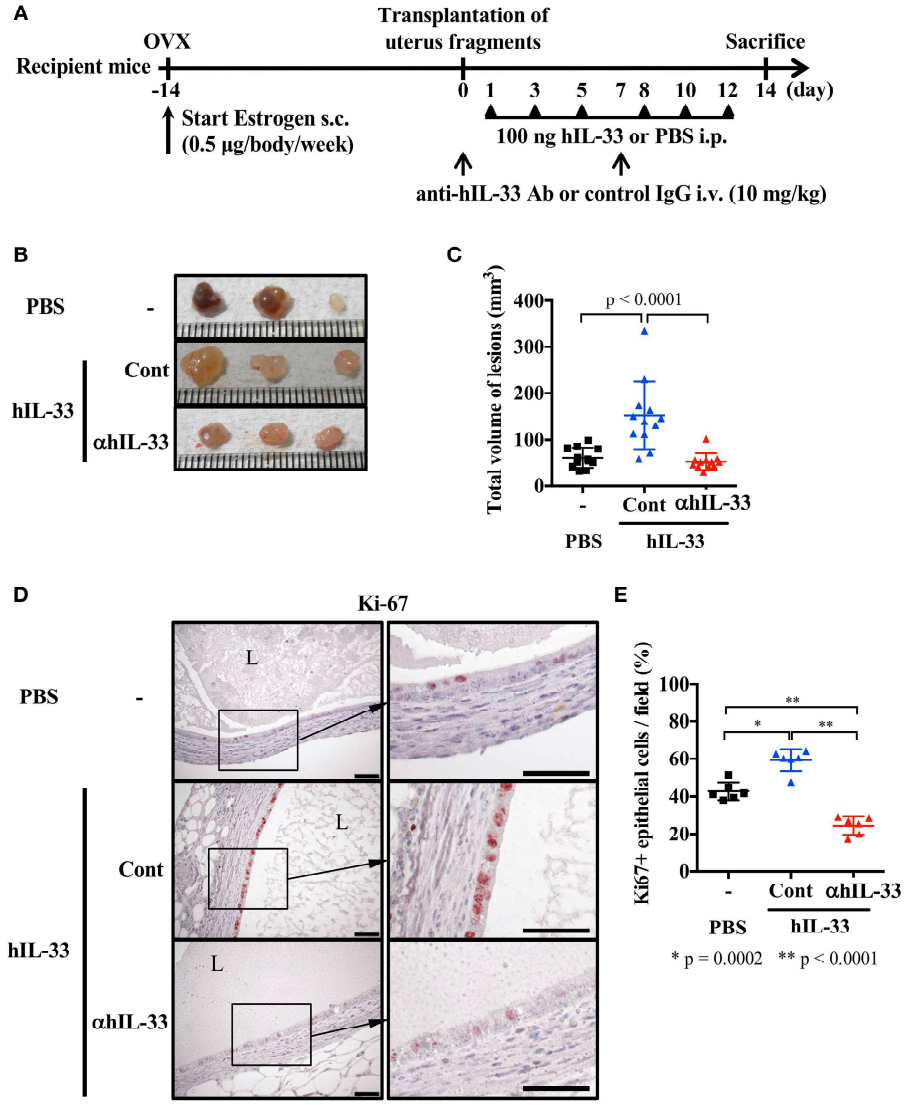


FIGURE 1 | Exogenous IL-33 exacerbates endometriosis. **(A)** Experimental workflow. Wild type BALB/c mice were ovariectomized (OVX) and administered estrogen subcutaneously (s.c.) for 2 weeks before transplantation of uterine fragments. After transplantation, mice received PBS or recombinant human IL-33 intraperitoneally (i.p.) at day 1, 3, 5, 8, 10, and 12. For the neutralization experiment, IL-33-treated mice were intravenously (i.v.) injected with control IgG (Cont) or anti-hIL-33 Ab (αhIL-33) at day 0 and 7. **(B)** Representative endometriosis lesions from each mouse. **(C)** Total volume of the lesions ($n = 12$ in each group). Pooled data from two independent experiments are shown (mean ± SD). **(D)** Immunohistological staining of Ki-67; Brown. L, lumen, Scale bar: 50 µm. **(E)** The proportion of Ki-67 positive epithelial cells lining the lumen of the cyst wall ($n = 6$, mean ± SD). Statistical analyses were performed using a one-way ANOVA with Tukey's *post-hoc* tests **(C,E)**.

examined 2 weeks after transplantation (**Figure 1A**). We found that the size of lesions was significantly increased by administration of rhIL-33 (**Figures 1B,C**). Simultaneously, Ki-67 staining of the diseased tissue revealed that rhIL-33 enhanced the proliferation of epithelial cells (**Figures 1D,E**). The increased lesion volume and epithelial cell proliferation induced by rhIL-33 were completely suppressed by administration of anti-human IL-33 antibody (**Figures 1B–E**), indicating that excessive levels of exogenous IL-33 exacerbated endometriosis in this mouse model.

Endogenous IL-33 Is Involved in Lesion Formation of Endometriosis

We examined the effect of endogenous IL-33 on the onset of endometriosis. First, we measured the amount of IL-33 in the peritoneal cavity after transplantation of uterine fragments. IL-33 was not detected in the control group treated with antibiotics alone after laparotomy, but was detected in the peritoneal cavity 4 h after transplantation (**Figure 2A**). It is known that IL-33 is released from necrotic cells, so we used *Il33*$^{-/-}$ mice as uterine donors to confirm that IL-33 was not derived from donor cells damaged by graft fragmentation. We found that IL-33 levels were

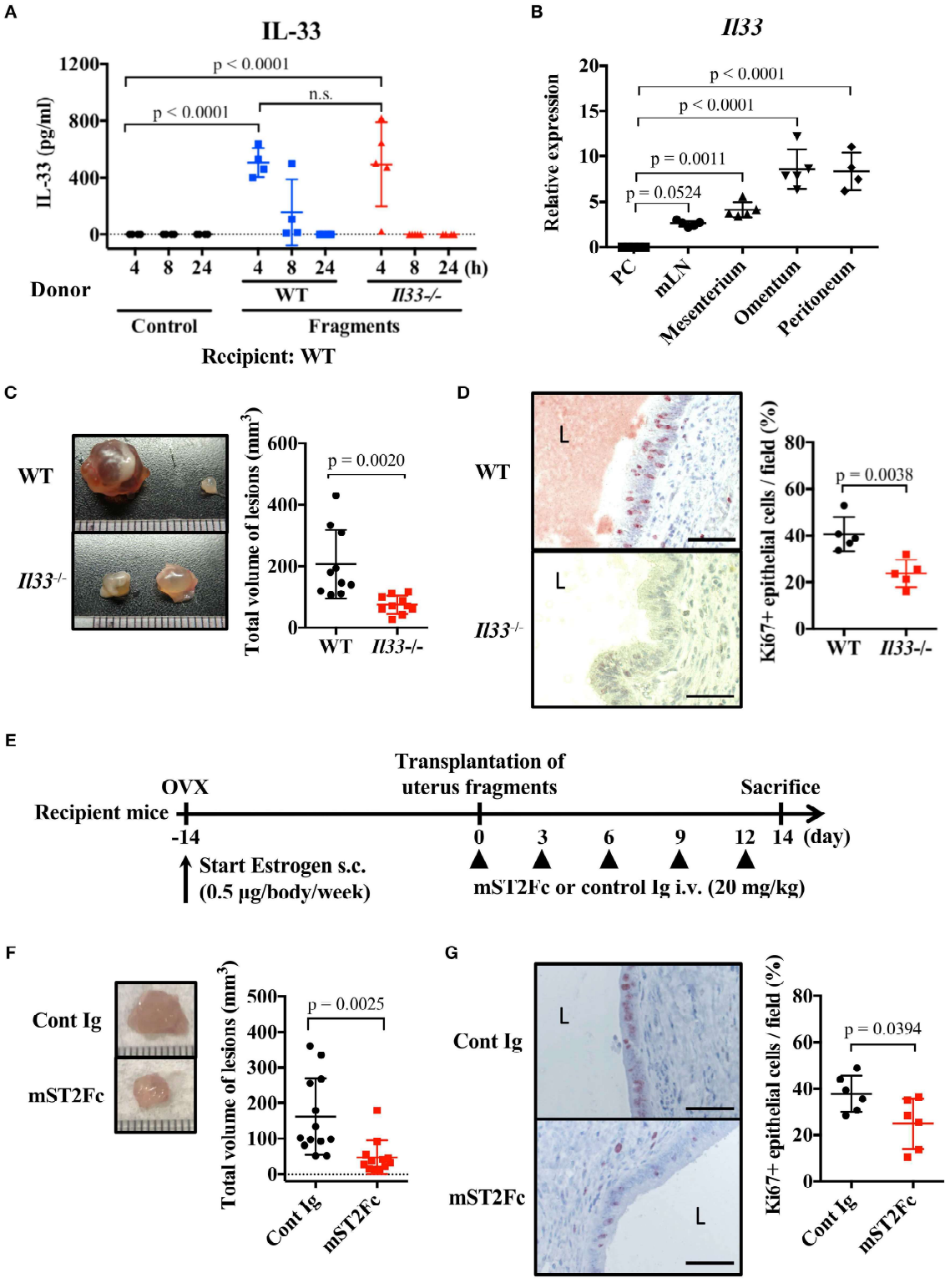


FIGURE 2 | Endogenous IL-33 contributes to lesion formation in endometriosis. **(A)** Concentration of IL-33 in peritoneal lavage fluid. Wild type (WT) BALB/c mice were injected with PBS or uterine fragments transplanted from WT or *Il33*$^{-/-}$ mice. Peritoneal lavage fluids were collected at indicated time points. **(B)** *Il33* mRNA

(Continued)

FIGURE 2 | expression in intraperitoneal tissues was measured by real-time RT-PCR. PC: peritoneal cells in peritoneal lavage fluid, mLN: mesenteric lymph node. **(C)** Mouse endometriosis model using WT mice or *Il33*$^{-/-}$ mice. (Left) Representative endometriosis lesions from each mouse. (Right) Total volume of the lesions ($n = 10$ in each group). Pooled data from two independent experiments are shown (mean ± SD). **(D)** (Left) Immunohistological staining of Ki-67; Brown. L: lumen, Scale bar: 50 μm. Low-power image was shown in **Supplementary Figure 2A**. (Right) The proportion of Ki-67 positive cells in epithelium ($n = 5$, mean ± SD). **(E)** Mouse endometriosis model performed with WT BALB/c mice. After uterine fragment transplantation, mice were treated with mST2Fc or control (Cont) Ig at the indicated days. **(F)** (Left) Representative endometriosis lesion. (Right) Total volume of the lesions (cont; $n = 13$, mST2Fc; $n = 12$). Pooled data from two independent experiments are shown (mean ± SD). **(G)** (Left) Immunohistological staining of Ki-67. Scale bar: 50 μm. Low-power image was shown in **Supplementary Figure 2B**. (Right) The proportion of Ki-67 positive cells in epithelium ($n = 6$, mean ± SD). Statistical analyses were performed using a one-way ANOVA with Tukey's *post-hoc* tests **(A,B)** or a Student's *t*-tests **(C,D,F,G)**.

comparable when using *Il33*$^{-/-}$ and WT donors (**Figure 2A**), indicating that IL-33 was produced from the recipient's cells after uterine transplantation.

To clarify the source of IL-33, we analyzed *Il33* mRNA expression in several tissues of the peritoneal cavity. We found strong *Il33* expression in the omentum and peritoneum, relatively weak expression in the mesenterium and mesenteric lymph node (mLN), and very low expression in peritoneal cells of peritoneal lavage fluid (**Figure 2B**). As the omentum and internal surface of the peritoneum are covered with mesothelial cells (34), uterine fragments may stimulate mesothelial cells lining the surface of the peritoneum cavity to release IL-33.

As uterine transplantation increased the concentration of IL-33 in the peritoneal cavity, we examined the influence of endogenous IL-33 on endometriosis-like lesion formation. Transplantation experiments resulted in a significant reduction in lesion size in *Il33*$^{-/-}$ mice compared with WT mice (**Figure 2C**). In addition, *Il33*$^{-/-}$ mice had a reduced proportion of Ki-67 positive epithelial cells in the lesion (**Figure 2D**, **Supplementary Figure 2A**). Furthermore, we neutralized the effects of endogenous IL-33 after transplantation by the administration of soluble mouse ST2 (mST2Fc), which contains the extracellular domain of mouse ST2 and Fc portion of human IgG (**Figure 2E**). We found that the lesion size and frequency of cell proliferation were suppressed by mST2Fc (**Figures 2F,G**, **Supplementary Figure 2B**). Since we observed the effect of IL-33 on the proliferation of endometrial epithelial cells, we examined the surface expression of ST2 on epithelial cells, considering that the epithelial cells may be direct target of IL-33. Unexpectedly, we could not detect ST2 expression on the surface of uterine endometrial epithelial cells (**Supplementary Figures 3A,B**). Taken together, these findings reveal that IL-33 was produced intraperitoneally at the onset of endometriosis, thereby promoting cell proliferation and increasing the lesion. In addition, neutralization of IL-33 suppressed the enlargement of the lesion.

IL-1 Exacerbates Endometriotic Lesions

IL-1β is thought to act on endometrial stromal cells and to be involved in the development of endometriosis (7, 35–37). However, this proposed action of IL-1 has not been confirmed *in vivo*. Therefore, we examined the role of IL-1 in a mouse endometriosis model. First, we measured the concentration of IL-1α and IL-1β in the blood and peritoneal cavity after transplantation of uterine fragments. A transient increase of IL-1β was observed in the abdominal cavity of both the control and transplantation groups 8 h after treatment, but in the transplanted group, IL-1β was increased from 4 h and remained higher 24 h after transplantation compared with the control group (**Figure 3A**). IL-1α was transiently increased at 4 h (**Supplementary Figure 4A**). Blood IL-1β was also increased 24 h after transplantation, and gradually decreased at 2 weeks, but increased again at 3 weeks after transplantation (**Figure 3B**). However, no significant increase in IL-1α concentration was found in the serum (**Supplementary Figure 4B**).

To clarify the role of IL-1 in lesion formation, we used C57BL/6-background IL-1R1-deficient mice for the endometriosis model. As previously reported, C57BL/6 mice exhibited smaller lesion formation than BALB/c mice (38). In *Il1r1*$^{-/-}$ mice, only very small lesions were observed compared with WT mice (**Figure 3C**). The number of Ki-67 positive epithelial cells was markedly decreased in *Il1r1*$^{-/-}$ relative to WT mice (**Figure 3D**, **Supplementary Figure 2C**). To block the action of IL-1, we administrated an anti-IL-1R1 antibody after transplantation (**Figure 3E**), and found it strongly suppressed lesion formation and the proportion of Ki-67 positive cells (**Figures 3F,G**). Furthermore, when the anti-IL-1R1 antibody was administered to *Il33*$^{-/-}$ mice to suppress both IL-33 and IL-1, lesion formation was strikingly inhibited compared with those in control mice (**Figure 3H**). These results reveal that IL-1 was involved in the development of endometriotic lesions.

MyD88 Signaling Is Essential for Endometriotic Lesion Formation

IL-1 and IL-33 act via MyD88, an adapter molecule for signal transduction. Therefore, we investigated the formation of endometriosis-like lesions in MyD88-deficient mice lacking the key signaling pathway of IL-1 family cytokines. Compared with WT mice, the formation of lesions were remarkably suppressed in *Myd88*$^{-/-}$ mice, with almost no cystic morphology observed (**Figure 4A**). Histological examination revealed lesions with small cystic morphology, but most epithelial cells were Ki-67 negative (**Figure 4B**, **Supplementary Figure 2D**). In addition, analysis of the frequency of apoptotic cells in endometriotic lesions found no difference between the *Myd88*$^{-/-}$ and WT mice (**Supplementary Figure 5**), indicating that MyD88 contributed to the growth of epithelial cells but not to their survival. These results reveal that MyD88 is essential for cystic endometriosis lesion formation.

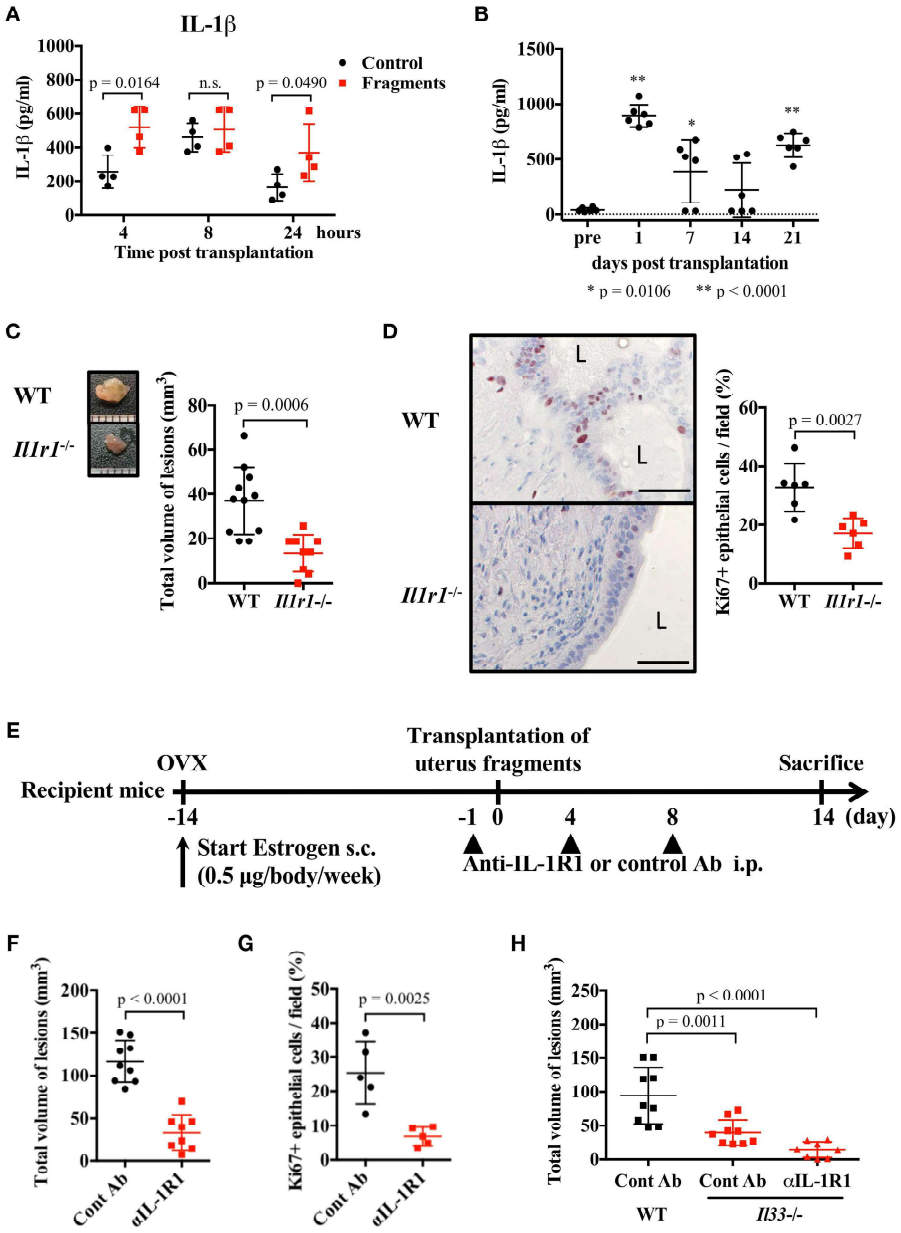


FIGURE 3 | IL-1 exacerbates endometriosis lesions. **(A,B)** Concentration of IL-1β in peritoneal lavage fluids (**A**, $n = 4$), or serum (**B**, $n = 6$). Wild type (WT) BALB/c mice were injected with PBS or transplanted with uterine fragments from WT mice. Peritoneal lavage fluids or serum were collected at indicated time points. Statistical analyses were performed using a two-way ANOVA with Holm-Sidak *post-hoc* tests **(A)** or a one-way ANOVA with Tukey's *post-hoc* tests. **(C)** Mouse endometriosis model using WT C57BL/6 mice or *Il1r1*$^{-/-}$ mice. (Left) Representative endometriosis lesion. (Right) Total volume of the lesions (WT; $n = 11$, *Il1r1*$^{-/-}$; $n = 9$). Pooled data from two independent experiments are shown (mean ± SD). **(D)** (Left) Immunohistological staining of Ki-67. Scale bar: 50 µm. Low-power image was shown in **Supplementary Figure 2C**. (Right) Proportion of Ki-67 positive cells in epithelium ($n = 6$, mean ± SD). **(E)** Mouse endometriosis model performed with WT BALB/c mice. One day before and 4 and 8 days after uterine fragment transplantation, mice were treated with anti-IL-1R1 Ab (αIL-1R1) or control (Cont) Ab. **(F)** Total volume of the lesions (cont; $n = 9$, anti-IL-1R1; $n = 8$). Pooled data from two independent experiments are shown (mean ± SD). **(G)** Immunohistological staining of Ki-67 was performed. Proportion of Ki-67 positive cells in epithelium are shown (n = 5, mean ± SD). Statistical analyses were performed using a Student's *t*-tests **(C,D,F,G)**. **(H)** Mouse endometriosis model performed as in **(E)** with WT and *Il33*$^{-/-}$ mice. Total volume of the lesions (WT: cont; n = 9, *Il33*$^{-/-}$: cont; $n = 9$, anti-IL-1R1; $n = 8$). Pooled data from two independent experiments are shown (mean ± SD). Statistical analyses were performed using a one-way ANOVA with Tukey's *post-hoc* tests.

MyD88 is also involved in other IL-1 family cytokine and Toll-like receptor (TLR) signaling, other than IL-1 and IL-33. Therefore, the involvement of other ligands and receptors was investigated. There was no difference in the size of endometriotic lesions in IL-18-deficient mice and TLR4-deficient mice compared with WT mice (**Figure 4C**).

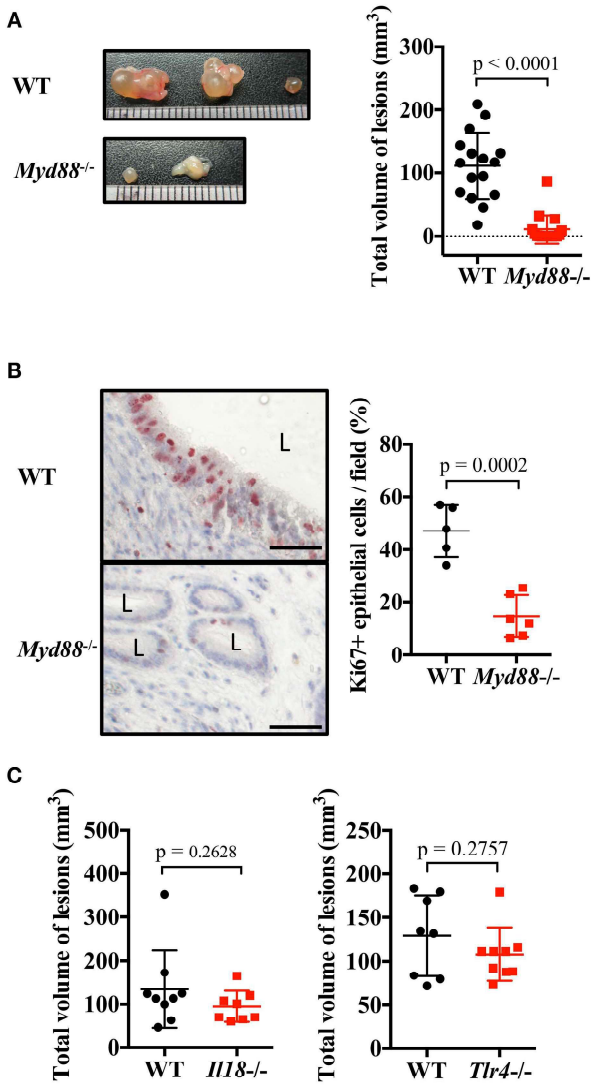


FIGURE 4 | MyD88 signaling is essential for endometriotic lesion formation. **(A)** Mouse endometriosis model performed with wild type (WT) BALB/c mice or *Myd88*$^{-/-}$ mice. (Left) Representative endometriosis lesions. (Right) Total volume of the lesions ($n = 20$). Pooled data from three independent experiments are shown (mean ± SD). **(B)** (Left) Immunohistological staining of Ki-67. Scale bar: 50 μm. Low-power image was shown in **Supplementary Figure 2D**. (Right) Proportion of Ki-67 positive cells in epithelium (WT; $n = 5$, *Myd88*$^{-/-}$; $n = 6$, mean ± SD). **(C)** Mouse endometriosis model performed with WT, *Il18*$^{-/-}$, and *Tlr4*$^{-/-}$ BALB/c mice. Total volume of the lesions (WT vs. *Il18*$^{-/-}$, WT: $n = 9$, *Il18*$^{-/-}$: $n = 8$; WT vs. *Tlr4*$^{-/-}$, WT: $n = 8$, *Tlr4*$^{-/-}$: $n = 9$). Pooled data from two independent experiments are shown (mean ± SD). Statistical analyses were performed using a Student's *t*-tests.

Blockade of MyD88 Signaling Suppresses Endometriosis

Because the signal transduction pathway mediated by MyD88 was essential for endometriosis lesion formation, we investigated whether inhibition of IRAK4 (an essential protein kinase for this pathway) provided an effective treatment. Daily oral administration of AS2444697, an IRAK4 inhibitor, for five consecutive days after the transplantation of uterine fragments was found to inhibit epithelial cell proliferation and markedly inhibit lesion growth (**Figures 5A–C**, **Supplementary Figure 2E**).

When considering a therapeutic approach to endometriosis, it would be beneficial to be effective against formed lesions. Therefore, we administered IRAK4 inhibitor 2 weeks after uterine implantation, when lesions had already formed, and examined its effect on lesion growth (**Figure 5D**). Compared with the control group, the AS2444697 treated group had a significantly smaller lesion size (**Figure 5E**). Epithelial cell proliferation was also suppressed by AS2444697 (**Figure 5F**, **Supplementary Figure 2F**). From these results, it is clear that MyD88 signaling was involved in the early stage of lesion formation, as well as the increase of lesion size after formation. Therefore, it is possible that blocking the MyD88 signaling pathway may provide an effective treatment for endometriosis.

DISCUSSION

In this study, we investigated the molecular mechanism involved in endometriosis and candidate pathways as treatment targets. Using an *in vivo* murine model with transplanted uterine fragments, we revealed that cytokines using MyD88 signaling, such as IL-1 and IL-33, were induced after transplantation and were important for ectopic endometrial cell proliferation. Furthermore, we demonstrated that neutralizing antibodies to IL-33 and/or IL-1R1, or an inhibitor of IRAK4 (involved in MyD88 signaling) were effective for the treatment of endometriosis in this model.

Previous reports showed that IL-33 was present in ascites of patients with endometriosis, and IL-33 concentrations were elevated in correlation with progression of the disease state, suggesting a relationship between IL-33 and the progression of endometriosis (9). Recently, another group reported that mouse endometriosis was exacerbated by administration of IL-33 (39), consistent with our results. Using IL-33-deficient mice, we clearly demonstrated that endogenous IL-33 plays an important role in lesion formation. The role of endogenous IL-33 was further confirmed by using mST2Fc, which mimics the IL-33 neutralizing function of soluble ST2. Furthermore, by using rhIL-33 and anti-human IL-33 antibody, we demonstrated that neutralization of IL-33 was effective at inhibiting the progression of endometriosis, and the use of anti-human IL-33 antibody may provide a beneficial clinical treatment.

In the present study, the source of IL-33 was likely to be the recipient cells, because IL-33 was detected in ascites even when uterine fragment transplants were prepared from *Il33*$^{-/-}$ mice. The expression of IL-33 in the peritoneal cavity was high in the peritoneum and omentum, which are tissues that contain a layer of mesothelial cells (34). Because IL-33 was released a short time after uterine transplantation, we propose that mesothelial cells first contact the transplanted fragments and provide the source of IL-33. Usually, IL-33 is released from the nucleus by the physical damage of tissues, but our model may not

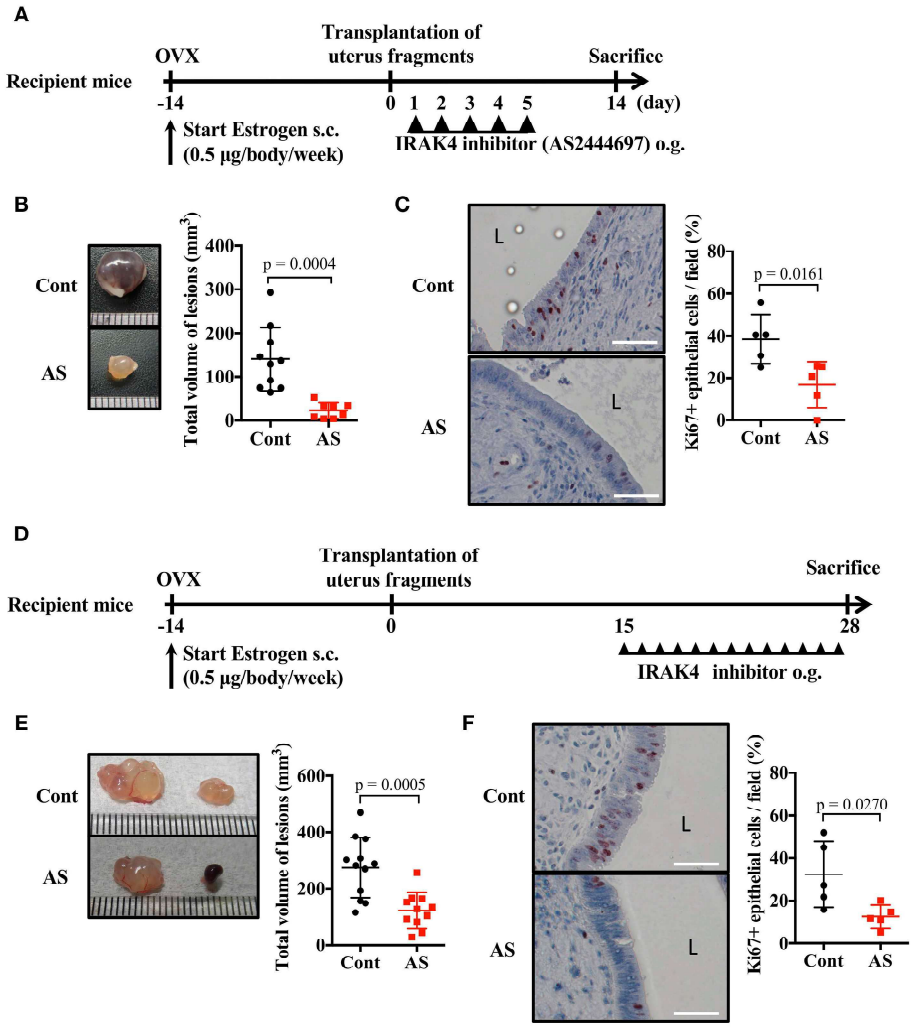


FIGURE 5 | Blockade of MyD88 signaling suppresses endometriosis. **(A)** Mouse endometriosis model performed with WT BALB/c mice. After uterine fragment transplantation, mice were treated with AS2444697 (AS) or sorbent (cont) at indicated time points. o.g., oral gavage. **(B)** Total volume of the lesions (cont; $n = 10$, AS; $n = 8$). Pooled data from two independent experiments are shown (mean ± SD). **(C)** (Left) Immunohistological staining of Ki-67. Scale bar: 50 μm. Low-power image was shown in **Supplementary Figure 2E**. (Right) Proportion of Ki-67 positive cells in epithelium ($n = 5$, mean ± SD). **(D)** Mouse endometriosis model performed with WT BALB/c mice as in **A**. Administration of inhibitor was started at 2 weeks after transplantation. **(E)** Total volume of the lesions (cont; $n = 12$, AS; $n = 11$). Pooled data from two independent experiments are shown (mean ± SD). **(F)** (Left) Immunohistological staining of Ki-67. Scale bar: 50 μm. Low-power image was shown in **Supplementary Figure 2F**. (Right) Proportion of Ki-67 positive cells in epithelium ($n = 5$, mean ± SD). Statistical analyses were performed using a Student's *t*-tests.

provide such a strong stimulus. We recently found that damage-associated molecular patterns (DAMPs) induce the expression of IL-33 (manuscript in preparation), and such DAMPs may be involved in the mechanism releasing IL-33, but the precise mechanism remains unknown. Here, we detected IL-33 only 4 h after transplantation, but we cannot exclude the possibility that low-level IL-33 is released continuously in the peritoneal cavity and contributes to the development of endometriosis.

Interleukin-33 uses the ST2 and IL-1RAcP as a receptor, and their expression is found in various immune system cells, such as mast cells, Th2 cells, and ILC2s (14). As ST2 was not detected on the surface of normal endometrial epithelial cells, it is possible that the immune system cells responding to IL-33 may produce secondary mediators which act on endometrial cells. Alternatively, some inflammatory conditions may induce ST2 expression on endometrial cells.

Interleukin-1β is produced by cleavage of precursor pro-IL-1β by caspase-1, which is activated via inflammasomes (40). It was reported that the NLRP3 inflammasome was involved in the exacerbation of endometriosis in a mouse model (41), consistent with our current findings showing smaller lesions in IL-1R1-deficient mice. The IL-1R1 is a common receptor for IL-1α and IL-1β. Our present work detected IL-1β in ascites and serum, and considering the involvement of inflammasomes, IL-1β may have an important role in endometriosis. However,

IL-1α is also released from the nucleus when the cell is damaged (like IL-33), and we indeed detected IL-1α in the ascites at 8 hours after transplantation, thus IL-1α may also contribute to the development of endometriosis.

There are caveats for using IL-1/IL-1R1 blocking reagents to treat patients with endometriosis wishing to give birth, such as the reported IL-1R antagonist (IL-1Ra)-induced inhibition of murine embryo implantation *in vivo*, and of increased trophoblast motility by urokinase plasminogen activator and plasminogen activator inhibitors *in vitro* (42, 43). However, recent case reports demonstrated successful pregnancy during treatment with IL-1Ra in patients with familial Mediterranean fever (44) and periodic syndromes, such as adult-onset Still's disease (45). In these diseases and gout, IL-1 is an important factor causing strong pain. Indeed, recently Yu et al. reported that a potential of IL-1β in neuroangiogenesis by the induction of brain-derived neurotrophic factor from eutopic endometriosis stromal cells (8). Suppression of IL-1 may provide effective treatment against pelvic pain caused by endometriosis.

It was reported that lipopolysaccharide (LPS) exacerbates endometriosis via NF-κB activation (46). Because LPS binds to TLR4 and activates MyD88 signaling (47), the current findings using *Myd88*$^{-/-}$ mice are consistent with LPS actions in endometriosis. Furthermore, TLR4 has been reported as a receptor for endogenous DAMPs (48). However, in our model, *Tlr4*$^{-/-}$ mice showed normal lesion formation, so the LPS-TLR4 pathway may not be essential for lesion formation. The target cells of LPS are unknown for endometriosis, but considering LPS acts on cells such as macrophages to produce IL-1β (47), it is possible that exacerbation of endometriosis by LPS may be mediated by induction of IL-1β. In addition, it was reported that the reflux of intrauterine flora exacerbated endometriosis by bacterial contamination of menstrual blood (49).

Recently, Kaabachi et al. reported that the concentration of anti-inflammatory cytokine IL-37 was increased in the peritoneal fluid of patients with endometriosis (50). IL-37 also has a capacity to affect the occurrence and development of endometriosis in a mouse model (51). Interleukin-37 is an IL-1 family cytokine that uses the IL-18R to inhibit IL-18 signaling. Like IL-1β, IL-18 is an IL-1 family cytokine that is cleaved and activated by caspase-1. Several groups reported that IL-18 concentrations were down-regulated in peritoneal fluid of patients with endometriosis (52, 53), but other groups did not observe this decrease (54, 55). Thus, the role of IL-18 in endometriosis remains controversial. In our experiment, lack of IL-18 did not affect lesion formation in mice, similar to the lack of TLR4. However, because IL-18 is also produced from macrophages by LPS stimulation (56) and is also an activator of MyD88 signaling, it may also influence the development of disease associated with bacterial infection.

Finally, we found that an IRAK4 inhibitor suppressed the onset and growth of endometriosis. The IRAK4 protein kinase is essential for NF-κB activation downstream of MyD88, and its inhibitor blocks most signals of IL-1 family cytokines and TLRs (26, 57). This inhibitor may provide a powerful therapeutic agent capable of suppressing the augmentation of endometriosis induced by IL-1β, IL-33, and LPS. Similar inhibitory effects were obtained using anti-IL-1R1 antibodies, and a stronger inhibitory effect may be expected by combining this treatment with an anti-IL-33 antibody or soluble ST2. Thus, our findings show that IL-1 family cytokines and signaling pathways may provide valuable targets for the treatment of endometriosis.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

TK, KY, and KI carried out the experiments. KY, HS, and TY planned the experiments and supervised the study. TK and KY wrote the manuscript. KY, KM, SH, and TY analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02021/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ー原著ー

近隣泌尿器科との連携による TESE-ICSIの成績と有用性の評価

藤井 雄太[1,2]，小林 眞一郎[1]，元石 睦郎[1]，山口 耕平[3]，石川 智基[3]，柴原 浩章[2]

[1] Kobaレディースクリニック　〒670-0935　兵庫県姫路市表情口2-18宮本ビル1F
[2] 兵庫医科大学 産科婦人科学講座　〒663-8501　兵庫県西宮市武庫川町1-1
[3] 石川病院 泌尿器科　〒671-0221　兵庫県姫路市別所町別所2-150

要 旨：　本研究は近隣泌尿器科との連携によるTESE-ICSIの有用性の評価を目的とした．無精子症の男性79人を対象とした．受精率は，NOA群58.2%，OA群82.2%，その他群78.0%であり，NOA群が低い傾向にあった．累積生児獲得率も，NOA群 40.0%，OA群 81.8%，その他群 70%と，NOA群が低い傾向にあった．また，TESE-ICSIの各群と比較し，TESEで得た精子の凍結融解処置，精巣組織の搬送の影響を評価する対照群として，新鮮射出精子を用いたICSI症例（fresh-ICSI群）を選択した．MⅡ卵子あたりの受精率は，fresh-ICSI群で83.1%であった．TESE-ICSI各群と比較して，NOA群の受精率のみが有意に低かった．臨床的妊娠率，分娩率においては有意差を認めなかった．TESE-ICSIを施行できた42組のカップルのうち30組（71.4%）が生児を獲得していた．
キーワード：TESE-ICSI, 閉塞性無精子症, 非閉塞性無精子症, 凍結融解精子, 新鮮射出精子

緒 言

男性不妊症の専門外来を受診する患者の約20%は無精子症であり，現在では無精子症患者に対してのTESE-ICSI（testicular sperm extraction-intracytoplasmic sperm injection）は標準的な治療となっている．日本のART（assisted reproductive technology）件数は世界有数であり，ICSIを行うことができる施設は年々増加傾向にあるが，TESEを実施できる施設はそう多くない．そのため，大半の施設では自施設内だけではTESE-ICSIを完遂できず，近隣泌尿器科と連携してTESE-ICSIを行っているのが実情と考えられる．

当クリニックでも，近隣泌尿器科と連携してTESE-ICSIに取り組んできた．その成績を後方視的に検討することで，近隣泌尿器科との連携によるTESE-ICSIを再評価し，TESEで得た精子の凍結融解処置や精巣組織の搬送の影響を明らかにすることを目的とした．

対 象

2003年12月～2017年12月の15年間に，当クリニックで無精子症と診断した男性のうち，インフォームドコンセントの得られた79人を対象とした．

方 法

無精子症患者を連携泌尿器科に診察依頼し，TESE当日は当クリニックの胚培養士を連携泌尿器科に派遣した．なお，確定診断のついた閉塞性無精子症（OA: obstructive azoospermia）患者にはconventional TESEを，それ以外の症例には，精液所見も加味し，micro TESEかconventional TESEかを個別に判断した．当院の胚培養士が手術室で精子の有無を確認した．精巣組織を培養液（Quinn's sperm washing medium）に浸して，当クリニックに持ち帰り，精細管を裁断して混濁液を作成し，検出できる運動精子数によって1本～数本のセラムチューブに分けて，液体窒素で蒸気冷却した後に凍結保存した．後日，採卵を施行し，凍結精子を融解しICSIを施行した．

TESEの適応を非閉塞性無精子症（NOA: non-obstructive azoospermia）群，OA群，その他群の3群に分けて，精子回収率，ICSIによる受精率，新鮮胚移植と凍結融解胚移植それぞれの臨床的妊娠件数・分娩件数，生児獲得率を比較した．

また，TESEで得た精子の凍結融解処置，精巣組織の搬送の影響を評価する対照群として，2016年1月～12月の1年間に当クリニックで施行した新鮮射出精子を用いたICSI症例（fresh-ICSI群：fresh ejaculatory

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責任著者：藤井 雄太　e-mail [deepspiritoverthetop@yahoo.co.jp]

sperm - ICSI) を選択し，受精率，臨床的妊娠率，分娩率を比較した．統計学的検討には，Mann-Whitney U 検定，χ^2検定を用い，p値<0.05を有意差ありとした．

結　果

1)TESE適応原因の内訳と精子回収率 (表1, 表2)

無精子症79例に対してTESEを施行した．TESE適応原因の内訳は，NOA群44例，OA群23例，その他群12例であった．TESEを複数回施行した症例もあったため，延べ症例数はNOA群45例，OA群23例，その他群14例であった．各々の精子回収率は，NOA群26.7% (12/45)，OA群100% (23/23)，その他群71.4% (10/14) であり，NOA群はOA群，その他群と比較して有意に低かった．

2)ICSI時の妻年齢と採卵，受精結果 (表3)

TESEの結果を受けて，採卵を施行した患者数は，NOA群10人，OA群22人，その他群10人であり，採卵件数は，NOA群20件，OA群53件，その他群21件であった．平均年齢は，NOA群35.7±5.2歳，OA群31.8±4.0歳，その他群37.2±4.8歳で，OA群が有意に低かった．1回採卵あたりの平均採卵数は，NOA群7.75個，OA群7.75個，その他群5.38個であり，各群に有意差は認めなかった．また1回採卵あたりの平均MⅡ卵子数も，NOA群6.50個，OA群6.15個，その他群4.71個であり，各群に有意差は認めなかった．

受精率は，NOA群58.2% (71/122)，OA群82.2% (249/302)，その他群78.0% (71/91) であり，年齢に有意差があるもののNOA群が有意に低かった．

3)妊娠・分娩結果 (表4)

新鮮胚移植周期において，臨床的妊娠率は，NOA群23.5% (4/17)，OA群31.1% (14/45)，その他群29.4% (5/17) であった．生産率は，NOA群17.6% (3/17)，OA群26.7% (12/45)，その他群29.4% (5/17) であった．

凍結融解胚移植周期において，臨床的妊娠率は，NOA群37.5% (3/8)，OA群47.1% (16/34)，その他群50.0% (5/10) であった．生産率も，NOA群37.5%

表1　TESE適応原因の内訳

	原　因	症例数	精子確認数
NOA 44例	原因不明	35	7
	Klinefelter 症候群	8	4
	AZFc 欠損	1	0
OA 23例	詳細不明	15	15
	先天性精管欠損症	6	6
	精管結紮術後	2	2
その他 12例	乏精子症	4	4
	精索静脈瘤	2	2
	逆行性射精	2	2
	精子死滅症	1	1
	射精障害	1	1
	抗がん剤治療後	1	0
	脊髄損傷	1	0

表2　精子回収率

	NOA	OA	その他	p値
TESE 施行人数	44	23	12	
TESE 施行件数	45	23	14	
精子確認件数	12	23	10	
精子回収率	26.7%[a]	100%[b]	71.4%[c]	a.c-b<0.05 a-c<0.05

表3　ICSI時の妻年齢と採卵、受精結果

	NOA	OA	その他	p値
女性年齢	35.7±1.2[d]	31.8±0.6[e]	37.2±1.0[f]	d.f-e<0.05
採卵個数／回	7.75	7.75	5.38	ns
MⅡ卵数／回	6.50	6.15	4.71	ns
受精率	58.2%[g] (71/122)	82.2%[h] (249/302)	78.0%[i] (71/91)	g-h.i<0.05

表4　妊娠・分娩結果

	NOA	OA	その他
新鮮胚移植件数	17	45	17
⇒臨床的妊娠件数	4	14	5
⇒分娩件数	3	12	5
凍結胚移植件数	8	34	10
⇒臨床的妊娠件数	3	16	5
⇒分娩件数	3	12	5
TESE 施行男性数	44	23	12
OPU-ICSI 患者数	10	22	10
生児獲得女性数	4	18	7
生児獲得率／患者	40%	81.8%	70%

表5　TESE-ICSI全体とfresh-ICSI（38歳以下）の比較

	TESE-ICSI	fresh-ICSI	p値
女性年齢	33.9±5.0	34.5±3.1	ns
採卵周期数	94	133	
平均採卵個数／回	7.22	6.21	ns
平均MII卵数／回	5.90	5.48	ns
受精率	75.9% (391/515)	83.1% (549/661)	<0.05
臨床的妊娠率	38.5% (47/122)	43.2% (79/183)	ns
分娩率	38.5% (47/122)	35.6% (65/183)	ns

表6　NOA-ICSI全体とfresh-ICSI（38歳以下）の比較

	NOA-ICSI	fresh-ICSI	p値
女性年齢	35.7±5.0	34.5±3.1	ns
採卵周期数	20	133	
平均採卵個数／回	7.74	6.21	ns
平均MII卵数／回	6.50	5.48	ns
受精率	58.2% (71/122)	83.1% (549/661)	<0.05
臨床的妊娠率	28.0% (7/25)	43.2% (79/183)	ns
分娩率	24.0% (6/25)	35.6% (65/183)	ns

(3/8)，OA群35.2% (12/34)，その他群50.0%(5/10)であった．

一連のTESEで得た凍結融解精巣精子を用いたICSI(以下，TESE-ICSIと表記)を受けた女性の累計生児獲得率は，NOA群40% (4/10)，OA群81.8% (18/22)，その他群70%(7/10)であり，NOA群はOA群，その他群に比して低い傾向にあった．

NOA群は生児獲得率も低い可能性が示唆された．

4)TESE-ICSI，NOA-ICSI，OA-ICSI，その他-ICSIと，マスタベーション法で得た新鮮射出精子を用いたICSI（以下，fresh-ICSIと表記）との比較

採卵時の妻年齢は，TESE-ICSI群33.9±5.0歳，NOA-ICSI群35.7±5.0歳，OA-ICSI群31.8±4.0歳，その他-ICSI群37.2±4.8歳であった．fresh-ICSI群と比較するにあたり各群の年齢が離れており，fresh-ICSI群が1つの年齢設定では全群を比較対象にできる年齢条件を整えることができなかった．そのため，それぞれの比較年齢設定を，TESE-ICSI群38歳以下，NOA-ICSI群38歳以下，OA-ICSI群35歳以下，その他-ICSI群31～38歳とすることで，各群において妻年齢の有意差をなくした上で，比較することとした．

5)TESE-ICSIとfresh-ICSI（38歳以下）の比較（表5）

採卵周期数は，TESE-ICSI群94周期，fresh-ICSI群133周期であった．TESE-ICSI，fresh-ICSIの順に，妻年齢は，33.9±5.0歳，34.5±3.1歳．平均採卵個数／回は，7.22個，6.21個．平均MII卵数／回は，5.90個，5.48個．受精率は，75.9% (391/515)，83.1% (549/661)．臨床的妊娠率は，38.5% (47/122)，43.2% (79/183)．分娩率は，32.8%(40/122)，35.6%(65/183)であった．受精率において，TESE-ICSI群が有意に低かった($p<0.05$)．その他は有意差を認めなかった．

6)TESE-ICSIとfresh-ICSI（38歳以下）の比較（表6）

採卵周期数は，NOA-ICSI群20周期，fresh-ICSI群133周期であった．NOA-ICSI，fresh-ICSIの順に，妻年齢は，35.7±5.0歳，34.5±3.1歳．平均採卵個数／回は，7.75個，6.21個．平均MII卵数／回は，6.50個，5.48個．受精率は，58.2% (71/122)，83.1% (549/661)．臨床的妊娠率は，28.0% (7/25)，43.2% (79/183)．分娩率は，24.0% (6/25)，35.6% (65/183)であった．受精率において，NOA-ICSI群が有意に低かった($p<0.05$)．その他は有意差を認めなかった．

表7　OA-ICSI 全体と fresh-ICSI (35 歳以下) の比較

	OA-ICSI	fresh-ICSI	p 値
女性年齢	31.8 ± 4.0	32.5 ± 2.5	ns
採卵周期数	53	75	
平均採卵個数／回	7.75	6.77	ns
平均 MⅡ卵数／回	6.15	5.81	ns
受精率	82.2% (249/302)	85.4% (339/397)	ns
臨床的妊娠率	42.9% (30/70)	41.1% (46/122)	ns
分娩率	34.2% (24/70)	24.1% (27/112)	ns

表8　その他 -ICSI 全体と fresh-ICSI (31 ～ 38 歳) の比較.

	その他-ICSI	fresh-ICSI	p 値
女性年齢	37.2 ± 4.8	35.3 ± 2.0	ns
採卵周期数	21	115	
平均採卵個数／回	5.38	6.06	ns
平均 MⅡ卵数／回	4.71	5.38	ns
受精率	78.0% (71/91)	81.7% (456/558)	ns
臨床的妊娠率	37.3% (10/27)	38.2% (58/152)	ns
分娩率	37.3% (10/27)	3.0% (35/152)	ns

7) OA- ICSI と fresh-ICSI (35歳以下) の比較 (表7)

採卵周期数は，OA-ICSI群53周期，fresh-ICSI群75周期であった．OA-ICSI，fresh-ICSIの順に，妻年齢は，31.8±4.0歳，32.5±2.5歳．平均採卵個数／回は，7.75個，6.77個．平均ＭⅡ卵数／回は，6.15個，5.81個．受精率は，82.2%(249/302)，85.4% (339/397)．臨床的妊娠率は，42.9% (30/70)，41.1% (46/112)．分娩率は，34.2% (24/70)，24.1% (27/112)であった．全てにおいて有意差を認めなかった．

8) その他- ICSI と fresh-ICSI (31～38歳以下) の比較 (表8)

採卵周期数は，その他-ICSI群21周期，fresh-ICSI群115周期であった．その他-ICSI，fresh-ICSIの順に，妻年齢は，37.2±4.8歳，35.3±2.0歳．平均採卵個数／回は，5.38個，6.06個．平均ＭⅡ卵数／回は，4.71個，5.38個．受精率は，78.0% (71/91)，81.7% (456/558)．臨床的妊娠率は，37.3% (10/27)，38.2% (58/152)．分娩率は，37.3% (10/27)，23.0% (35/152) であった．全てにおいて有意差を認めなかった．

考　察

現在，無精子症患者に対してTESE-ICSIは標準治療となっているが，TESEを実施できる施設はARTを実施できる施設に比して少ない[1]．2018年4月時点で，生殖医療専門医725人のうち泌尿器領域の専攻者は58人の登録となっている．また，2015年の湯村らの報告[1]では「泌尿器生殖医療専門医所属施設の半数で自施設の婦人科とは連携がなく，患者診察が有効に機能していないと考えられた」とされている．もちろん泌尿器生殖医療専門医のみがTESEを行っているわけではないが，自施設内でTESE-ICSIを完遂している施設は多くないと予想され，近隣泌尿器科との連携は非常に重要であると考えられる．

近隣泌尿器科との連携での懸念事項は主に2つある．1つは，TESE-ICSIに凍結融解精子を使用することが多くなる点である．TESEを他施設に依頼するため，TESE同日に採卵を調整できたとしてもTESEの手術時間，採卵時間，精巣組織の輸送時間などの兼ね合いで，新鮮精巣精子を用いた同日のICSIが困難な状況となることがある．当クリニックでのTESE-ICSIはそれらの理由のため全例で凍結融解精子を用いた．もう1つは，近隣泌尿器科での精巣組織採取後に自施設までにかか

る培養液中での輸送時間の影響である.

これまでTESEによる精子凍結に関しては,様々な報告がある.一般的にTESEによって採取した精巣精子は,運動精子数が少なく,運動率も低く,それは非閉塞性無精子症で顕著であり,そこに凍結融解操作が加わることは受精率の低下などが懸念されてきた[2)3)].

しかし,Waldら[4]は,TESEで回収した凍結融解精巣精子,新鮮精巣精子を用いたICSIにおいて,受精率,妊娠率に有意差は認めなかったとしている.また,Habermannら[5]は,凍結融解精巣精子,新鮮精巣精子を用いたICSIにおいて,妊娠率,胚分割率,着床率,臨床的妊娠率,妊娠率,分娩率,自然流産率の全てにおいて,有意差を認めなかったとしている.

一方で,凍結融解精巣精子では受精率が低いとする報告[6)-8)]もある.しかし,これらの報告も分娩率には有意差を認めなかったとしている.少なくとも,凍結融解精巣精子は分娩率には影響しないことが示唆されてきた.ただし,これらの報告は全てOA,NOAなどの原因については論じられていない.

OAに限ると以下のような報告がある.

Wuら[9]は,OAにおいて新鮮精巣精子を使用したICSI群と凍結融解精巣精子を使用したICSI群とを比較したところ,受精率,良好胚率,妊娠率において有意差を認めなかったと報告している.

小山ら[10]は,凍結融解精巣精子を使用したICSI群と新鮮精巣精子を使用したICSI群を比較すると,凍結融解精巣精子を使用したICSI群において,5日目の胚盤胞到達率が有意に高かった以外は,受精率,良好胚率,臨床妊娠率,分娩率などに有意差を認めなかったと報告している.

NOAに限ると,Hauserらの報告[11]があり,凍結融解操作によって運動性は低下するものの,受精率,着床率,妊娠率には有意差は認めなったとしている.

今回,我々はTESEによって採取した凍結融解精巣精子と新鮮射出精子を比較した.それは近隣泌尿器科との連携に使用する凍結融解精巣精子が,新鮮射出精子と成績が遜色なければ,TESE,精巣組織の輸送,精子の凍結融解操作に関する懸念を解消できると考えたからである.

結果は,NOA群の受精率が有意に低いことを認めたのみであった.NOA群は精子の質という点でどうしても受精率が低下してしまうことが示唆された.

それを除くOA群とその他群において,受精率,臨床的妊娠率,分娩率に悪影響がないことを示せたのは,近隣泌尿器科との連携における悪影響がないことを確認できたと考える.

一方で,近隣泌尿器科での精巣組織採取後に自施設までにかかる培養液中での輸送時間の影響に関しては検討できなかった.当クリニックでは,現在まで3施設との連携をとってきたものの,最長の輸送時間でも約90分と恵まれた立地であった.90分程度なら問題ないことは示唆できるが,逆にどの程度の輸送時間まで許容可能かということは不明である.しかし,TESEで得た精子は貴重であり,処理までの時間を延長することは許容出来ず,それを検証することは困難とも考えられた.

結　語

TESE-ICSIを施行できた42組のうち,30組(71.4%)が生児を獲得していた.

同施設内でなくても,近隣の泌尿器科と連携することで,女性を転院させずとも,男性不妊カップルへの治療が十分可能であることが再確認された.

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—Original Article—

Effects of 5-hydroxytryptamine on spermatozoal hyperactivation and *in vitro* fertilization in mice

Yukiko SUGIYAMA[1)], Masakatsu FUJINOKI[2, 3)] and Hiroaki SHIBAHARA[1)]

[1)]*Department of Obstetrics and Gynecology, Hyogo College of Medicine, Hyogo 663-8501, Japan*
[2)]*Department of Physiology, School of Medicine, Dokkyo Medical University, Tochigi 321-0293, Japan*
[3)]*Laboratory Animal Research Center, Dokkyo Medical University, Tochigi 321-0293, Japan*

Abstract. In this study, we examined the effects of 5-hydroxytryptamine (5-HT) on the motility and hyperactivation of mouse spermatozoa. In addition, we examined whether 5-HT increases the success of *in vitro* fertilization (IVF) in mice. Interestingly, 5-HT and agonists of the 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors significantly increased the percentage of hyperactivated spermatozoa but did not affect the percentage of motile spermatozoa. Moreover, agonists of the 5-HT_2, 5-HT_3, and 5-HT_4 receptors significantly affected the velocities, linearity, straightness, wobbler coefficient, amplitude and/or frequency of spermatozoa. In particular, the improvement of hyperactivation by 5-HT was strongly inhibited by antagonists of the receptors 5-HT_4 and 5-HT_7 and was completely inhibited by a mixture of the four 5-HT-receptor antagonists. The increase in hyperactivation by the agonists was significantly inhibited by the corresponding 5-HT-receptor antagonist. Moreover, 5-HT significantly increased the percentage of two-cell embryos. The increase in the IVF success rate by 5-HT was significantly inhibited by a 5-HT_4-receptor antagonist. These results suggest that 5-HT increased hyperactivation through the 5-HT receptors and increased the success of IVF in mice.

Key words: 5-Hydroxytryptamine (5-HT), Hyperactivation, *In vitro* fertilization (IVF), Spermatozoa

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Spermatozoa are activated after ejaculation, and activated mammalian spermatozoa are capacitated before fertilization [1, 2]. Activated spermatozoa exhibit small-bend amplitude in flagellar movement and linear swimming patterns [3, 4]. However, capacitation is inhibited by the binding of a decapacitation factor [2] and cannot occur until the decapacitation factor is lost. Capacitated spermatozoa then show hyperactivation of flagellar movement, and acrosome reactions (AR) occur in the head [1, 2]. Hyperactivated spermatozoa have a large amplitude and large asymmetric flagellar beating pattern [3–5]; therefore, hyperactivation provides increased motility and propulsive force to facilitate passage of the spermatozoa through the zona pellucida and the cumulus cell layers [3]. Whereas, AR is a form of exocytosis that exposes an enzyme system that promotes binding to the oocyte [1]. Finally, acrosome-reacted spermatozoa are able to fertilize the oocyte.

In order for spermatozoa to be capacitated and hyperactivated *in vitro*, three essential components, albumin, Ca^{2+}, and, HCO_3^-, are required in the capacitation medium. Albumin removes cholesterol from the spermatozoal plasma membrane [6], and Ca^{2+} and HCO_3^- stimulate soluble adenylate cyclase (sAC) and induce cAMP production [2, 7, 8]. Moreover, Ca^{2+} and cAMP activate several protein kinases and phosphatases, thereby inducing protein phosphorylation and de-phosphorylation [2, 5, 9, 10]. It has been reported that the spermatozoal capacity for hyperactivation is correlated with the success rate of *in vitro* fertilization (IVF) [11].

Several oviductal hormones have been observed to affect hyperactivation processes [4], including the induction of hyperactivation by progesterone (P_4), melatonin, and 5-hydroxytryptamine (5-HT) [12–14]. In contrast, 17β-estradiol suppresses the effects of P_4 and melatonin [15–17]. Moreover, γ-aminobutyric acid (GABA) induces hyperactivation in humans [18], although it suppresses the effects of P_4 and 5-HT in hamsters [19, 20].

The hormone 5-HT is known to regulate numerous functions by activating specific receptors [21]. The receptors for 5-HT consist of seven subtypes (5-HT_1, 5-HT_2, 5-HT_3, 5-HT_4, 5-HT_5, 5-HT_6, and 5-HT_7). The receptors 5-HT_1 and 5-HT_5 suppress transmembrane adenylate cyclase (tmAC); while 5-HT_4, 5-HT_6, and 5-HT_7 stimulate tmAC. The 5-HT_2 receptors stimulate phospholipase C (PLC), which regulates the release of Ca^{2+} from Ca^{2+}-stores. Whereas, the 5-HT_3 receptor is a ligand-gated cation channel receptor.

The spermatozoal functions of several mammal species are affected by 5-HT. In hamster spermatozoa [13, 22], 5-HT induces AR and hyperactivation through 5-HT_2 and 5-HT_4 receptors. In addition, 5-HT dose-dependently induces hyperactivation [13]. Low concentrations (fM to pM) of 5-HT increased hyperactivation through the 5-HT_2 receptor, whereas high concentrations (nM) improved hyperactivation through the 5-HT_4 receptor. The hormone receptors, 5-HT_1, 5-HT_2, and 5-HT_3, have been found in human and stallion spermatozoa [23, 24]. Furthermore, it has been suggested that 5-HT increases straight-line velocity (VSL), curvilinear velocity (VCL), and average-path

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Correspondence: M Fujinoki (e-mail: fujinoki@dokkyomed.ac.jp)
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velocity (VAP) of human spermatozoa [23]. It is assumed that 5-HT also affects fertilization, as 5-HT and the 5-HT_1, $5HT_2$, and $5HT_7$ receptors are found in oocytes, cumulus-oocyte complexes (COCs), follicular fluid, oviducts, and embryos of humans and mice [21]. In this study, we examined whether 5-HT affects the motility and hyperactivation of mouse spermatozoa and investigated the effects of 5-HT on mouse IVF.

Materials and Methods

Chemicals

We purchased 5-HT, sumatriptan succinate (Sumatriptan), α-methylserotonin maleate salt (MS), 1-(3-chlorophenyl)biguanide hydrochloride (mCPBG), 5-methoxytryptamine (MT), WAY-208466 dihydrochloride (WAY), LP12 hydrochloride hydrate (LP12), cyproheptadine hydrochloride sesquihydrate (Cypro), dolasetron mesylate hydrate (DS), GR113808 (GR), and SB-258719 (SB25) from Sigma-Aldrich (St. Louis, MO, USA). Pregnant mare serum gonadotropin (PMSG) (Serotropin®) and human chorionic gonadotropin (hCG) (Gonatropin®) were purchased from ASKA Pharmaceutical (Tokyo, Japan). Other reagent-grade chemicals were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan).

Animals

ICR mice were bred in the Laboratory Animal Research Center of Dokkyo Medical University. The present study was approved by the Animal Care and Use Committee of the university (experimental permission number: 0107) and performed in accordance with the University's Guidelines for Animal Experimentation.

Preparation of hyperactivated spermatozoa

Hyperactivated spermatozoa were prepared according to the method described previously [9] with some modifications. Modified Tyrode's albumin lactate pyruvate (mTALP) medium [25] was used as a capacitation medium. Spermatozoa were collected from the cauda epididymis of male mice (10–20 weeks of age). One drop (approximately 3 µl) of spermatozoa was placed on a culture dish (35 mm diameter), and 3 ml mTALP medium was carefully added to the dish. The spermatozoa were incubated for 5 min at 37°C to allow diffusion into the medium, and the supernatant containing motile spermatozoa were collected and placed in a new dish containing a vehicle or antagonists. After incubation for 5 min, the supernatant was transferred to a new dish containing the vehicle, 5-HT, or agonists. The spermatozoa were incubated for 2 h at 37°C in an atmosphere containing 5% CO_2 to induce hyperactivation. Stock solutions of 5-HT (100 µM), sumatriptan (100 µM), MS (100 pM), mCPBG (100 mM), WAY (7.3 µM), LP12 (0.13 µM), and DS (3.8 µM) were dissolved in de-ionized and distilled water. Stock solutions of MT (10 nM) and Cypro (1 mM) were dissolved in ethanol. Stock solutions of GR (1 mM) and SB25 (10 mM) were dissolved in dimethyl sulfoxide (DMSO). In all experiments, the maximum concentration of the vehicle was 0.5%.

Measurement of motile and hyperactivated spermatozoa

The analyses of motile and hyperactivated spermatozoa were performed as follows: motile spermatozoa were recorded on a Blu-ray disc recorder (DIGA DMR-BRW520; Panasonic, Osaka, Japan) using a CCD camera (Model XC-77; Kyoshin, Tokyo, Japan) attached to a microscope (CX41, Olympus, Tokyo, Japan) with phase-contrast illumination and a warm plate (MP-1000; Kitazato, Shizuoka, Japan). The suspension containing motile spermatozoa (50 µl) was transferred to an observation chamber (0.125 mm deep, 16 mm wide, and 18 mm long) made of vinyl tape attached to the glass slide in two parallel strips, and then covered with a cover glass. Observations were performed for 1 min at 37°C. Visual analyses of the movies comprised manual counts of the numbers of total spermatozoa, motile spermatozoa, and hyperactivated spermatozoa. The analyses were performed in a blinded manner for all experiments. Motile spermatozoa that exhibited asymmetric flagellar movement and moved in a circular and/or figure of eight pattern were defined as hyperactivated [1, 4] (see Supplemental Movie). The percentages of motile and hyperactivated spermatozoa were defined as the number of motile spermatozoa/number of total spermatozoa × 100, and the number of hyperactivated spermatozoa/number of total spermatozoa × 100, respectively. Each experiment was performed four times on four different mice. When the proportion of motile spermatozoa was equal to or below 70% of levels seen before incubation, the experiment was repeated. The data were statistically analyzed by Student's *t*-test using Microsoft Excel (Microsoft Japan, Tokyo, Japan) or by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using Microsoft Excel with the Statcel2 (OMS Publishing, Saitama, Japan) add-on. Statistical significance was considered for P values of < 0.05.

Motility assay by the Sperm Motility Analysis System (SMAS)

The motility assay was evaluated by using SMAS for animals (Ver. 3.18) with the loaded parameter file mouse_BM10×_640nm_Bright59_150fps-shutter200.ini (Ditect, Tokyo, Japan) [26]. The suspension containing motile spermatozoa (20 µl) was transferred to an observation chamber (0.1 mm deep, 18 mm wide, and 18 mm long) made of mending tape attached to the glass slide in two parallel strips, and then covered with a cover slips. Spermatozoal movement was recorded for 1 sec on the hard disk drive of the SMAS via a high-speed digital camera (HAS-L2; Ditect) attached to a microscope (ECLIPSE E2000; Nikon, Tokyo, Japan) with phase-contrast illumination, a 650 nm band-pass filter, and a warm plate (MP10DM; Kitazato). The SMAS analyzed 150 consecutive images obtained from a single field at 10 × magnification in a negative phase-contrast. VSL (µm/sec), VCL (µm/sec), VAP (µm/sec), linearity (LIN; defined as VSL/VCL), straightness (STR; defined as VSL/VAP), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz), were automatically calculated by the SMAS; wobbler coefficient (WOB; defined as VAP/VCL) was calculated manually [27]. The SMAS analysis was repeated five times on five different mice. In each experiment, ≥ 300 spermatozoa were detected. Only motile spermatozoa judged as significant were analyzed. The effects of 5-HT and agonists were statistically analyzed by a Student's *t*-test performed using Microsoft Excel or by one-way ANOVA with Tukey's multiple comparisons test using Microsoft Excel with the add-in software Statcel2. Differences were considered significant for P values of < 0.05.

IVF

IVF was performed in accordance with the method described by Takeo *et al*. [28] with some modifications. For the superovulation treatment, female mice (8–12 weeks of age) were intraperitoneally (i.p.) administered 10 units PMSG at 1600 h, three days before IVF was conducted, followed by 10 units hCG (i.p.) at 1630 h, one day before IVF was performed. At 0950 h on the day IVF was performed, one drop (approximately 3 µl) of the dense mass of spermatozoa obtained from the cauda epididymis of male mice (10–20 weeks of age) were introduced to 300 µl drops of mTALP medium in the presence and absence of a vehicle, 100 pM 5-HT, and/or antagonists. Spermatozoa at 20 × 10^6 cell/ml were incubated for 1 h at 37°C in an atmosphere containing 5% CO_2. The eggs were collected at 1030 h on the day of IVF (18 h after the hCG injection) from PMSG/hCG-treated female mice. COCs were dissected from the ampullae of both oviducts and incubated in 300 µl drops of the mTALP medium in the presence and absence of a vehicle, 100 pM 5-HT, and/or antagonists. At 1050 h on the day of IVF, 10 µl of the suspension containing the pre-incubated spermatozoa was added to the medium containing the COCs to start the insemination process. The spermatozoa and COCs were incubated together for 0.5 or 5 h at 37°C in an atmosphere containing 5% CO_2. After incubation, the eggs or COCs were collected and washed with the mTALP medium to remove spermatozoa and cumulus cells from the medium, and insemination was stopped. The washed eggs or COCs were observed after incubation at 1600 h on the day of IVF using an inverted microscope (Leica DM IRB; Leica Microsystems, Wetzlar, Germany), and the total number of eggs was manually counted. The eggs or COCs were observed again at 1600 h after re-incubation on the day after IVF using an inverted microscope. The two-cell embryos were manually counted. The percentage of two-cell embryos was defined as the number of two-cell embryos/total number of eggs × 100. The analysis was performed in a blinded manner. Experiments were performed four times on four different male and four different female mice. The data were statistically analyzed by Student's *t*-test using Microsoft Excel or one-way ANOVA with Tukey's multiple comparisons test using Microsoft Excel with the add-in software Statcel2, with differences considered significant for P values of < 0.05.

Results

Effects of 5-HT and 5-HT-receptor agonists on percentages of motile and hyperactivated spermatozoa, and the motility assay

Whether 5-HT affects the percentages of motile and hyperactivated spermatozoa was investigated (Fig. 1A). When spermatozoa were exposed to 100 fM, 100 pM, and 100 nM 5-HT, all concentrations of 5-HT significantly increased the percentage of hyperactivated spermatozoa but did not affect the percentage of motile spermatozoa. Moreover, spermatozoa treated with 5-HT were evaluated using SMAS (Table 1A). All concentrations of 5-HT did not affect VSL, VCL, VAP, LIN, STR, WOB, ALH, and BCF.

In the next step, the effects of 5-HT-receptor agonists on the percentages of motile and hyperactivated spermatozoa were examined (Figs. 1B–G). Sumatriptan (5-HT_1-receptor agonist) and WAY (5-HT_6-receptor agonist) did not affect the percentages of motile and hyperactivated spermatozoa (Figs. 1B and 1F). However, MS (5-HT_2-receptor agonist), mCPBG (5-HT_3-receptor agonist), MT (5-HT_4-receptor agonist), and LP12 (5-HT_7-receptor agonist) significantly increased the percentage of hyperactivated spermatozoa, but they did not affect the percentage of motile spermatozoa (Figs. 1C–E and 1G).

Spermatozoa treated with 5-HT-receptor agonists were evaluated by SMAS. MS significantly decreased VSL, VCL, LIN, STR, and ALH but it did not affect VAP, WOB, and BCF (Table 1B). Treatment by mCPBG significantly decreased VSL, LIN, WOB, and BCF but it did not affect VCL, VAP, STR, and ALH (Table 1C). MT significantly decreased VCL and ALH and significantly increased WOB, whereas it did not affect VSL, VAP, LIN, STR, and BCF (Table 1D). LP12 did not affect VSL, VCL, VAP, LIN, STR, WOB, ALH, and BCF (Table 1E).

Effects of 5-HT-receptor antagonists on the increase of hyperactivation by 5-HT and 5-HT-receptor agonists

As the 5-HT and four 5-HT-receptor agonists increased hyperactivation (Fig. 1), the effects of the 5-HT-receptor antagonists on these increases were investigated (Fig. 2). Cypro (5-HT_2-receptor antagonist) at 1 µM [13, 22] and 3.8 nM DS (5-HT_3-receptor antagonist) [29] did not significantly suppress the improvement of hyperactivation by any concentration of 5-HT (Figs. 2A and B). As shown in Fig. 2C, 1 µM GR (5-HT_4-receptor antagonist) [13, 22] significantly suppressed the increase of hyperactivation by 100 fM and 100 pM 5-HT, although 1 µM GR did not suppress the increase of hyperactivation by 100 nM 5-HT. Although 10 µM SB25 (5-HT_7-receptor antagonist) [30] did not suppress the improvement of hyperactivation by 100 fM and 100 nM 5-HT, it significantly suppressed the increase by 100 pM 5-HT (Fig. 2D). In addition, a mixture of the four 5-HT-receptor antagonists significantly suppressed the increase of hyperactivation by all concentrations of 5-HT (Fig. 2E). As shown in Fig. 3, the increase of hyperactivation by each agonist was significantly suppressed by the corresponding antagonists.

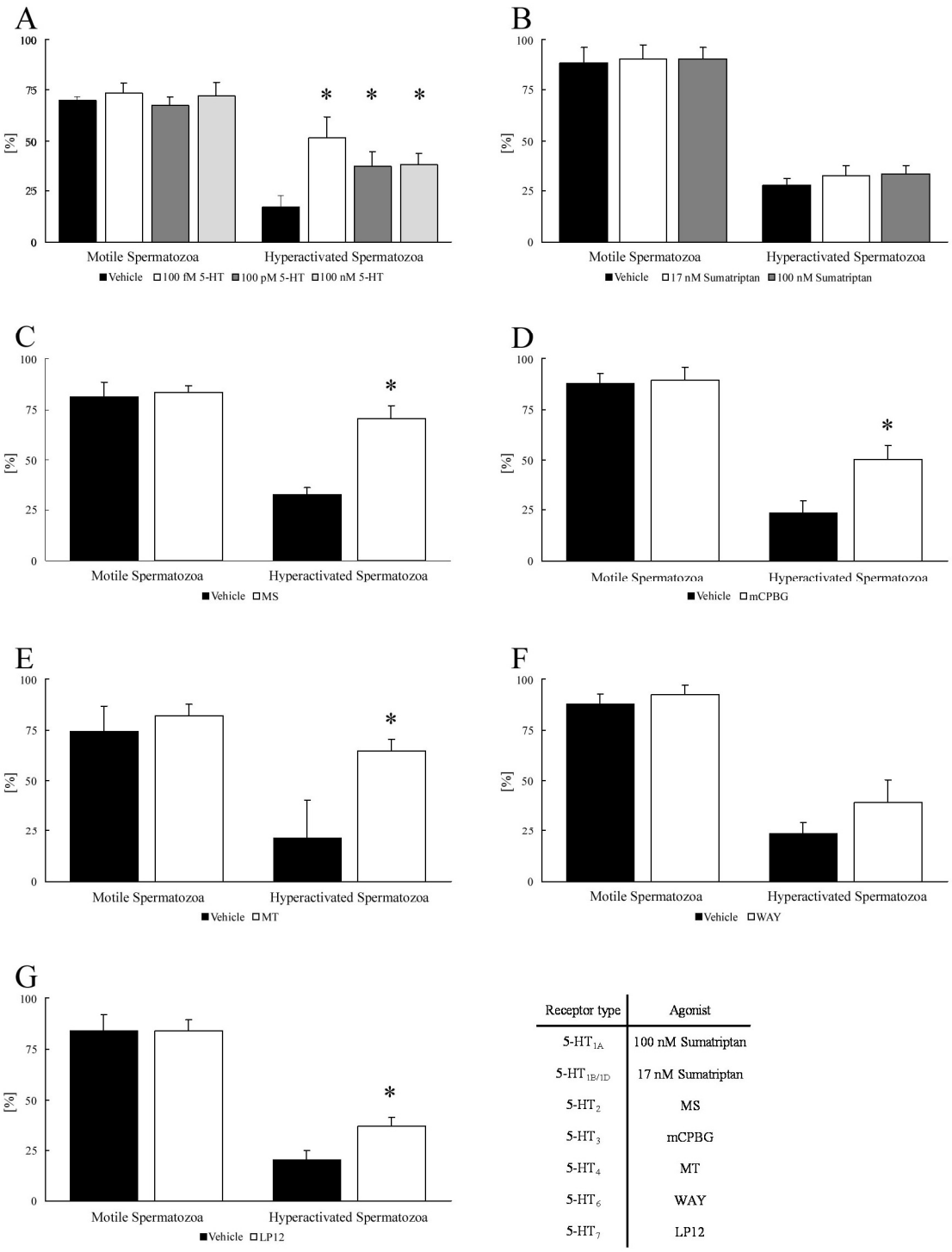
Effects of 5-HT on IVF in mice

Finally, the effect of 5-HT on the success rate of IVF was examined (Table 2). The addition of 100 pM of 5-HT to the medium did not affect the percentage of two-cell embryos when spermatozoa were co-incubated with COCs for 5 h. However, 100 pM 5-HT significantly increased the percentage of two-cell embryos when spermatozoa were co-incubated with COCs for 0.5 h.

We then investigated the 5-HT-receptor antagonists effects on the increase of two-cell embryos by 5-HT (Table 3). Cypro at 1 µM (Table 3A), 3.8 nM DS (Table 3B), 10 µM SB25 (Table 3D), and the mixture of antagonists (Table 3E) did not suppress the percentage increase of two-cell embryos by 100 pM 5-HT. In contrast, 1 µM GR significantly suppressed the effect of 5-HT (Table 3C). Additionally, 1 µM GR significantly decreased the percentage of two-cell embryos.

Discussion

In mammalian reproduction, 5-HT (serotonin) regulates several important functions of gametes and organs [13, 21–24]. Because 5-HT and 5-HT receptors are found in female reproductive organs [21], serotonergic signaling is thought to be involved in the regulation of



Receptor type	Agonist
5-HT$_{1A}$	100 nM Sumatriptan
5-HT$_{1B/1D}$	17 nM Sumatriptan
5-HT$_{2}$	MS
5-HT$_{3}$	mCPBG
5-HT$_{4}$	MT
5-HT$_{6}$	WAY
5-HT$_{7}$	LP12

Fig. 1. Effects of 5-hydroxytryptamine (5-HT) and 5-HT-receptor agonists on percentages of motile and hyperactivated spermatozoa. The percentages of motile and hyperactivated spermatozoa are shown after spermatozoa were exposed to 100 fM, 100 pM, or 100 nM 5-HT (A), 17 nM or 100 nM sumatriptan succinate (Sumatriptan) (B), 100 fM α-methylserotonin maleate salt (MS) (C), 100 μM 1-(3-chlorophenyl)biguanide hydrochloride (mCPBG) (D), 10 pM 5-methoxytryptamine (MT) (E), 7.3 nM WAY-208466 dihydrochloride (WAY) (F), and 0.13 nM LP12 hydrochloride hydrate (LP12) (G). Data represent the mean ± SD. (A) (Vehicle) the medium with 0.1% (v/v) pure water as vehicle; (respective concentrations of 5-HT) the medium with indicated concentration of 5-HT and vehicle. (B) (Vehicle) same as above; (respective concentrations of Sumatriptan) the medium with indicated concentrations of Sumatriptan and vehicle. (C) (Vehicle) same as above; (MS) the medium with MS and vehicle. (D) (Vehicle) same as above; (mCPBG) the medium with mCPBG and vehicle. (E) (Vehicle) medium with 0.1% (v/v) ethanol as vehicle; (MT) medium with MT and vehicle. (F) (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (WAY) medium with WAY and vehicle. (G) (Vehicle) same as above; (LP12) medium with LP12 and vehicle. * Significant difference compared with "Vehicle" ($P < 0.05$).

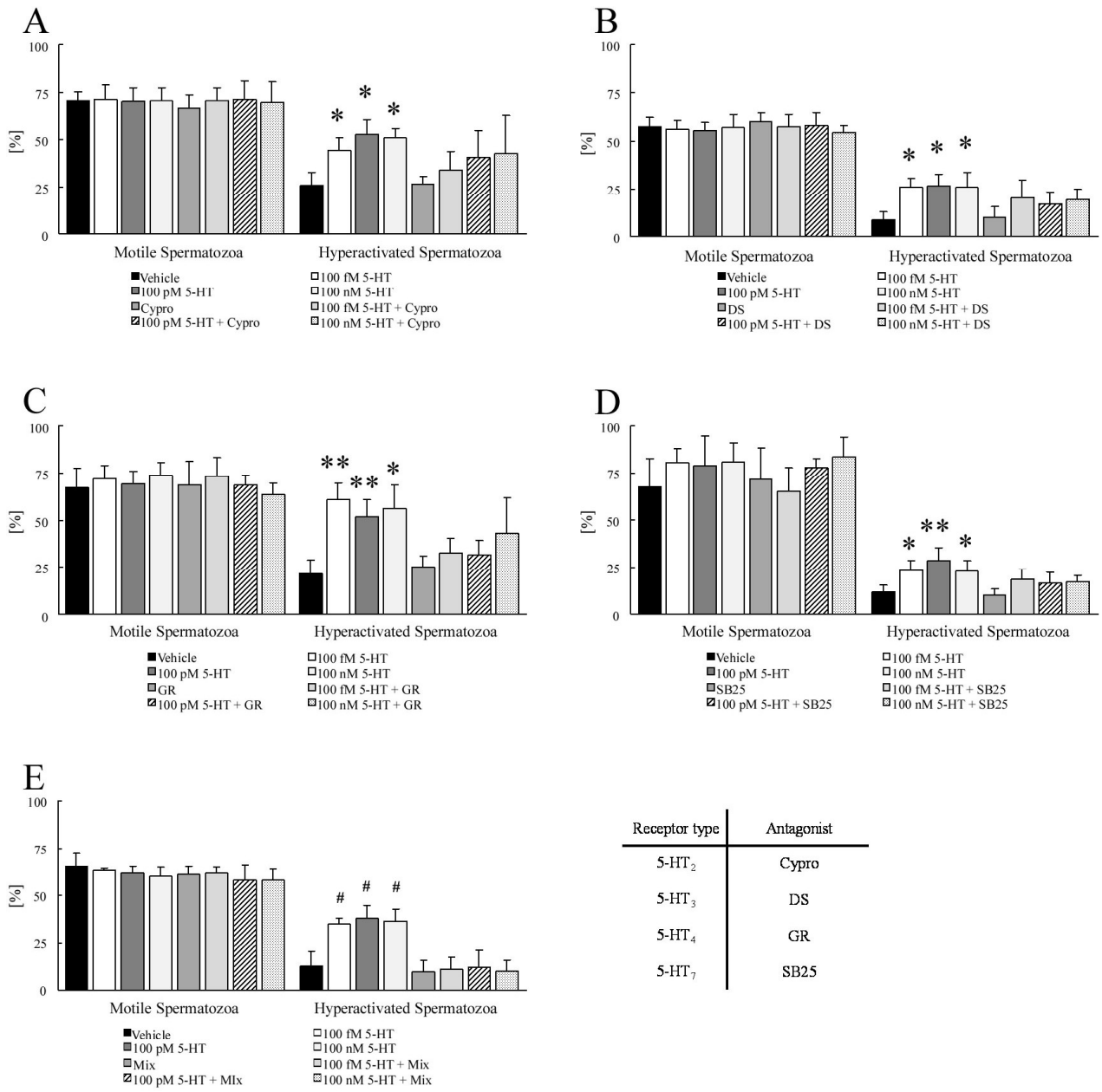
Table 1. Effects of 5-hydroxytryptamine (5-HT) and 5-HT-receptor agonists on motility assay by Sperm Motility Analysis System (SMAS)

		VSL (μm/sec)	VCL (μm/sec)	VAP (μm/sec)	LIN
A	Vehicle	84.27 ± 13.67	314.31 ± 32.18	142.65 ± 21.96	0.27 ± 0.04
	100 fM 5-HT	83.57 ± 10.96	306.68 ± 23.74	143.82 ± 24.20	0.29 ± 0.05
	100 pM 5-HT	66.41 ± 13.65	295.51 ± 48.02	137.68 ± 22.24	0.23 ± 0.06
	100 nM 5-HT	85.88 ± 16.68	296.61 ± 20.17	141.89 ± 10.85	0.30 ± 0.05
B	Vehicle	84.92 ± 9.21	322.45 ± 17.50	152.97 ± 11.02	0.27 ± 0.03
	MS	64.04 ± 4.05 *	293.27 ± 17.27 *	142.12 ± 15.50	0.22 ± 0.01 *
C	Vehicle	78.75 ± 4.15	313.24 ± 12.88	150.75 ± 26.40	0.26 ± 0.02
	mCPBG	57.95 ± 10.67 *	327.22 ± 84.36	129.49 ± 38.58	0.21 ± 0.03 *
D	Vehicle	75.67 ± 18.79	287.99 ± 29.08	137.33 ± 14.02	0.27 ± 0.05
	MT	68.86 ± 21.91	262.32 ± 28.38 *	138.90 ± 13.31	0.26 ± 0.07
E	Vehicle	76.00 ± 14.89	317.11 ± 9.74	130.51 ± 21.00	0.24 ± 0.05
	LP12	74.26 ± 15.24	334.24 ± 32.32	137.80 ± 37.73	0.23 ± 0.04

		STR	WOB	ALH (μm)	BCF (Hz)
A	Vehicle	0.59 ± 0.10	0.47 ± 0.10	7.41 ± 0.40	8.78 ± 1.20
	100 fM 5-HT	0.61 ± 0.09	0.48 ± 0.05	7.40 ± 0.91	8.29 ± 0.48
	100 pM 5-HT	0.51 ± 0.14	0.48 ± 0.04	7.72 ± 1.31	7.85 ± 0.35
	100 nM 5-HT	0.62 ± 0.11	0.48 ± 0.03	7.48 ± 0.29	8.36 ± 0.66
B	Vehicle	0.57 ± 0.09	0.48 ± 0.04	8.19 ± 0.74	7.99 ± 0.84
	MS	0.48 ± 0.08 *	0.49 ± 0.06	7.41 ± 0.78 *	8.44 ± 0.48
C	Vehicle	0.54 ± 0.05	0.49 ± 0.06	7.25 ± 1.09	9.88 ± 1.19
	mCPBG	0.49 ± 0.07	0.42 ± 0.07 *	7.60 ± 2.20	7.53 ± 0.49 *
D	Vehicle	0.56 ± 0.10	0.48 ± 0.04	7.70 ± 0.95	7.37 ± 0.70
	MT	0.51 ± 0.14	0.53 ± 0.02 *	7.05 ± 0.74 *	8.34 ± 0.84
E	Vehicle	0.57 ± 0.09	0.42 ± 0.08	7.35 ± 1.35	8.45 ± 1.24
	LP12	0.55 ± 0.05	0.42 ± 0.09	8.00 ± 2.00	7.53 ± 0.90

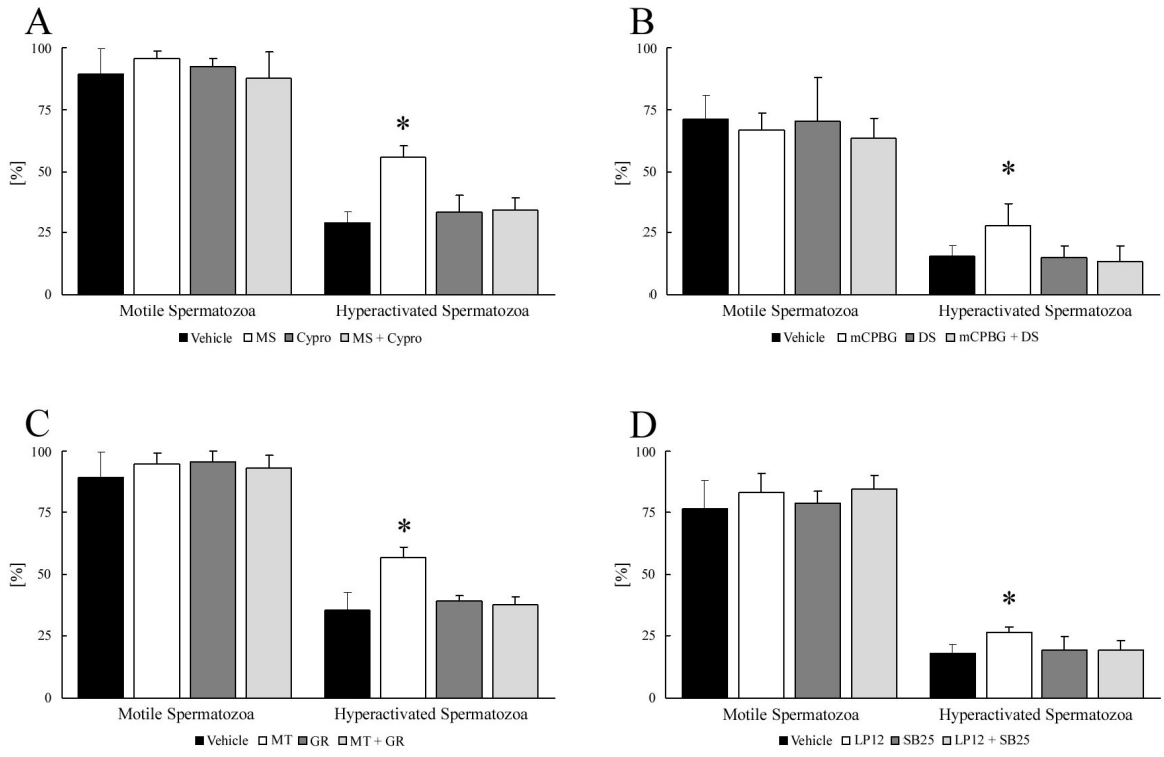
Straight-line velocity (VSL), curvilinear velocity (VCL), average-path velocity (VAP), linearity (LIN), straightness (STR), wobbler coefficient (WOB), amplitude of lateral head displacement (ALH), and beat-cross frequency (BCF) are shown after spermatozoa were exposed to 100 fM, 100 pM, or 100 nM 5-HT (A), 100 fM α-methylserotonin maleate salt (MS) (B), 100 μM 1-(3-chlorophenyl)biguanide hydrochloride (mCPBG) (C), 10 pM 5-methoxytryptamine (MT) (D), and 0.13 nM LP12 hydrochloride hydrate (LP12) (E). Data represent the mean ± SD. (A) (Vehicle) the medium with 0.1% (v/v) pure water as vehicle; (respective concentrations of 5-HT) the medium with indicated concentration of 5-HT and vehicle. (B) (Vehicle) same as above; (MS) the medium with MS and vehicle. (C) (Vehicle) same as above; (mCPBG) the medium with mCPBG and vehicle. (D) (Vehicle) the medium with 0.1% (v/v) ethanol as vehicle; (MT) medium with MT and vehicle. (E) (Vehicle) the medium with 0.1% (v/v) pure water as vehicle; (LP12) medium with LP12 and vehicle. * Significant difference compared with "Vehicle" in the same condition ($P < 0.05$). Experiments were repeated five times on five different mice.

steroidogenesis, oocyte maturation, and embryonic development. For example, the $5HT_2$ receptor has been detected in mouse cumulus cells [21]. The concentration of 5-HT in rat oviducts is between 2.06 and 3.34 μg/g fresh tissue [31]; whereas, in humans, the concentration of 5-HT in the preovulatory follicles and the cystically degenerated follicles is 14.3 ± 8.9 μg/100 ml and 12.2 ± 6.2 μg/100 ml, respectively [32]. Moreover, 5-HT induces the release of P_4 from granulose cells [33]. In hamsters, 5-HT and MT induce the AR in the spermatozoa, and this induction can be inhibited by Cypro [22], and 5-HT dose-dependently enhances hyperactivation through the 5-HT_2 and 5-HT_4 receptors [13]. In addition, the $5HT_{1B}$, $5HT_{2A}$, and $5HT_3$ receptors have been found in human and stallion spermatozoa [23, 24], and 5-HT increases the VSL, VCL, and VAP of human spermatozoa [23]. It is assumed that the velocities of human spermatozoa are regulated by the 5-HT_2 and 5-HT_3 receptors because the 5-HT_1 receptor suppresses tmAC and decreases cAMP concentration [21]. In mice, 5-HT and LP12 did not affect the VSL, VCL, and VAP of the spermatozoa (Tables 1A and E), but MS decreased VSL and VCL (Table 1B). Moreover, mCPBG and MT decreased VSL and VCL, respectively (Tables 1C and D). In either case, the 5-HT_2 receptor appears to be associated with the regulation of spermatozoa velocities in humans and mice. The 5-HT_2 receptor stimulates Ca^{2+} signals related to the PLC [21] and, Ca^{2+} stimulates the sAC to induce motility and hyperactivation via the production of cAMP [8]. Therefore, it is likely that 5-HT regulates spermatozoal velocities and hyperactivation through the activation of sAC by Ca^{2+} signals associated with the 5-HT_2 receptor. As for the other parameters, 5-HT and LP12 had no effects (Tables 1A and E). As MS decreased VSL



Receptor type	Antagonist
5-HT$_2$	Cypro
5-HT$_3$	DS
5-HT$_4$	GR
5-HT$_7$	SB25

Fig. 2. Suppressive effects of 5-hydroxytryptamine (5-HT)-receptor antagonists on the increase of hyperactivation by 5-HT. The percentages of motile and hyperactivated spermatozoa are shown after spermatozoa were exposed to 100 fM, 100 pM, or 100 nM 5-HT after exposure to 5-HT-receptor antagonists (A–D) or a mixture of antagonists (E) for 5 min. The mixture contained 1 μM cyproheptadine hydrochloride sesquihydrate (Cypro), 3.8 nM dolasetron mesylate hydrate (DS), 1 μM GR113808 (GR), and 10 μM SB-258719 (SB). The data represent the mean ± SD. (A) (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (respective concentrations of 5-HT) medium with respective concentration of 5-HT and vehicle; (Cypro) the medium with 1 μM Cypro and vehicle; (respective concentrations of 5-HT + Cypro) medium with respective concentration of 5-HT, 1 μM Cypro, and vehicle. (B) (Vehicle) medium with 0.2% (v/v) pure water as vehicle; (respective concentrations of 5-HT) medium with respective concentration of 5-HT and vehicle; (DS) medium with 3.8 nM DS and vehicle; (respective concentrations of 5-HT + DS) medium with respective concentration of 5-HT, 3.8 nM DS, and vehicle. (C) (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide (DMSO) as vehicle; (respective concentrations of 5-HT) medium with respective concentration of 5-HT and vehicle; (GR) medium with 1 μM GR and vehicle; (respective concentrations of 5-HT + GR) medium with respective concentration of 5-HT, 1 μM GR, and vehicle. (D) (Vehicle) same as above; (all concentrations of 5-HT) medium with respective concentration of 5-HT and vehicle; (SB25) medium with 10 μM SB-258719 (SB25) and vehicle; (respective concentrations of 5-HT + SB25) medium with respective concentration of 5-HT, 10 μM SB25, and vehicle. (E) (Vehicle) medium with 0.2% (v/v) pure water, 0.1% (v/v) ethanol, and 0.2% (v/v) DMSO as vehicle; (respective concentrations of 5-HT) medium with the respective concentrations of 5-HT and vehicle; (Mix) medium with the mixture of four antagonists and vehicle; (respective concentrations of 5-HT + Mix) medium with all concentrations of 5-HT, the mixture of four antagonists, and vehicle. * Significant difference compared with "Vehicle" and "antagonist" ($P < 0.05$). ** Significant difference compared with "Vehicle", "antagonist", and "respective concentrations of 5-HT + antagonist" ($P < 0.05$). # Significant difference compared with "Vehicle", "Mix", and "respective concentrations of 5-HT + Mix" ($P < 0.05$).



Receptor type	Agonist	Antagonist
5-HT_2	MS	Cypro
5-HT_3	mCPBG	DS
5-HT_4	MT	GR
5-HT_7	LP12	SB25

Fig. 3. Inhibition of the effect of 5-hydroxytryptamine (5-HT)-receptor agonists by 5-HT-receptor antagonists. The percentages of motile and hyperactivated spermatozoa are shown when spermatozoa were exposed to 5-HT-receptor agonists after exposure to 5-HT-receptor antagonists for 5 min. The data represent the mean ± SD. (A) (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (MS) medium with 100 fM α-methylserotonin maleate salt (MS) and vehicle; (Cypro) medium with 1 μM cyproheptadine hydrochloride sesquihydrate (Cypro) and vehicle; (MS + Cypro) the medium with 100 fM MS, 1 μM Cypro, and vehicle. (B) (Vehicle) medium with 0.2% (v/v) pure water as vehicle; (mCPBG) medium with 100 μM 1-(3-chlorophenyl)biguanide hydrochloride (mCPBG) and vehicle; (DS) medium with 3.8 nM dolasetron mesylate hydrate (DS) and vehicle; (mCPBG + DS) medium with 100 μM mCPBG, 3.8 nM DS, and vehicle. (C) (Vehicle) medium with 0.1% (v/v) ethanol and 0.1% (v/v) dimethyl sulfoxide (DMSO) as vehicle; (MT) medium with 10 pM 5-methoxytryptamine (MT) and vehicle; (GR) medium with 1 μM GR113808 (GR) and vehicle; (MT + GR) medium with 10 pM MT, 1 μM GR, and vehicle. (D) (Vehicle) medium with 0.1% (v/v) pure water and 0.1% (v/v) DMSO as vehicle; (LP12) medium with 0.13 nM LP12 hydrochloride hydrate (LP12) and vehicle; (SB25) medium with 10 μM SB-258719 (SB25) and vehicle; (LP12 + SB25) medium with 0.13 nM LP12, 10 μM SB25, and vehicle. * Significant difference compared with "Vehicle", "antagonist", and "agonist + antagonist" for the same incubation time ($P < 0.05$).

and VCL, it decreased LIN and STR, which are calculated as VSL/VCL and VSL/VAP, respectively (Table 1B). As mCPBG decreased VSL, LIN also decreased (Table 1C). In addition, mCPBG decreased WOB, which is calculated as VAP/VCL, although it did not affect VCL or VAP (Table 1C). Therefore, as MT decreased VCL, WOB increased (Table 1D). Both MS and MT decreased ALH, and mCPBG decreased BCF (Tables 1B, C and D).

Previously, we found that 100 fM, 100 pM, and 100 nM 5-HT significantly improved the hyperactivation of hamster spermatozoa through the 5-HT_2 and 5-HT_4 receptors [13]. Although not all concentrations of 5-HT affected the percentage of motile mouse spermatozoa, they significantly increased the percentage of hyperactivated spermatozoa through the 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors (Figs. 1 and 3). In hamster spermatozoa, 100 fM and 100 pM 5-HT enhanced hyperactivation through the 5-HT_2 receptor only [13]. In contrast, hyperactivation was not always improved through the receptors in mouse spermatozoa (Fig. 2A). The improvement by 100 nM 5-HT was not affected by Cypro in either hamsters [13] or

Table 2. Effects of 5-hydroxytryptamine (5-HT) on *in vitro* fertilization (IVF)

		No. of total eggs	No. of two-cell embryos	Two-cell embryo (%)
A	5 h insemination			
	Vehicle	120	39	34.35 ± 14.11
	100 pM 5HT	112	33	30.17 ± 9.06
B	0.5 h insemination			
	Vehicle	112	11	9.23 ± 4.62
	100 pM 5HT	101	27	26.59 ± 10.37 *

The percentages of the two-cell embryos are shown when IVF was performed in the medium in the presence and absence of 100 pM 5-HT. The data represent the mean ± SD. The insemination times of IVF were 5 h (A) and 0.5 h (B). (Vehicle) medium with 0.1% (v/v) pure water as vehicle; (100 pM 5-HT) medium with 100 pM 5-HT and vehicle. * Significant difference compared with "Vehicle" ($P < 0.05$). Experiments were performed four times on four different male and four different female mice.

Table 3. Inhibition of the increase in *in vitro* fertilization (IVF) by 5-hydroxytryptamine (5-HT)-receptor antagonists

		No. of total eggs	No. of two-cell embryos	Two-cell embryo (%)
A	Vehicle	154	25	14.96 ± 5.29
	100 pM 5HT	164	57	35.96 ± 10.69 *
	Cypro	73	19	25.25 ± 8.58
	100 pM 5HT + Cypro	89	20	22.09 ± 5.81
B	Vehicle	128	13	6.64 ± 10.59
	100 pM 5HT	107	39	34.76 ± 7.79 **
	DS	67	2	3.35 ± 3.88
	100 pM 5HT + DS	102	30	25.71 ± 19.39
C	Vehicle	138	29	21.33 ± 3.73
	100 pM 5HT	130	48	35.91 ± 6.75 $^{\#}$
	GR	91	5	4.96 ± 4.53 $^{\#\#}$
	100 pM 5HT + GR	97	16	18.25 ± 6.04
D	Vehicle	103	14	12.15 ± 8.11
	100 pM 5HT	129	52	40.55 ± 9.96 **
	SB25	109	3	3.11 ± 2.98 $^{\$}$
	100 pM 5HT + SB25	110	26	22.31 ± 12.31
E	Vehicle	141	15	9.43 ± 6.47
	100 pM 5HT	144	51	35.18 ± 11.53 **
	Mix	51	4	6.25 ± 12.50
	100 pM 5HT + Mix	60	13	17.39 ± 14.58

The percentages of the two-cell embryos are shown when IVF was performed in the medium with 100 pM 5-HT and 5-HT-receptor antagonists (A–D) or a mixture of antagonists (E). The mixture contained 1 µM cyproheptadine hydrochloride sesquihydrate (Cypro), 3.8 nM dolasetron mesylate hydrate (DS), 1 µM GR113808 (GR), and 10 µM SB-258719 (SB25). The data represent the mean ± SD. (A) (Vehicle) medium with medium with 0.1% (v/v) pure water and 0.1% (v/v) ethanol as vehicle; (100 pM 5-HT) medium with 100 pM 5-HT and vehicle; (Cypro) medium with 1 µM Cypro and vehicle; (100 pM 5-HT + Cypro) medium with 100 pM 5-HT, 1 µM Cypro, and vehicle. (B) (Vehicle) medium with medium with 0.2% (v/v) pure water as vehicle; (100 pM 5-HT) medium with 100 pM 5-HT and vehicle; (DS) medium with 3.8 nM DS and vehicle; (100 pM 5-HT + DS) medium with 100 pM 5-HT, 3.8 nM DS, and vehicle. (C) (Vehicle) medium with medium with 0.1% (v/v) pure water and 0.1% (v/v) dimethyl sulfoxide (DMSO) as vehicle; (100 pM 5-HT) medium with 100 pM 5-HT and vehicle; (GR) medium with 1 µM GR113808 (GR) and vehicle; (100 pM 5-HT + GR) medium with 100 pM 5-HT, 1 µM GR, and vehicle. (D) (Vehicle) same as above; (100 pM 5-HT) medium with 100 pM 5-HT and vehicle; (SB25) medium with 10 µM SB-258719 (SB25) and vehicle; (100 pM 5-HT + SB25) medium with 100 pM 5-HT, 10 mM SB25, and vehicle. (E) (Vehicle) medium with medium with 0.2% (v/v) pure water, 0.1% (v/v) ethanol, and 0.2% (v/v) DMSO as vehicle; (100 pM 5-HT) medium with 100 pM 5-HT and vehicle; (Mix) medium with the mixture of antagonists and vehicle; (100 pM 5-HT + Mix) medium with 100 pM 5-HT, the mixture of antagonists, and vehicle. * Significant difference compared with "Vehicle" ($P < 0.05$). ** Significant difference compared with "Vehicle", and "antagonist" ($P < 0.05$). $^{\#}$ Significant difference compared with "Vehicle", "antagonist", and "100 pM 5-HT + antagonist" ($P < 0.05$). $^{\#\#}$ Significant difference compared with "Vehicle", "100 pM 5-HT", and "100 pM 5-HT + antagonist" ($P < 0.05$). $^{\$}$ Significant difference compared with "100 pM 5-HT", and "100 pM 5-HT + antagonist" ($P < 0.05$). Experiments were performed four times on four different male and four female mice.

mice (Fig. 2A). In hamsters, 100 nM 5-HT enhanced hyperactivation through the 5-HT_4 receptor only [13]; however, the same experiment had no effect in mice, although 100 fM and 100 pM 5-HT did increase hyperactivation (Fig. 2C). In addition, the 5-HT_3 receptor was associated with improved hyperactivation by 5-HT, but 5-HT_3 was not the main regulatory receptor for this effect (Figs. 1D and 2B). Moreover, 100 pM 5-HT increased hyperactivation through the 5-HT_7 receptor (Fig. 2D). Furthermore, the mixture of Cypro, DS, GR, and SB25 completely inhibited the improvement by 5-HT in all conditions (Fig. 2E). These results suggest that 5-HT simultaneously stimulated these receptors to increase hyperactivation in mice (Fig. 2). In addition, it is likely that the Ca^{2+} signals associated with PLC and the cAMP signals associated with the tmAC were simultaneously stimulated in the increase of hyperactivation of mouse spermatozoa by 5-HT. However, it was not possible to show how the 5-HT_3 receptor regulated the increase of hyperactivation by 5-HT in mice in this study.

In human studies, it has been suggested that the capability for hyperactivation is correlated with the success of IVF [11]. P_4 is an inducer of hyperactivation in human spermatozoa [34, 35], but P_4-induced hyperactivation did not increase the success of IVF [11]. Several hormones induce hyperactivation in mammals [12–14, 18], although, no studies have reported an increase in the success rate of IVF. In the present study, 5-HT increased the success rate of IVF in mice (Tables 2 and 3). Because 5-HT significantly improved hyperactivation of mouse spermatozoa, it is likely that 5-HT increases IVF success through an increase in hyperactivation. In addition, the percentage of two-cell embryos tended to decrease when the increase of hyperactivation by 5-HT was suppressed by 5-HT-receptor antagonists (Fig. 2 and Table 3). In particular, the 5-HT_4-receptor antagonists inhibited the increase of hyperactivation by 5-HT, resulting in a decrease in the success of IVF (Fig. 2C and Table 3).

In this study, Cypro was used as a 5-HT_2-receptor antagonist (Figs. 2 and 3 and Table 3A). Cypro significantly suppressed the increase of hyperactivation of mouse spermatozoa by MS (Fig. 3A) but tended to suppress the 5-HT-increased hyperactivation and IVF success (Fig. 2A and Table 3A). We found that a mixture of Cypro and other antagonists significantly suppressed the increase of hyperactivation and slightly suppressed the increase in IVF success by 5-HT (Fig. 2E and Table 3E). In hamsters [13, 22], Cypro significantly suppressed the induction of AR and hyperactivation by 5-HT. These results suggest that Cypro may induce infertility in rodents. It is thought that Cypro suppresses the effects of 5-HT on the reproductive functions in humans because the 5-HT_2 receptor is expressed in the spermatozoa and oocytes [21, 23]. Cypro has been approved for use in humans and is used as an antihistamine for the treatment of allergic reactions [36].

In conclusion, this study investigated whether 5-HT regulates spermatozoal hyperactivation and IVF in mice. The experimental results indicate that 5-HT significantly increases hyperactivation through the 5-HT_2, 5-HT_3, 5-HT_4, and 5-HT_7 receptors. Moreover, the results show that 5-HT substantially increases the success rate of IVF through these receptors. It is expected that 5-HT could be used as a stimulatory agent to increase the success rate of IVF, as the spermatozoal ability to hyperactivate is correlated with the success rate of IVF in humans [11].

Declaration of conflict of interest: The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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ORIGINAL RESEARCH ARTICLE

Time from referral to ovarian tissue cryopreservation in a cohort of Danish women

Stine Gry Kristensen[1] | Susanne Elisabeth Pors[1] | Liv la Cour Poulsen[2] | Signe Taasti Andersen[1,3] | Yu Wakimoto[1,4] | Claus Yding Andersen[1,3]

[1]Laboratory of Reproductive Biology, The Juliane Marie Center for Women, Children and Reproduction, University Hospital of Copenhagen, Copenhagen, Denmark

[2]The Fertility Clinic, Department of Gynecology and Obstetrics, Zealand University Hospital, Køge, Denmark

[3]Faculty of Health Science, University of Copenhagen, Copenhagen, Denmark

[4]Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Correspondence
Stine G. Kristensen, Laboratory of Reproductive Biology, Copenhagen University Hospital – Rigshospitalet, Copenhagen, Denmark.
Email: stine.gry.kristensen@regionh.dk

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Abstract

Introduction: Young women with a cancer diagnosis often have very little time to decide whether or not to commence fertility-preserving strategies before initiating potentially sterilizing cancer treatment. Minimizing the interval from opting for fertility preservation to completion of the procedure will reduce the potential risk of delaying cancer treatment. In the current study, we have evaluated the period of time from referral to ovarian tissue cryopreservation (OTC) to actual freezing of the tissue in a cohort of Danish women.

Material and methods: The study population comprised 277 consecutive patients with both malignant and nonmalignant diseases referred for OTC from four centers in the Danish network. Statistical analysis was conducted to analyze the impact of age, diagnosis, and referring center on the time from OTC-referral to OTC. A literature search for "random start" protocols for controlled ovarian stimulation (COS) for fertility preservation in cancer patients was performed.

Results: The time from OTC-referral to OTC was significantly influenced by diagnosis, age, and referring center. Women with malignant diseases other than breast cancer, such as sarcomas, pelvic cancers, and hematological cancers, experienced a significantly shorter interval to OTC (5 days) than women with breast cancer (7 days) and nonmalignant diseases including systemic, ovarian, and hereditary conditions (13-17.5 days). Women over the age of 30 years experienced a significantly longer time to OTC ($P < 0.03$), and the diagnosis determined the length of the interval ($P < 0.001$). According to the literature, fertility preservation by oocyte vitrification requires 13-14 days, as the average time for 1 round of COS was 11 days and oocyte collection can be performed 2 days later.

Conclusions: It is in the interest of both cancer patients and clinicians to perform fertility preservation as quickly and safely as possible. In a Danish setting, OTC provides a short interval of around 6 days from the patient choosing this option to completion of the procedure. This is considerably less time than what is needed to perform COS and oocyte vitrification, and therefore OTC might be considered the preferred choice of fertility preservation when urgency is needed.

Abbreviations: COS, controlled ovarian stimulation; FP, fertility preservation; IVF, in vitro fertilization; OTC, ovarian tissue cryopreservation; RC, referring center.

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KEYWORDS
cancer, fertility preservation, oocyte vitrification, ovarian tissue cryopreservation, random start ovarian stimulation

1 | INTRODUCTION

In parallel with the increasing survival rates for most forms of cancer, the interest in fertility preservation (FP), especially for young women, has increased. Loss of fertility is considered one of the most significant late effects of cancer treatment and this concern is an additional stress factor for patients.[1] Making time-pressured decisions about FP while simultaneously planning urgent cancer treatment are demanding because women have to trade-off the immediate consequences of starting cancer treatment with the long-term chances of having a biological child.

Currently, several types of FP are offered to women. Some of the most prominent include cryopreservation of mature oocytes or embryos, if the woman has a partner. Due to the widespread knowledge on controlled ovarian stimulation (COS) and in vitro fertilization (IVF) these methods are currently regarded as the preferred options for postpubertal patients.[2,3] The established technique of COS secures multiple follicles growing to the preovulatory stage, enabling aspiration of mature oocytes, which are subsequently vitrified before or after fertilization. Alternatively, one ovary, or part of an ovary, can be excised through surgery after which the cortical region of the ovary, containing >90% of the follicles, is isolated and frozen. Ovarian tissue cryopreservation (OTC) is the only fertility-preserving option for prepubertal patients, and this method is often considered as the first option for FP when there is not enough time to perform COS before cancer treatment. It is now well documented that autologous transplantation of the preserved frozen/thawed tissue will revive ovarian function in the vast majority of women (ie >95%), and the births of >130 children following this treatment have been reported.[4,5]

The two fertility-preserving strategies differ profoundly in the period of time required to obtain either mature oocytes or cortical tissue. This difference has attracted little attention despite the fact that newly diagnosed women wanting FP often urgently needs gonadotoxic treatment. COS and oocyte aspiration are usually considered to take around 2 weeks, even with random-start protocols,[6,7] whereas ovarian tissue does not need any pretreatment and can be excised as soon as an operation can be scheduled.[8] Most studies on FP, irrespective of whether focus is on freezing mature oocytes/embryos or ovarian tissue, state that the procedure was performed without delaying cancer treatment.[9-11] However, it is obviously of interest to the patient to keep the time interval between referral to OTC and the actual procedure as short as possible. The aim of the present study was to determine the period of time from referral to OTC to the excision and cryopreservation of ovarian tissue in a cohort of Danish women undergoing OTC. Further, we wanted to evaluate differences in relation to diagnostic categories, age of the patient and between each of the four referring hospitals in our network. Moreover, the time interval from OTC-referral to OTC was related to the recommended time frames for diagnosis and treatment of cancer patients provided by the Danish Health Authority, and the reported duration of COS and collection of mature oocytes for FP in oncological patients.

Key message
Fertility preservation is of great importance to cancer patients. In a cohort of Danish cancer patients, time from ovarian tissue cryopreservation referral to freezing was 6 days, which is considerably less than the 14 days needed for oocyte vitrification.

2 | MATERIAL AND METHODS

2.1 | Study population

The study population included all patients referred for OTC at the Laboratory of Reproductive Biology in Copenhagen, Denmark from June 2015 to September 2018, who subsequently underwent oophorectomy.

A total of 277 consecutive patients referred for OTC from four centers (of which three are located in Denmark and one in south Sweden) were included; Aarhus University Hospital (referring center 1 [RC1]; n = 124), University Hospital of Copenhagen (RC2; n = 100), Odense University Hospital (RC4; n = 36), and Skaane University Hospital (RC3; n = 17). Time from OTC-referral to OTC was defined as the period of time, measured in days, from first contact between the referring center and Laboratory of Reproductive Biology to schedule a date for OTC, to the day of ovarian excision and cryopreservation. The laboratory was always able to accommodate the requested dates for OTC from the referring centers and did not delay the procedure.

The patient diagnoses were categorized based upon Donnez & Dolmans,[5] with five groups of malignant diseases (n = 242) and two groups of nonmalignant diseases (n = 35) (Table 1). Diagnosis at the time of cryopreservation included breast cancer (n = 118), pelvic cancers (n = 24), hematological cancers (n = 53), sarcomas (n = 24), central nervous system and other anatomically cranially placed cancers (n = 23), systemic conditions with need of gonadotoxic chemo- or radiotherapy (n = 25), and a heterogeneous group of ovarian and hereditary conditions with the risk of primary ovarian insufficiency

TABLE 1 Time from OTC-referral to OTC in relation to diagnosis

Diagnosis	Patients (n)	Days from OTC-referral to OTC	
		Mean ± SD	Median [range]
Malignant diseases			
Pelvic cancers	24	5.8 ± 5.5	5.0 [1-27]
Kidney-related	6	7.0 ± 9.9	3.5 [1-27]
Genital-related	14	5.6 ± 3.5	6.0 [1-13]
Intestine-related (- esophagus)	4	4.5 ± 3.5	4.5 [1-8]
Hematological cancers	53	6.6 ± 6.3	5.0 [1-34]
Leukemia	17	8.2 ± 6.6	7.0 [1-22]
Lymphoma	36	5.9 ± 6.1	5.0 [1-34]
Sarcomas	24	5.4 ± 3.7	4.5 [1-18]
CNS and other cranial cancers	23	9.4 ± 10.8	6.0 [1-53]
CNS cancer	21	9.7 ± 11.3	6.0 [1-53]
Esophagus cancer	1	5.0	5.0
Cancer of nasal cavity	1	7.0	7.0
Total malignant not breast cancer	124	6.7 ± 6.9	5.0 [1-53]
Breast cancers	118	9.9 ± 7.2	7.0 [1-36]
Total malignant conditions	242	8.3 ± 7.2	6.0 [1-53]
Nonmalignant diseases			
Systemic conditions	25	12.6 ± 8.6	13.0 [2-29]
Hematological conditions	11	9.9 ± 7.0	7.0 [3-24]
Sclerosis	7	16.7 ± 9.9	20.0 [4-20]
Autoimmune	7	12.7 ± 9.2	13.0 [2-29]
Ovarian and hereditary conditions (risk of POI)	10	25.2 ± 18.9	17.5 [1-57]
Ovarian torsion	1	1.0	1.0
POI	3	16.3 ± 2.5	16.0 [14-19]
Turner syndrome	2	44.0 ± 18.4	44.0 [31-57]
Syndromes/other hereditary	4	28.5 ± 20.8	22.5 [12-57]
Total nonmalignant conditions	35	16.2 ± 13.4	14.0 [1-57]
Patients in total	277	9.3 ± 8.6	6.0 [1-57]

CNS, central nervous system; OTC, ovarian tissue cryopreservation; POI, primary ovarian insufficiency.

(n = 10). Table 1 shows a detailed list of the diagnoses of the patients included in this study. The age of the study population ranged from 3 days to 41 years, and the median age for the cohort was 27 years.

Oocyte vitrification is now gaining ground as an established procedure in Denmark but has only been offered for FP in a small number of patients. Hence, there are insufficient data to perform a comparison between oocyte vitrification and OTC within our own data.

2.2 | Literature search

A systematic search on PubMed was performed for original studies evaluating "random start"-protocols for COS for FP in cancer patients. Case reports of a single or few women were excluded.

2.3 | Statistical analyses

A negative binomial regression model was used to analyze the impact of age, diagnosis, and referring center on the time from OTC-referral to OTC. *P*-values below 0.05 were considered significant. Analysis was carried out using R version 3.4.3 (https://cran.r-project.org/).

2.4 | Ethical approval

Cryopreservation of ovarian tissue for fertility preservation has been approved by the Minister of Health in Denmark and by the ethical committee of the municipalities of Copenhagen and Frederiksberg (H-2-2011-044).

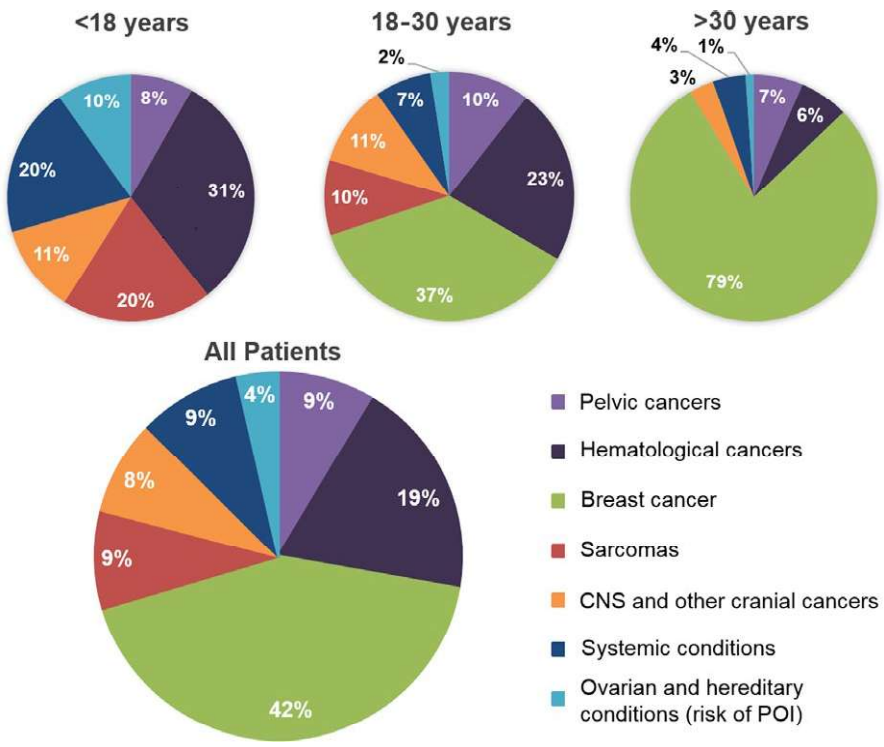


FIGURE 1 Distribution of diagnosis among the referred patients overall and according to age. Top panel depicts the distribution of diagnosis within three different age groups. Patients <18 years, n = 61. Patients between 18 and 30 years, n = 123. Patients >30 years, n = 93. Large diagram depicts the diagnosis within the whole cohort of patients (n = 277). CNS, central nervous system; POI, primary ovarian insufficiency [Colour figure can be viewed at wileyonlinelibrary.com]

3 | RESULTS

3.1 | Diagnosis

Figure 1 depicts the percentages of each diagnostic category within three age groups (<18, 18-30 and >30) and of the whole cohort of patients. Overall, the majority of patients referred for OTC were breast cancer patients (42%), followed by hematological cancers (19%) with the remaining 5 groups comprising <10% each. No breast cancer patients were present in the group of girls <18 years of age, but it was the most frequent diagnosis in patients >18 years. This was especially pronounced in the women over the age of 30 (79% breast cancer). Among the girls under the age of 18 hematological cancers (31%), including both Morbus Hodgkin, non-Hodgkin lymphomas and B-cell lymphomas, sarcomas (20%), and systemic conditions (20%) were the main reasons for FP.

3.2 | Days from referral to OTC to freezing of the tissue

The number of days from OTC-referral to OTC (both mean and median) are given for each of the diagnostic groups and underlying diagnoses (Table 1). Between the seven diagnostic groups, the median number of days to OTC ranged from 4.5 days (mean 5.4 days) for sarcomas to 17.5 days (mean 25.2 days) for ovarian and hereditary conditions, with an overall median time interval of 6 days (mean 9.3 days) for all diagnostic groups. Specifically, the shortest time intervals to OTC were observed in pelvic cancers, hematological cancers and sarcomas, while the longest intervals were found within the nonmalignant Turner syndrome and other hereditary disorders and syndromes. Breast cancer patients experienced a median number of days to OTC of 7 days (mean 9.9 days), ranging from 1 to 53 days.

3.3 | Diagnosis, age and referring center impact the time to OTC

The time from OTC-referral to OTC was found to be significantly influenced by age, diagnosis, and referring center with each factor independently impacting the time interval (Table 2). Women over the age of 30 experienced a significantly longer time interval to OTC than patients with an age <18 ($P < 0.03$). The diagnosis of the patient determined the length of the interval to OTC ($P < 0.001$). Patients with an ovarian and hereditary condition experienced a significant longer time interval to OTC than patients with breast cancer and other malignant conditions. Furthermore, patients with breast cancer experienced a longer time to OTC than patients with other malignant conditions. When diagnoses were grouped into 3 large categories (Table 1); "malignant conditions not breast cancer" (n = 124), breast cancer (n = 118), and nonmalignant conditions (n = 35), the median numbers of days to OTC were 5 days (mean 6.7 days), 7 days (mean 9.9 days), and 14 days (mean 16.2 days), respectively (Figure 2). Patients with malignant diseases other than breast cancer experienced a significantly shorter time interval to OTC than the remaining patients. Finally, the referring center also impacted on the number of days from OTC-referral to OTC ($P < 0.001$). RC4 provided a significantly shorter time interval to OTC when compared with RC2 and RC1, whereas RC1 provided the significantly longest interval to OTC compared with all other centers (Table 2 and Figure 3).

TABLE 2 Estimates of the number of days from OTC-referral to OTC according to age of patients, diagnosis, and referring center. Relative time was estimated using a negative binomial regression

Parameter	Days from OTC-referral to OTC	Confidence interval	*P*-value
Age (y)			
<18	1[a]		0.03
18-30	1.11[ab]	0.90-1.35	
>30	1.41[b]	1.06-1.90	
Diagnosis			
Breast cancer	1[a]		<0.001
Malignant conditions			
Pelvic cancers	0.45[b]	0.32-0.63	
Sarcomas	0.45[b]	0.32-0.64	
Hematological cancers	0.54[b]	0.42-0.69	
CNS and other cranial	0.62[bc]	0.45-0.87	
Nonmalignant conditions			
Systemic conditions	1.13[acd]	0.84-1.54	
Ovarian and hereditary conditions	2.20[d]	1.42-3.41	
Referring center			
RC1	1[a]		<0.001
RC2	0.73[b]	0.62-0.88	
RC3	0.60[bc]	0.40-0.91	
RC4	0.45[c]	0.35-0.59	

CNS, central nervous system; OTC, ovarian tissue cryopreservation; RC, referring center.
P-values <0.05 were considered significant.
[a,b,c,d]indicates significant differences between groups.

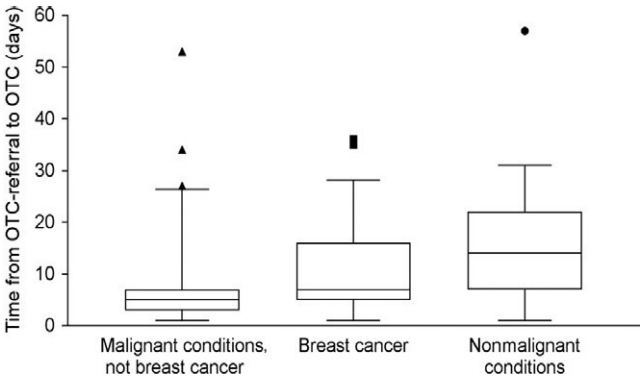


FIGURE 2 Time from OTC-referral to OTC according to the diagnosis. The different diagnoses were grouped according to Table 1 into "malignant conditions not breast cancer", "breast cancer", and "nonmalignant conditions". Patients with ovarian and hereditary conditions had a significantly longer time to OTC than patients with breast cancer and other malignant conditions. Patients with malignant disease other than breast cancer had a significantly shorter time to OTC than the remaining patients (Table 2)

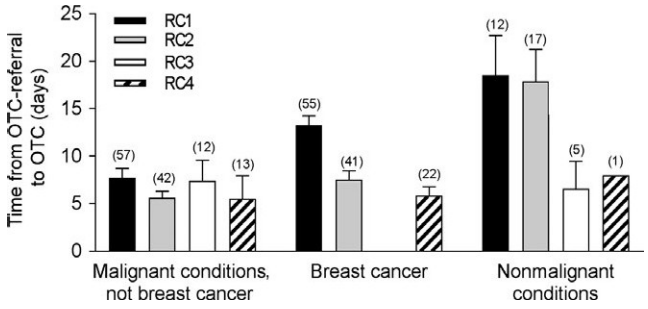


FIGURE 3 Time from OTC-referral to OTC according to diagnosis and referring center (RC). RC4 provided significantly shorter time intervals to OTC when compared with RC2 and RC1. RC1 provided the significantly longest interval compared with all other centers (Table 2)

3.4 | Duration of ovarian stimulation in random start protocols

The concept of random start COS is based on the emergence of evidence that follicular growth is not only initiated once per menstrual cycle, but rather in 2 or 3 follicular waves.[12] This allows recruitment of a follicle cohort under the influence of applied COS regardless of the timing of initiation, which is exploited for FP when there is a lack of time. Table 3 shows an overview of peer-reviewed, original articles (n = 9) reporting the duration of "random start" protocols for COS for FP in cancer patients. A total of 1150 patients were included in the 9 studies, and the average time for 1 round of COS was 11 days, irrespective of where in the menstrual cycle stimulation was initiated. In addition to this, 2 days should be added to allow for ovulation induction and subsequent oocyte collection 36 hours later. Hence, COS and oocyte pick up add around 13-14 days in total for these cancer patients.

TABLE 3 Reported duration of a "random start controlled ovarian stimulation" protocol for fertility preservation in cancer patients

Reference	Design	Patients (n)	Initiation	Duration of stimulation (mean no. of days)
Muteshi et al[7]	Retrospective cohort	127	COS	11.5 (11.2-12.0)
			rsCOS	12.2 (10.7-13.7)
Campos et al[13]	Prospective cohort	26	Follicular phase	10.6 ± 2.1
			Luteal phase	10.0 ± 0.4
Cavagna et al[14]	Cross-sectional	40	COS	10.00 ± 1.41
			Late follicular phase	9.70 ± 0.49
			Luteal phase	10.21 ± 1.23
von Wolff et al[6]	Retrospective cohort	684	COS	10.8 ± 2.4
			Late follicular phase	10.6 ± 2.7
			Luteal phase	11.5 ± 2.2
Kim et al[15]	Retrospective case/control	66	COS	10.3 (9-14)*
			rsCOS	11.4 (9-14)*
Simi et al[16]	Retrospective cohort	25	COS	10.53 ± 2.66
			Late follicular or luteal phase	9.5 ± 1.16
Rashidi et al[17]	Prospective cohort	14	COS	7.8 ± 1
			rsCOS	8.7 ± 2
Cakmak et al[18]	Retrospective cohort	128	COS	9.3 (9.0-9.5)
			Late follicular phase	10.5 (9.6-11.4)
			Luteal phase	11.5 (10.5-12.0)
von Wolff et al[19]	Prospective cohort	40	Follicular phase	10.6 ± 2.5
			Luteal phase	11.4 ± 2.6

The duration of stimulation is reported in mean (95% CI or ±SD, *in one case median [range]). Controlled ovarian stimulation (COS, early follicular phase, initiated at cycle day 2-5), random start controlled ovarian stimulation (rsCOS, initiated at any day in the cycle, late follicular phase day 6-13, luteal phase day 14-28).

4 | DISCUSSION

In this study, we reported the time from OTC-referral to the actual freezing of ovarian tissue in relation to diagnosis, age, and referring center in a cohort of Danish women undergoing FP. This period of time approximates the potential delay in cancer treatment caused by FP, that is, the time from opting for FP to completion of the procedure. The number of days from referral to OTC to freezing of the tissue within all diagnostics groups in the cohort was a median of 6 days. Overall, the shortest time intervals to OTC (4.5-5 days) were observed in patients with sarcomas, pelvic cancers, and hematological cancers, whereas the longest intervals (13-17.5 days) were present within the nonmalignant diseases including systemic, ovarian, and hereditary conditions. It was expected that the time interval from OTC-referral to OTC was longest in the nonmalignant group compared with the malignant group, but it confirms that this parameter is a crucial determinant of the speed with which OTC is executed.

Diagnosis, age, and referring center were found to significantly impact the time to OTC. The time interval from OTC-referral to OTC was found to be longer in the group of women >30 years of age; however, this may be primarily due to the large percentage of breast cancer patients in this group (79%), and not a direct age-effect. Furthermore, significant differences were found in the time to OTC between the 4 referring centers, but some of those differences were due to the lack of specific patient groups within one center. RC3 only referred young patients and did not refer any women with breast cancer for OTC. RC1 and RC2 are the two largest referral centers and represented patients with the same age and diagnosis, and in this case the difference in prolonged time to OTC was directly dependent on the referring center. These differences between centers could be due to small differences in policies regarding the indications and referral for OTC, or management of the logistics at the individual hospitals. Collectively, small differences in the time intervals to OTC exist between the four referring centers in the Danish OTC network, but current data reflect that OTC for FP is performed quickly and efficiently without causing any obvious delay in cancer treatments for the patients who need it the most.

We also found that women with malignant diseases other than breast cancer experienced a significantly shorter time to OTC (5 days) than women with breast cancer (7 days) and nonmalignant diseases (14 days). In Denmark, the Danish Health Authority provide clinically recommended and justified time frames from diagnosis of cancer to primary treatment is initiated ("Cancer pathways", Table 4).[20] In the

TABLE 4 The Danish Health Authority Cancer Pathways—clinically justified time frames

Type of cancer	Standardized duration from diagnosis to primary treatment (d)			Choice of primary treatment
	Chemotherapy	Radiation	Surgery	
Without subcategory				
Cancer in children	Individually planned			
Sarcoma	11	15	14	Predominantly surgery, but 20% (especially children and young adults) are treated with neoadjuvant chemotherapy or radiation
Breast cancer	13	13	13	Predominantly surgery with adjuvant chemotherapy, but neoadjuvant or primary chemotherapy occur
Hematological				
Acute leukemia	3	—	—	Chemotherapy is the only option
Lymphoma	3	15	—	85% receive chemotherapy
Multiple myeloma	Individually planned	—	—	70% receive chemotherapy
Pelvic				
Bowel cancer	10	14	10	Both primary surgery and neoadjuvant chemotherapy may be applied
Ovarian cancer	11	—	8	Predominantly surgery and adjuvant chemotherapy
Cervical cancer	11	15	8	50% neoadjuvant radiation or chemotherapy
Anal cancer	15	15	—	Radiation and chemotherapy concomitantly
Uterine cancer	—	—	8	Predominantly surgery and perhaps adjuvant chemotherapy
CNS and other cranial				
Head or neck cancer	11	11	7	75% radiation possibly combined with chemotherapy
Gastric and esophageal cancer	18	—	10	Surgery, often combined with neoadjuvant and adjuvant chemotherapy
Brain cancer	21	—	7	Predominantly surgery with adjuvant radiation and chemotherapy

The pathways are based on national evidence-based clinical guidelines and describe all steps of the optimal diagnosis and treatment procedures ensuring uniform, high quality across the five Danish hospital regions, including unnecessary waiting times. The success of the pathways is centrally monitored. Neoadjuvant chemotherapy: chemotherapy prior to surgery. Adjuvant chemotherapy: chemotherapy following primary surgery. Source: The Danish Health Authority—Cancer pathways and follow-up plans 2016-2018. Available at: https://www.sst.dk/da/sygdom-og-behandling/kraeft/kraeftpakker-og-opfoelgningsprogrammer.

group of hematological conditions, including leukemia and lymphomas, the choice of primary treatment in the clear majority of cases is chemotherapy with the recommendation that treatment is initiated within 3 days. The median number of days from OTC-referral to OTC for hematological conditions in the current study was 5 days (mean 6.6 days), which implies that FP would cause delay in initiation of cancer treatment in this group of patients. However, some patients with hematological conditions would need to undergo hematopoietic stem cell transplant, in which primary ovarian insufficiency rates are 70%-100% and post-treatment parenthood rates are as low as 3%-8%.[21,22] As a result, if in any way permissible in the individual case, FP would be strongly recommended in these women, and the choice of OTC would minimize the delay in cancer treatment.

In Denmark, OTC is only performed in patients with leukemia once they are in complete remission, which allows more time for FP.[23] For sarcomas, breast cancer, and pelvic cancers the recommended time frames are approximately 10-13 days, when the primary treatment is chemotherapy or neoadjuvant chemotherapy (Table 4). In these cases, the median numbers of days to OTC range between 4.5 and 7 days (mean 5.4-9.9 days), which in the majority of cases would allow time for OTC with no or a minimal delay in initiation of cancer treatment. The recommended "Cancer pathways" from the Danish Health Authority were developed to ensure that all patients receive treatment of uniform, high quality. However, the recommendations underline that every patient should be treated individually and concrete circumstances, such as complex disease or a patient's wish for extra time to consider treatment options, should be taken into account.[20] In cases where surgery is the first choice of treatment, which is the case for most brain cancers and many breast cancer patients, the time frame for the following adjuvant chemotherapy is not specified and would allow sufficient time for COS and oocyte vitrification for FP. Nonetheless, the recommended time frames for initiation of cancer treatment in most cancers highlight the urgency of treatment and the fact that these women have very little time in which to commence fertility-preserving strategies without affecting their cancer treatment.

In the current study, we found that women with malignant diseases experienced a median number of days from OTC-referral to OTC of no more than 6 days (mean 8.3 days), which is considerably less than the 13-14 days required to perform COS and oocyte vitrification. Expressed differently, 69% of breast cancer patients and 89% of the patients with the remaining malignant diseases experienced a time interval to OTC of <14 days. Even among patients without a medical indication for urgency (ie nonmalignant disease), 47% experienced a time interval to OTC <14 days in the Danish cohort. These data highlight the advantage of OTC as being a strategy with a very short time frame. This aspect is obviously of great importance concerning cancer treatment, but it may be even more important for the patient. Studies have found that one of the biggest concerns for patients with malignant diseases is the time from cancer diagnosis to the initiation of treatment, as well as the concern for FP.[24] Hence, minimizing the interval between FP-referral and FP is not merely a matter of treatment demand, but also of great value to the psychological well-being of the patients who are juggling thoughts of survival and the wish for future motherhood.

With the emergence of random start protocols for COS, many patients who previously did not have time for conventional COS before chemotherapy are now having their mature oocytes vitrified for FP. A literature review on "random start" protocols used for cancer patients showed that the duration of COS can be limited to around 11 days, which will add up to approximately 13-14 days when including ovulation induction and oocyte collection. However, in the vast majority of cancer patients, only 1 round of stimulation will be possible, and the number of oocytes retrieved has been found to be negatively correlated with age, but not with the type of cancer.[25] Although oocyte/embryo vitrification is currently used routinely for young women requiring FP,[2,3,8,26] the evidence regarding outcomes of IVF for cancer patients who have returned to use their stored oocytes, is scarce. The hitherto largest study, counting a cohort of 80 cancer patients, recently reported a cumulative live birth rate in young cancer patients (≤35 years) of only around 40%, whereas the percentage was markedly higher (around 70%) in the age-matched group of elective freezers.[26] Hence, poorer IVF outcomes achieved by cancer patients combined with a significantly lower oocyte survival rates in this group indicate that the underlying disease in cancer patients could potentially impair reproductive outcome.[26] This means that despite the advancement of random start protocols for oocyte vitrification, it is not completely straightforward. Moreover, oocyte vitrification is only now being implemented as a standard procedure in Danish fertility clinics, and cryopreservation of embryos is still considered the most established and reliable procedure in many centers. The superiority of oocyte vitrification also needs to be proven in the context of OTC, and so far, only 1 study by Diaz-Garcia et al,[8] have compared the efficacy of oocyte vitrification with that of OTC in a single-center setting. Diaz-Garcia et al[8] found a trend toward higher clinical pregnancy rates and live birth rates after oocyte vitrification compared with OTC in breast cancer patients undergoing FP. They did not find the same trend in patients with Hodgkin lymphoma or other conditions, including sarcoma, leukemia, autoimmune disease and gynecological cancers, but the number of women returning to attempt pregnancy is still low in these groups and a limiting factor to the overall conclusions in the study.[8] Moreover, Diaz-Garcia and co-workers highlight that OTC allowed for natural conception in almost half of the women and restored ovarian function in 93% of the women after 3 months.[8]

Ovarian tissue cryopreservation is currently gaining ground as a valid method to preserve and restore not only fertility, but also ovarian function in young women and girls. With OTC the concerns of the patients are met at multiple levels: by allowing a short interval between diagnosis and initiation of cancer treatment, having no concern about aggravating a hormone-sensitive cancer, and providing restoration of fertility with the chance of multiple conceptions—the natural way—and revival of circulating hormones. The latter can provide natural endogenous hormone therapy for puberty induction in childhood cancer survivors and for women who enter menopause prematurely.[27-29] In addition, the collection and in vitro maturation of immature oocytes in connection with OTC provides an additional fertility potential, which could be essential especially for patients with cancers originating in the ovary.[29] Irrespective of all the above, it should always be prioritized to treat patients as quickly and safely as possible, and in our view OTC in most instances qualifies to be the preferred option for FP, which is in accordance with the current recommendations from international societies like the American Society of Clinical Oncology and the American Society for Reproductive Medicine for women with restricted time to undergo FP.[3,30] Finally, the choice of any fertility-preserving strategy should be an individual assessment of each woman depending on the specific indication, age, gonadotoxic treatment, psychological well-being, and future wishes for motherhood or ovarian function.

5 | CONCLUSION

In conclusion, the time from opting for FP to completion of the procedure is of importance for many cancer patients, who are in need of urgent cancer treatment. Keeping the time interval for FP as short as possible will minimize any potential delay in treatment and potentially relieve the time-pressured decision-making for some patients. In this cohort study of Danish women, we found that the time from OTC-referral to freezing was considerable less than the time needed for COS and oocyte/embryo vitrification. Thus, for many women with malignant diseases, OTC would probably be the preferred choice for FP from a time-restricted perspective.

CONFLICT OF INTEREST

The authors have no conflict of interest.

ORCID

Stine Gry Kristensen https://orcid.org/0000-0003-1194-8393

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Check for updates

The precise ovarian volume is significantly associated with serum concentrations of antimüllerian hormone, the luteinizing hormone/follicle-stimulating hormone ratio, and total testosterone

Yu Wakimoto, M.D., Ph.D.,[a,b] Susanne Elisabeth Pors, Ph.D.,[a] Jesús Cadenas, Ph.D.,[a] Lotte Colmorn, M.D., Ph.D.,[c] Erik Ernst, M.D., Ph.D.,[d] Margit Dueholm, M.D., Ph.D.,[e] Jens Fedder, M.D., Ph.D.,[f,g] Linn S. Mamsen, Ph.D.,[a] Stine Gry Kristensen, Ph.D.,[a] and Claus Yding Andersen, D.M.Sc.[a]

[a] Laboratory of Reproductive Biology, The Juliane Marie Centre for Women, Children and Reproduction, University Hospital of Copenhagen, Rigshospitalet, Copenhagen, Denmark; [b] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan; [c] The Fertility Clinic, University Hospital of Copenhagen, Rigshospitalet, Copenhagen; [d] Department of Gynecology and Obstetrics, Horsens Regional Hospital, Horsens; [e] Department of Gynecology and Obstetrics, Aarhus University Hospital, Aarhus; and [f] Department D, Centre of Andrology and Fertility Clinic, Odense University Hospital; and [g] Research Unit of Human Reproduction, Department of Clinical Research, University of Southern Denmark, Odense, Denmark

Objective: To evaluate potential associations between concentrations of antimüllerian hormone (AMH) and T as well as the LH/FSH ratio and the unbiased precise ovarian volume obtained after unilateral ovariectomy.
Design: Cohort study.
Setting: University hospital.
Patient(s): A total of 765 patients having one ovary surgically removed for fertility preservation. Inclusion criteria were age >15 years and ovarian volume <25 mL; 386 women had one or more hormone parameter (AMH, LH, FSH, or total T) determined before oophorectomy.
Intervention(s): None.
Main Outcome Measure(s): A precise weight of the ovary was equated with ovarian volume. Associations between ovarian volume and AMH, the LH/FSH ratio, T concentrations, and body mass index (BMI) were evaluated. Patient characteristics in relation to ovarian volume cutoff values between 8 and 12 mL were also examined.
Result(s): Ovarian volume was significantly positively associated with concentrations of AMH, the LH/FSH ratio, and T. Ovarian volume, concentrations of AMH and LH, and the LH/FSH ratio were significantly augmented in women having ovarian volumes above a threshold of 8, 9, and 10 mL compared with those below. Average age, FSH, and T concentrations did not differ between below and above the 10 mL threshold. There was a significant association between BMI and ovarian volume and BMI and T, while other hormone parameters were nonsignificant.
Conclusion(s): The precise ovarian volume reflected ovarian activity measured as circulating concentrations of AMH and T as well as the LH/FSH ratio. These significant associations showed continuous progression, and a 10 mL threshold offered no clear difference compared with other volume threshold values. (Fertil Steril® 2020;113:453-9. ©2019 by American Society for Reproductive Medicine.)
El resumen está disponible en Español al final del artículo.

Key Words: Precise ovarian volume, ovarian tissue cryopreservation, antimüllerian hormone, LH/FSH ratio, total testosterone

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Reprint requests: Claus Yding Andersen, Laboratory of Reproductive Biology, Section 5712/5701, Copenhagen University Hospital, Rigshospitalet, Blegdamsvej 9, DK–2100 Copenhagen, Denmark (E-mail: yding@rh.dk).

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Evaluation of the ovarian reserve and assessing the response to exogenous hormone administration in connection with ovarian stimulation and assisted reproductive technology are important for a successful outcome of fertility treatment. Antimüllerian hormone (AMH) measurements have been instrumental in improving information on these topics in recent years, but AMH also has its limitations because it varies considerably during the menstrual cycle, different assays do not measure the same AMH concentration, and specific samples may behave differently depending on assay (1, 2). Alternative markers like FSH and inhibin-B also provide some information but are often of limited value due to their pronounced cycle variability. Ovarian volume based on ultrasound measurements has been used as an additional marker, but volume measured by transvaginal ultrasound assuming a prolate ellipsoid shape is subject to considerable interobserver variability (3). Furthermore, it has been reported that the estimated volume obtained by ultrasound was reduced at least 27% compared with the true ovarian volume obtained by weight, given that 1 g tissue corresponds to a volume of 1 mL (3). These shortcomings have obviously limited the use of ultrasound-determined ovarian volumes. However, ovarian volume is an established parameter in the diagnosis of polycystic ovary syndrome (PCOS), where a cutoff value of 10 mL is one among other parameters used to distinguish women with PCOS from women with normal ovaries according to the Rotterdam criteria (4). One study found that the LH concentrations and the LH/FSH ratio, but not AMH concentrations, correlated to ultrasound-based ovarian volume in women with PCOS (5). Moreover, it has been reported that ultrasound-based ovarian volume is significantly positively associated with T concentrations in women with PCOS (6, 7).

In this study, a possible association between the precise weight of one intact ovary, obtained from women having one ovary surgically removed for fertility preservation by cryopreservation of ovarian cortex, and the concentration of AMH, the LH/FSH ratio, and the concentration of total T at ovariectomy were evaluated. We also evaluated an ovarian volume cutoff value using 8, 9, 10, 11, or 12 mL in relation to hormone concentrations to evaluate the strength of using an ovarian volume of 10 mL as one of several parameters to distinguish women with polycystic ovaries from women with normal ovaries (4). Thus, the aim of this study was to evaluate the impact of accurate ovarian volume determination on its use as an additional marker in fertility treatment and potentially encourage development of new more precise methods to determine ovarian volume.

MATERIAL AND METHODS

Study Population

This retrospective study examined a cohort of women with a diagnosis for which treatment introduced a risk of becoming menopausal, most often a cancer diagnosis or treatment for an autoimmune or genetic disease. A total of 765 women underwent unilateral ovariectomy followed by ovarian tissue cryopreservation (OTC) for fertility preservation. Included were women in whom the ovarian volume was recorded, an age >15 years, an ovarian volume <25 mL, and a disease unrelated to the ovary. Excluded from the analysis were ovaries from women who had received cancer therapy before ovariectomy, which is well known to affect ovarian function. OTC was performed at the Laboratory of Reproductive Biology in Copenhagen, Denmark (8, 9). The OTC schemes and collection of patient data were approved by the Ministry of Health (J. no. 30-1372) and by the Danish authorities to comply with the European Union.

Ovarian Volume, Body Mass Index (BMI), and Hormone Measurements

One ovary was surgically removed for OTC, and the ovarian weight was recorded with a precision weight (Mettler PJ 300, Mettler Toledo). Each ovary was trimmed free of adhering tissue including part of the oviduct and ovarian ligament before weighing. We previously investigated an association between the ovarian weight and the ovarian volume (3). From 11 women, pieces of ovarian tissue were immersed into saline and the precise volume was recorded and associated with the weight. In this way a density of 1.0 g/mL was established independent of the woman's age (3). Thus, in this study the volume of each entire ovary without adhering nonovarian tissue was determined using the conversion of 1 g of tissue corresponding to 1 mL (3).

In a subgroup of the patients (n = 386), one or more of the serum hormone concentrations of AMH, LH, FSH, and T as well as the BMI were measured before OTC as part of the routine workup using the clinical biochemical department in one of the three hospitals from which ovaries were received. Thus, the specific assays used in the different hospitals may not have been identical. The average concentration measured of AMH, LH, FSH, and T (including all available data) did not, however, differ significantly among the three hospitals, suggesting that no major differences in concentrations were present. Hormone values were obtained on a variable day during the patients' menstrual cycle, and the precise cycle day was not monitored. Therefore, LH measurements >20 IU/L were omitted due to the possibility of obtaining the sample during a midcycle surge of gonadotropins. Hormone measurements below the detection limits were not included. During the first decade of our fertility preservation program, concentrations of AMH were not monitored. Different cutoff values for ovarian volumes of 8, 9, 10, 11, and 12 mL were evaluated for the frequency of ovaries above or below the cutoff and the effect on hormone concentrations because an ovarian volume of 10 mL is used as a cutoff to distinguish women with polycystic ovaries from women with normal ovaries.

Statistical Analysis

The associations between ovarian volume and hormone concentrations of BMI, AMH, and T and LH/FSH were evaluated using a generalized linear model. Differences between the hormone concentrations, age, and volumes above and below the cutoff values were analyzed using a two-sided *t* test. Effect of gonadotoxic treatment on ovarian volume was analyzed using a generalized linear model with age as a covariate.

Optimal volume cutoff levels predicted on the basis of hormone levels were calculated using receiver operating characteristic curves with the Youden index (R-package: OptimalCutpoints). All analysis was done using R version 3.4.4. $P < .05$ was considered statistically significant.

RESULTS

Ovarian Volume

The mean ovarian volume in the 765 women was 7.2 ± 3.7 mL (range, 2–21 mL). The frequency of an ovarian volume above or below 10 mL was 21% and 79%, respectively (Table 1). The frequencies and characteristics of patients with ovaries below and above the cutoff values of 8, 9, 10, 11, and 12 mL are listed in Table 1. Decreasing the ovarian volume cutoff value from 12 to 8 mL increased the frequency of large ovaries from 13% to 36%.

Association between Ovarian Volume and BMI or Serum Hormone Levels

The hormone characteristics of patients before OTC (n = 386) and ovaries below and above the cutoff values of 8, 9, 10, 11, and 12 mL are shown in Table 1.

The serum concentrations of AMH and the LH/FSH ratio were significantly higher in patients with ovarian volumes above all the cutoff levels (from 8 to 12 mL; Table 1). Moreover, the levels of LH were significantly higher in patients with ovaries above the cutoff volumes of 8, 9, and 10 mL, but no differences were found above 11 mL. For all five different cutoff levels of ovarian volume, no differences were found between concentrations of FSH and T (Table 1). The ratio of LH/FSH was the only parameter that predicted a volume cutoff level, however, with a low area under the curve (Table 1).

The ovarian volume was positively associated with AMH levels ($P < .001$; Fig. 1A), the LH/FSH ratio ($P < .001$; Fig. 1B), and the T levels ($P < .001$; Fig. 1C).

A total of 292 patients had a BMI available (age, mean ± SD, 24 ± 4.9 years [range, 15.0–46.3 years]). There was a significant association between BMI and ovarian volume ($P = .045$) and between BMI and T ($P = .042$), while there was no significant association with the other hormone parameters measured (Fig. 2).

DISCUSSION

The current study has measured the ovarian volume accurately in connection with unilateral ovariectomy of normal ovaries excised for fertility preservation. Thus, it was possible to accommodate for the highly variable shapes and forms that normal ovaries take, which make accurate volume measurements in a clinical evaluation via ultrasound difficult.

The present study found highly significant associations between hormone concentrations and ovarian volume. The significant association between AMH and ovarian volume as determined in the present study contrasts with a previous study, which was based on transvaginal ultrasonography and found no association between ovarian volume and circulating AMH concentrations (5). This discrepancy may be explained by the more precise measurement of ovarian volume in our study. However, two previous studies in women with PCOS showed that the positive association between serum AMH and ovarian volume reflects the fact that the volume is primarily determined by the number of growing follicles rather than the amount of stromal tissue (10–12). This is in accordance with the fact that most AMH in circulation is produced by small antral follicles with diameters of around 5–8 mm (13). It is well known that circulating levels of AMH and the number of antral follicles are significantly associated (5, 11), and an association between AMH and the ovarian volume in the present study strengthens the premise that ovarian volume is primarily determined by the number of antral follicles. However, it is a new finding that an accurate measurement of the ovarian volume also predicts the AMH concentration.

It has been reported that LH is modified by BMI, and a study in patients with PCOS showed an inverse relationship between LH and BMI (14). However, most patients included in our study were of normal weight, which may explain this difference. Further, BMI and T were significantly associated, potentially reflecting that at a higher BMI the possibility of PCOS is increased.

The LH/FSH ratio is a useful diagnostic marker for PCOS, and an increased ratio indicates a higher possibility of the presence of multiple follicles. Like the AMH concentration, the LH/FSH ratio is also significantly associated with ovarian volume irrespective of ovarian volume cutoff. As LH receptors in the normal ovary become expressed on granulosa cells around follicular selection (15) and most of the ovaries in the present study had follicles below this threshold, the effect of LH is likely to result from stimulation of the theca cells, where LH receptors are constitutively expressed (16). LH may indirectly affect follicle growth via androgen synthesis, which in turn may augment FSH receptor expression of the granulosa cells and enhance responsiveness. This hypothesis is supported by the significant association between levels of T and ovarian volume, which also indicates that the enhanced synthesis of T results in increased follicular activity.

It is also noticeable that associations between ovarian volume and the hormone concentrations showed a continuous slope across the range of volumes. Increasing the volume above 10 mL does not appear to cause any specific changes in the slope of the associations for any of the hormone parameters. In fact, the hormone concentrations using different cutoff levels for ovarian volume (i.e., 8, 9, 10, 11, and 12 mL) showed only modest differences. Based on these results, there is no specific support for a limit of a 10 mL ovarian volume as the threshold for determining PCOS, which may be expected as this condition is a genetically multifactorial associated and polygenic disorder (17). It appears that the change of biochemical parameters takes place progressively as the volume enlarges and that development of polycystic ovaries is a gradual process. A similar conclusion was reached by Balen and coworkers, who found that not all polycystic ovaries show a volume exceeding 10 mL (12).

There are different methods for measuring ovarian volume. The standard technique is by transvaginal ultrasound using a prolate ellipsoid formula (longitudinal diameter ×

TABLE 1

Descriptive data (mean ± SD [range]) for patients who did not receive gonadotoxic treatment before ovarian tissue cryopreservation, above 15 years of age and with ovarian volume < 25 mL.

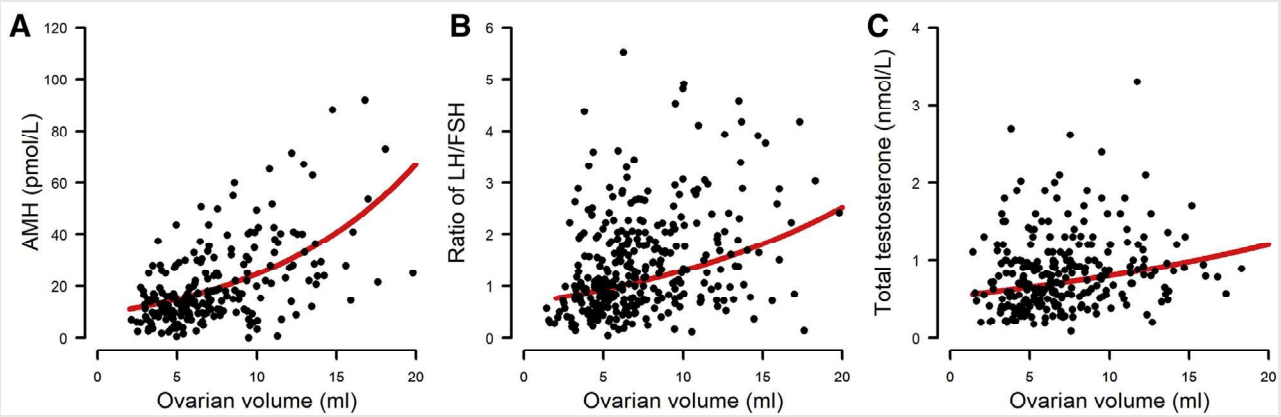
		Volume cutoff, mL									
		8		9		10		11		12	
	Total	≤	>	≤	>	≤	>	≤	>	≤	>
No. of patients (%)	765	491 (64)	274 (36)	546 (71)	219 (29)	603 (79)	162 (21)	633 (83)	132 (17)	667 (87)	98 (13)
Age, years [range]	28.2 ± 6.0 [15.1–43.1]	27.9 ± 6.2	28.8 ± 5.6[a]	28.1 ± 6.1	28.7 ± 5.7	28.1 ± 6.1	28.8 ± 5.3	28.1 ± 6.1	28.8 ± 5.5	28.1 ± 6.1	28.9 ± 5.5
Volume, mL [range]	7.4 ± 3.6 [1.2–20.90]	5.2 ± 1.6	11.4 ± 2.7[c]	5.5 ± 1.8	12.1 ± 2.5[c]	5.9 ± 2.1	13.0 ± 2.3[c]	6.1 ± 2.2	13.6 ± 2.1[c]	6.4 ± 2.5	14.3 ± 2.0[c]
		Cutoff points		Cutoff points		Cutoff points		Cutoff points		Cutoff points	
Hormone		AUC		AUC		AUC		AUC		AUC	
LH, IU/L (n = 316)	5.7 ± 4.3	7.2 ± 4.1[b]		5.8 ± 4.2	7.5 ± 4.3[b]	5.8 ± 4.2	7.5 ± 4.2[b]	6.1 ± 4.3	6.9 ± 3.9	6.1 ± 4.4	6.9 ± 3.7
FSH, IU/L (n = 341)	6.1 ± 9.3	5.4 ± 4.6		6.0 ± 8.9	5.4 ± 5	5.9 ± 8.6	5.8 ± 5.8	5.7 ± 8.4	5.8 ± 6.3	5.8 ± 8.2	6.0 ± 6.9
LH/FSH ratio (n = 310)	1.2 ± 0.8	1.7 ± 1.0[c]	1.3, 66%	1.2 ± 0.8	1.7 ± 1.1[c]	1.2 ± 0.8	1.7 ± 1.1[b]	1.3, 67%	1.3 ± 0.9	1.6 ± 0.9[a]	1.6, 67%	1.3 ± 0.9	1.7 ± 0.9[a]	1.2, 67%
Antimüllerian hormone, pmol/L (n = 225)	15 ± 9.6	31 ± 21.8[c]		16 ± 10.8	32 ± 22.8[c]	17 ± 11.4	35 ± 24.5[c]	17 ± 12.2	37 ± 25.3[c]	18 ± 14.2	37 ± 23.3[c]
T, nmol/L (n = 269)	0.8 ± 0.6	0.9 ± 0.5		0.8 ± 0.6	0.9 ± 0.5	0.8 ± 0.6	1.0 ± 0.5	0.8 ± 0.6	0.9 ± 0.5	0.8 ± 0.6	0.9 ± 0.4

Note: Comparison of hormone concentrations above and below the cutoff were done using a *t* test. Significantly different values are marked with letters. Estimated cutoff points and area under the curves (AUC) for receiver operating characteristic curves are provided when above 50%.

[a] $P < .05$.
[b] $P < .01$.
[c] $P < .001$.

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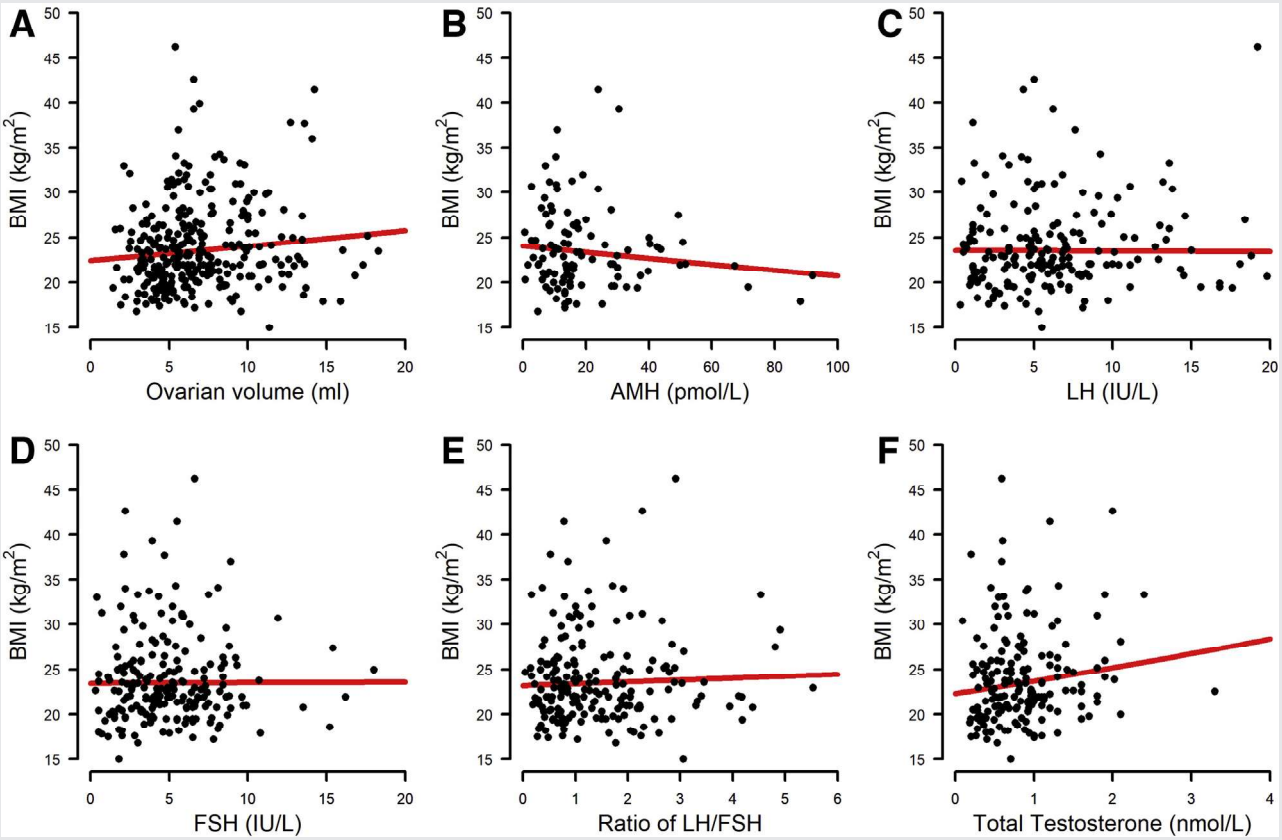
FIGURE 1



Association between ovarian volume and hormone parameters. Predicted values of hormone measurements (redline) and observed value (black dots) from patients before ovariectomy. **(A)** Antimüllerian hormone (AMH), **(B)** LH/FSH, **(C)** total T. Ovarian volume is significantly associated with levels of AMH ($P < .001$), the LH/FSH ratio ($P < .001$), and the T concentration ($P < .001$).

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FIGURE 2



Association between body mass index (BMI), ovarian volume, and hormone parameters. Predicted values of hormone measurements (redline) and observed value (black dots) from patients before ovariectomy. **(A)** ovarian volume, **(B)** antimüllerian hormone (AMH), **(C)** LH, **(D)** FSH, **(E)** ratio of LH/FSH, **(F)** total T. There were significant positive associations between BMI and ovarian volume ($P = .045$) and between BMI and T concentration ($P = .042$), while no significant associations between BMI and LH ($P = .90$) and LH/FSH ($P = .41$) and AMH ($P = .15$) were found.

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anteroposterior diameter × transverse diameter × $\pi/6$) (12, 18). However, using a three-dimensional rather than a two-dimensional approach will improve accuracy, but whether this is sufficient to qualify a more widespread use of the determination of ovarian volume needs further investigation (18, 19).

This study has some limitations. First, ovarian volume was measured at a variable day during the menstrual cycle, and the cycle day was not monitored. Ovarian volume has been shown to be associated with the day of the menstrual cycle, and the biggest volume occurs on cycle day 19 (20). Second, serum hormone levels including the AMH, LH, FSH, and total T were also measured on a random day during the menstrual cycle. It has been shown that levels of AMH, LH, and FSH in serum vary during the normal menstrual cycle. Furthermore, it cannot be excluded that the clinical biochemical departments that analyzed hormone values used different assays that did not provide exactly similar values. This is especially relevant in connection with AMH measurements where the commonly used platforms and assays have changed over time. We have been unable to adjust for the different assays used, but the average concentration of the measured hormone concentrations in each of the different hospitals did not differ significantly between the hospitals, which indicates that this is not a major problem. In connection with the excision of one of the two ovaries, the surgeon may preferentially have chosen the ovary without a preovulatory follicle and therefore on average recovered the ovary with the smallest volume compared with completely randomized selection of either one of the ovaries. In most instances the ovary was bisected into two halves to facilitate access of medium to the tissue when transported; this may in some instances have cut open an antral follicle and thereby reduced the recorded volume. In addition, when an organ is excised and placed in culture medium it is likely to lose some blood and thereby achieve a reduced weight (21). Collectively this suggests that the volume measured by weight underestimates the true in vivo volume of the ovary. It is unlikely to have affected the recorded volume significantly, but we cannot exclude it. However, it puts the ultrasound-measured ovarian volume in perspective, since the estimated volume obtained by ultrasound already underestimates the precise volume as determined by weight by at least 27% (3) and suggests that this difference perhaps may be even bigger.

Further, it would have strengthened the study if a diagnostic examination for PCOS was performed in each woman, but this was unrelated to the fertility preservation procedure and was not performed.

Collectively, eliminating the bias in obtaining measurements of ovarian volume via ultrasonography demonstrated that hormone concentrations including the levels of AMH and T and the LH/FSH ratio are highly significantly associated with accurate ovarian volume. This information should encourage the use of more precise methods for determining ovarian volume in vivo, taking the limitations of this study into account, or the development of new methods, which could constitute a valuable new clinical tool in evaluating ovarian activity and ovarian response to stimulation with exogenous gonadotropins.

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El volumen ovárico preciso está asociado significativamente con la concentración sérica de la hormona antimülleriana, el cociente de la hormona luteinizante/ hormona estimulante del folículo, y testosterona total

Objetivo: Evaluar las posibles asociaciones entre las concentraciones de hormona antimülleriana (AMH) y Testosterona (T), así como la relación LH/FSH y el volumen ovárico preciso imparcial obtenido después de la ovariectomía unilateral.

Diseño: Estudio de cohorte.

Entorno: Hospital universitario

Paciente (s): Un total de 765 pacientes a quienes se les extirpó un ovario quirúrgicamente para preservar la fertilidad. Los criterios de inclusión fueron edad > 15 años y volumen ovárico < 25 ml; 386 mujeres tenían uno o más parámetros hormonales (AMH, LH, FSH o T total) determinados antes de la ooforectomía.

Intervención (es): Ninguna

Principales medidas de resultado: Un peso preciso del ovario se equiparó con el volumen ovárico. Se evaluaron las asociaciones entre el volumen ovárico y AMH, la relación LH/FSH, las concentraciones de T y el índice de masa corporal (BMI). También se examinaron las características de las pacientes en relación con los valores de corte del volumen ovárico entre 8 y 12 ml.

Resultado (s): El volumen ovárico se asoció positiva y significativamente con las concentraciones de AMH, la relación LH/FSH y T. El volumen ovárico, las concentraciones de AMH y LH, y la relación LH/FSH se incrementaron significativamente en mujeres que tenían volúmenes ováricos por encima de un umbral de 8, 9 y 10 ml en comparación con valores inferiores. El promedio de edad, las concentraciones de FSH y T no diferían entre valores por debajo y por encima del umbral de 10 ml. Hubo una asociación significativa entre el BMI y el volumen ovárico y el BMI y T, mientras que otros parámetros hormonales no fueron significativos.

Conclusión (es): El volumen ovárico preciso reflejó la actividad ovárica medida como concentraciones circulantes de AMH y T, así como la relación LH/FSH. Estas asociaciones significativas mostraron una progresión continua, y un umbral de 10 ml no ofreció una diferencia clara en comparación con otros valores de umbral de volumen.

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The β_2-Glycoprotein I/HLA–DR Complex As a Major Autoantibody Target in Obstetric Antiphospholipid Syndrome

Kenji Tanimura,[1] Shigeru Saito,[2] Mikiya Nakatsuka,[3] Takeshi Nagamatsu,[4] Tomoyuki Fujii,[4] Atsushi Fukui,[5] Masashi Deguchi,[6] Yuki Sasagawa,[1] Noriko Arase,[7] Hisashi Arase,[8] and Hideto Yamada[6]

Objective. The clinical manifestations of antiphospholipid syndrome (APS) include vascular thrombosis and pregnancy morbidity as well as recurrent pregnancy loss (RPL). However, in more than half of patients with RPL, the cause is never determined. Recently, β_2-glycoprotein I (β_2GPI) complexed with HLA class II molecules (β_2GPI/HLA-DR) was found to be a major autoantibody target in APS. The present study was undertaken to assess the serum levels of autoantibodies against the β_2GPI/HLA II complex as a potential risk factor for RPL in women.

Methods. Serum levels of antiphospholipid antibodies (aPLs), including IgG/IgM anticardiolipin antibodies, IgG/IgM anti–β_2GPI antibodies, and lupus anticoagulant as well as anti-β_2GPI/HLA–DR antibodies, were measured in 227 women with RPL. In this prospective, multicenter, cross-sectional study, women with RPL and their partners underwent HLA–DR immunotyping and analysis to identify potential causes and risk factors associated with RPL. The normal range for anti-β_2GPI/HLA–DR antibody levels was determined using serum samples obtained from a control population of female subjects (208 women of childbearing potential).

Results. Of the 227 women with RPL, aPL antibodies were detected in 19.8%, and 52 (22.9%) tested positive for anti-β_2GPI/HLA–DR antibodies. Among the 227 women, 121 (53.3%) had no risk factors for RPL, and among these women with unexplained RPL, 24 (19.8%) were positive for anti-β_2GPI/HLA–DR antibodies. Of the 112 women who had clinical symptoms of APS but did not have levels of aPLs that met the diagnostic criteria for APS, 21 (18.8%) were positive for anti-β_2GPI/HLA–DR antibodies.

Conclusion. The anti-β_2GPI/HLA–DR antibody is frequently associated with RPL. Detection of these autoantibodies is useful in understanding the pathogenesis of RPL. Our findings may provide potential new therapeutic strategies for addressing RPL in patients with obstetric APS.

INTRODUCTION

The clinical manifestations of antiphospholipid syndrome (APS) are vascular thrombosis and pregnancy morbidity (1). Pregnancy morbidity, as defined by the revised Sapporo classification of APS (1), includes the following: unexplained fetal death beyond 10 gestational weeks; premature births of normal neonates prior to 34 gestational weeks due to eclampsia, severe eclampsia, or recognized features of placental insufficiency; and recurrent pregnancy loss (RPL). Laboratory criteria for APS include antiphospholipid antibodies (aPLs), lupus anticoagulant (LAC), anticardiolipin antibodies (aCLs), and anti–β_2-glycoprotein I (anti-β_2GPI) antibodies (1–4). Antiphospholipid antibodies mainly target β_2GPI, a phospholipid-binding molecule (5,6). It is thought that

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[1]Kenji Tanimura, MD, PhD, Yuki Sasagawa, MD, PhD: Kobe University Graduate School of Medicine, Kobe, Japan, and Osaka University, Suita, Japan; [2]Shigeru Saito, MD, PhD: University of Toyama, Toyama, Japan; [3]Mikiya Nakatsuka, MD, PhD: Okayama University, Okayama, Japan; [4]Takeshi Nagamatsu, MD, PhD, Tomoyuki Fujii, MD, PhD: University of Tokyo, Tokyo, Japan; [5]Atsushi Fukui, MD, PhD: Hyogo College of Medicine, Nishinomiya, Japan; [6]Masashi Deguchi, MD, PhD, Hideto Yamada, MD, PhD: Kobe University Graduate School of Medicine, Kobe, Japan; [7]Noriko Arase, MD, PhD: Osaka University, Suita, Japan; [8]Hisashi Arase, MD, PhD: World Premier International Immunology Frontier Research Center and Research Institute for Microbial Diseases, Osaka University, Suita, Japan.

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Address correspondence to Hideto Yamada, MD, PhD, Kobe University Graduate School of Medicine, Department of Obstetrics and Gynecology, 7-5-1 Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan. Email: yhideto@med.kobe-u.ac.jp.

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circular β_2GPI is converted to linear β_2GPI, exposing the major epitope for aPLs when β_2GPI binds to anionic phospholipids (7). Antiphospholipid antibodies are usually detected by enzyme-linked immunosorbent assay (ELISA) methods, using plates containing solid-phase, negatively charged phospholipids or plates with β_2GPI (8,9).

However, autoantibodies against β_2GPI that are bound to negatively charged phospholipids or plates are detected in less than half of patients with clinical manifestations of APS (2–4). Additionally, β_2GPI is secreted from the cell and is usually not expressed on the cell surface. How aPLs bind to vascular endothelial cells to induce thrombosis and/or pregnancy morbidity remains unclear.

A series of studies have demonstrated that misfolded proteins generated in the endoplasmic reticulum (ER), which generally tend to be eliminated by ER-associated degradation, can be rescued from degradation and transported to the cell surface without being processed into peptides. This process seems to occur in the ER through an association between the misfolded proteins and the peptide-binding groove of HLA class II molecules (10). In addition, misfolded proteins complexed with HLA class II molecules of disease-susceptible alleles have been found to be involved in the pathogenesis of several autoimmune diseases, serving as autoantibody targets.

IgG heavy chain/HLA–DR complexes in patients with rheumatoid arthritis, myeloperoxidase/HLA–DR complexes in patients with microscopic polyangiitis, and β_2GPI/HLA–DR complexes in patients with APS were found to be major targets for autoantibodies (11–13). Our previous study used stored sera from 120 patients with APS, with the majority of patients having a history of vascular thrombosis. The study showed that 83% of 120 patients had autoantibodies that targeted β_2GPI/HLA–DR complexes (13). Approximately 50% of the APS patients with autoantibodies targeting β_2GPI/HLA–DR complexes were negative for IgG aCLs or IgG anti-β_2GPI (13). A recent study also demonstrated that 27% of 111 patients with idiopathic chronic limb ulcers who were negative for aPLs had autoantibodies that targeted β_2GPI/HLA–DR complexes (14). These results suggest that autoantibodies that target β_2GPI/HLA–DR complexes are associated with APS manifestations, even in patients who are negative for aPLs and therefore do not meet the diagnostic criteria for APS.

RPL is defined as the loss of ≥2 pregnancies (15,16) and has a prevalence of 0.8–1.4% among women (17). RPL dramatically impacts countries with declining birth rates and increasingly aging populations, such as certain Western countries and Japan. In more than half of these patients, the causes of RPL are unknown (18–20). RPL is one of the clinical manifestations of APS and, additionally, we have found that β_2GPI/HLA class II complexes exist in the vascular endothelial cells of placental decidual tissue obtained from women with APS who have experienced spontaneous miscarriages (13). Therefore, we hypothesize that the novel autoantibody targeting β_2GPI/HLA–DR complexes may be involved in the pathogenesis of not only vascular thrombosis, but also RPL. In this prospective, multicenter, cross-sectional study, we evaluated whether women of childbearing potential and women with RPL have autoantibodies targeting β_2GPI/HLA–DR complexes, and we extensively addressed whether women with unexplained RPL possess these novel autoantibodies.

PATIENTS AND METHODS

The present study was approved by the Institutional Review Boards of 5 medical centers (reference no. 1566 at Kobe University Hospital), and all participants enrolled in the study provided written informed consent. In this study, RPL is defined according to the definition of the American Society for Reproductive Medicine, which is the occurrence of ≥2 consecutive pregnancy losses (15,16). Pregnancy losses at ≥10 gestational weeks were diagnosed if the dead fetus had a crown rump length (CRL) exceeding the average CRL typically observed at 10 gestational weeks. Women with RPL who visited 1 of the 5 centers between December 2016 and November 2018 underwent serum measurements for autoantibodies against the β_2GPI/HLA–DR complex (anti-β_2GPI/HLA–DR antibodies) and HLA–DR typing, as well as conventional assessments to identify the cause of and risk factors for RPL. Partners of the participating women also underwent HLA–DR typing.

Serum levels of anti-β_2GPI/HLA–DR antibodies in 208 healthy women of childbearing potential were used as controls to establish a normal range and cutoff values for measurement of these antibodies. All of the control population had a history of normal-term delivery and no pregnancy losses or autoimmune diseases. The serum samples, purchased from the National Center for Child Health and Development BioBank were collected between July 2014 and July 2017 from women who had a history of normal-term delivery and no pregnancy losses or autoimmune disease and who were already known to be negative for all aPLs (in accordance with the diagnostic criteria for APS). The samples were frozen immediately after blood collection and stored at –80°C until being used as controls in our assessments of the associations of anti-β2GPI/HLA–DR antibodies with the causes of and risk factors for RPL. The associations of anti-β_2GPI/HLA–DR antibody with the causes of and risk factors for RPL were then assessed.

Measurement of serum levels of autoantibodies targeting the β_2GPI/HLA–DR complex. To analyze autoantibody levels specific to the β_2GPI/HLA–DR complex and not HLA–DR alone, we modified a previously described method (13) that we then used to determine anti-β_2GPI/HLA–DR antibody levels. Complementary DNA (cDNA) was prepared from pooled human peripheral blood mononuclear cells (3H Biomedical) and cloned into pME18S or pCAGGS expression vectors. For

transient infections of 293T cells, Polyethylenimine Max reagent (PolyScience) was used. For the generation of green fluorescent protein (GFP)–labeled β_2GPI/HLA–DR complex–expressing cells and DsRed-labeled HLA–DR–expressing cells, 293T cells were cotransfected with GFP, β_2GPI, and HLA–DRA*01:01 and DRB1*07:01 or with DsRed and HLA–DRA*01:01 and DRB1*07:01. A single lot of β_2GPI/HLA–DR–transfected cells and HLA–DR–transfected cells were aliquoted (3×10^6 cells per tube) with 500 μl of the cryroprotectant medium (90% fetal bovine serum and 10% DMSO) and stored at −80°C until used. A serum sample from a woman with RPL in whom anti-β_2GPI/HLA–DR antibodies were detectable after a 10^6-fold dilution was used as a standard throughout the present study. The anti-β_2GPI/HLA–DR antibody level of a standard serum was defined as 1,000 anti-β_2GPI/HLA–DR antibody units.

The mean fluorescence intensity (MFI) of IgG binding to transfected cells in the sample sera was analyzed by flow cytometry. Specific IgG binding to the β_2GPI/HLA–DR complex was calculated by subtracting the MFI of IgG binding to cells transfected with HLA–DR alone from cells transfected with both β_2GPI and HLA–DR (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41410/abstract).

Anti-β_2GPI/HLA–DR antibody levels in each serum sample were calculated from the standard curve, which was generated by measuring specific IgG binding to the β_2GPI/HLA–DR complex in serially diluted standard serum. All measurements were performed in duplicate, and the mean values were defined as the anti-β_2GPI/HLA–DR antibody level of the sample.

RPL causes and risk factors identified during clinical evaluations. All women with RPL enrolled in this study attended evaluations to identify causes of and risk factors for RPL. Ultrasound examinations were performed to detect uterine malformations, including septate uteri, bicorporeal uteri, and uterine myomas that deform the uterine cavity. Magnetic resonance imaging (MRI) or hysterosalpingography were also performed if necessary.

Serum levels of thyroid-stimulating hormone (TSH) (normal range 0.449–3.809 μIU/ml) and free thyroxine (normal range 0.82–1.22 ng/dl) were measured using an Architect i2000SR Analyzer (Abbott Japan). Serum TSH levels were used to diagnose hyperthyroidism and hypothyroidism (<0.4 μIU/ml for hyperthyroidism and ≥2.5 μIU/ml for hypothyroidism). Measurements of serum IgG aCL levels (normal threshold <10 units/ml), IgM aCL levels (normal threshold <8 units/ml), β_2GPI-dependent anticardiolipin antibody (aCL/β_2GPI) levels (normal threshold <1.8 units/ml), and tests for LAC based on dilute Russell's viper venom time (normalized ratio <1.3) were performed (Special References Laboratories). IgG anti-β_2GPI levels (normal threshold <20 units/ml) and IgM anti-β_2GPI levels (normal threshold <20 units/ml) were measured using a Quanta Flash Antiphospholipid Assay Panel (Inova Diagnostics).

In the present study, the 99th percentile cutoff values established by diagnostic laboratories in healthy control subjects were used as the upper limit of normal for each aPL, and aPL positivity was determined by repeated tests conducted ≥12 weeks apart. Hemostatic molecular markers, protein S (PS) activity (normal range 60–150%), protein C (PC) activity (normal range 67–127%), and coagulation factor XII (normal range 50–150%) were also measured. Fresh blood specimens were used for testing aPL and hemostatic molecular markers. Serum samples used for measuring anti-β_2GPI/HLA–DR antibody levels, which were collected at the same time as blood specimens used for other tests, were immediately frozen after blood collection and stored at −80°C until analyzed.

Chromosomal karyotyping (Special References Laboratories) of peripheral blood samples obtained from women with RPL and their partners, and HLA–DR typing using Luminex-based HLA class II single-antigen bead assays (HLA Laboratory) were performed.

Statistical analysis. Clinical characteristics were compared between women with RPL and control female subjects of childbearing potential. Student's *t*-test, Mann–Whitney U test, chi-square test, and Fisher's exact test with Bonferroni adjustment for multiple comparisons were used to analyze differences between the 2 groups. *P* values less than 0.05 were considered significant. All statistical analyses were performed using SPSS software version 19.

Table 1. Clinical characteristics of the women with recurrent pregnancy loss and the control subjects (healthy women of childbearing potential)*

	Women with recurrent pregnancy loss (n = 227)	Control subjects (n = 208)	*P*
Age, years	35.0 ± 4.6	36.1 ± 4.3	<0.05
Gravidity, no.	3.6 ± 1.8	1.4 ± 0.6	<0.0001
Parity, no.	0.6 ± 0.8	1.4 ± 0.6	<0.0001
Spontaneous miscarriages, no.	3.0 ± 1.6	0.0 ± 0.0	<0.0001
Stillbirths at 22 gestational weeks or later			
No.	0.1 ± 0.3	0.0 ± 0.0	<0.001
% of women	5.7	0	<0.001

* Except where indicated otherwise, values are the mean ± SD.

RESULTS

Study population. During the study period, 227 couples who had experienced RPL were enrolled (117 at Kobe University Hospital, 71 at University of Toyama Hospital, 26 at Okayama University Hospital, 10 at the University of Tokyo Hospital, and 3 at Hyogo College of Medicine College Hospital). Of the women who experienced RPL, 185 (81.5%) had ≥2 pregnancy losses prior to 10 gestational weeks, and 11 (4.8%) had ≥2 pregnancy losses at 10 gestational weeks or later. Serum samples obtained from control subjects (208 women of childbearing potential) were purchased from the National Center for Child Health and Development BioBank.

Age and parity among women with RPL were significantly lower than what was observed among control subjects ($P < 0.05$ and $P < 0.0001$, respectively) (Table 1). Gravidity ($P < 0.0001$), number of spontaneous miscarriages ($P < 0.0001$), number of stillbirths at ≥22 gestational weeks ($P < 0.001$), and percentage of those who had a history of stillbirth at ≥22 gestational weeks ($P < 0.001$) were significantly higher in women with RPL than in control subjects. However, these differences are not believed to have significantly influenced the study results.

Distribution of serum anti-β_2GPI/HLA–DR antibody levels in control subjects. To establish the cutoff value for anti-β_2GPI/HLA–DR antibodies, serum levels of autoantibodies in 208 control subjects were measured as described in Patients and Methods. The normal range of serum anti-β_2GPI/HLA–DR antibody levels (<52.6 anti-β_2GPI/HLA–DR antibody units) was set at the 99th percentile for control subjects (Figure 1).

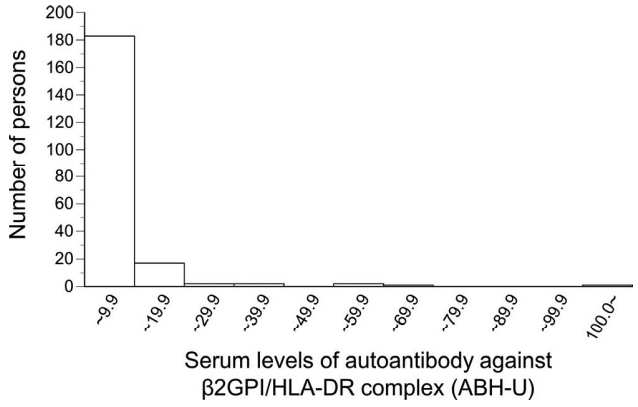


Figure 1. Distribution of serum anti–β_2-glycoprotein I (anti-β_2GPI)/HLA–DR antibody levels in 208 control subjects (healthy women of childbearing potential). Control subjects enrolled in this study were women who met the following criteria: 1) history of delivering ≥1 living infant who had survived to term, 2) no history of miscarriages or stillbirths, 3) no history of autoimmune diseases, and 4) absence of antiphospholipid antibodies. ABH-U = anti-β_2GPI/HLA–DR antibody units.

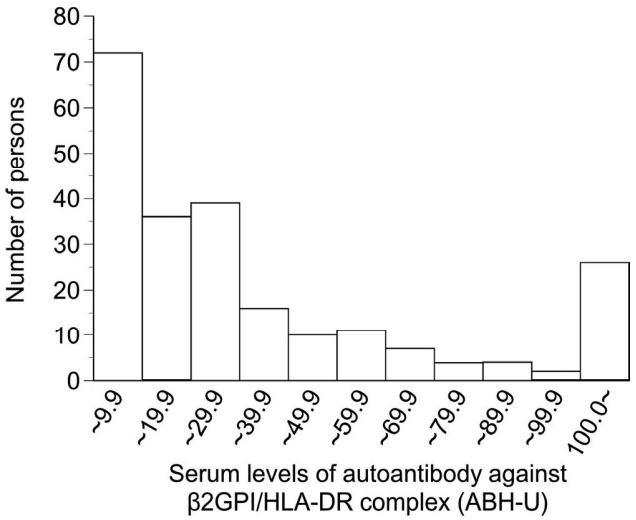


Figure 2. Distribution of serum anti–β_2-glycoprotein I (anti-β_2GPI)/HLA–DR antibody levels in 227 women with recurrent pregnancy loss (RPL). The upper limit of normal for anti-β_2GPI/HLA–DR antibody levels, which was established using values measured in 208 control subjects (healthy women of childbearing potential) was 52.6 anti-β_2GPI/HLA–DR antibody units (ABH-U). Of the 227 women with RPL, 52 (22.9%) were positive for anti-β_2GPI/HLA–DR antibodies.

Anti-β_2GPI/HLA–DR antibodies in women with RPL. To assess whether anti-β_2GPI/HLA class II antibodies are involved in the pathogenesis of RPL, serum levels of anti-β_2GPI/HLA–DR antibodies in 227 women with RPL were measured. The median serum level of anti-β_2GPI/HLA–DR antibody in women with RPL was 21.0 anti-β_2GPI/HLA–DR antibody units (range 0–1,952), which was significantly higher than the level measured in control subjects (2.9 anti-β_2GPI/HLA–DR antibody units [range 0–308.2]) ($P < 0.00005$) (Figure 2). In total, 52 (22.9%) of the 227 women with RPL were positive for anti-β_2GPI/HLA–DR antibodies.

The proportion of women with RPL who were positive for anti-β_2GPI/HLA–DR antibodies was significantly higher among the 11 women who had ≥2 pregnancy losses at 10 gestational weeks or later than among the 185 women who had pregnancy losses before 10 gestational weeks (54.5% versus 21.1%) ($P < 0.05$). This proportion also tended to be higher than that of the 31 women who had pregnancy losses both before 10 gestational weeks and at 10 gestational weeks or later (22.6%) ($P = 0.07$).

Next, the relationship between anti-β_2GPI/HLA–DR antibody levels and aPLs was assessed (Figure 3 and Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41410/abstract). Of the 227 women with RPL, 45 (19.8%) were positive for at least 1 of the 5 aPLs subtypes meeting the diagnostic criteria for APS (with positivity being defined as greater than or equal to the 99th percentile in control subjects). Rates of positivity for each aPL were as follows: 8.8% for IgG aCL (≥10 units/ml), 6.2% for IgM aCL (≥8 units/ml), 3.1% for IgG anti–β_2GPI (≥20 units/ml), 1.3% for IgM anti-β_2GPI

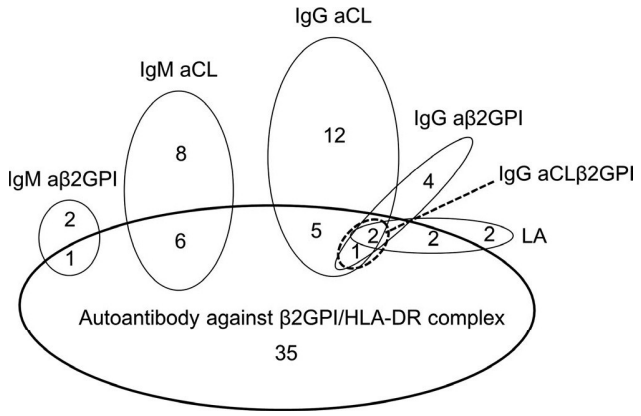


Figure 3. Positivity for anti–β_2-glycoprotein I (aβ_2GPI)/HLA–DR antibodies and antiphospholipid antibodies (aPLs) in 227 women with recurrent pregnancy loss (RPL). Of the 227 with RPL, 52 were positive for anti-β_2GPI/HLA–DR antibodies. Positivity for antibodies among women with RPL was as follows: 20 had IgG anticardiolipin antibodies (aCLs), 14 had IgM aCLs, 7 had IgG anti-β_2GPI, 3 had IgM anti-β_2GPI, 3 had IgG aCL/β_2GPI, and 6 had lupus anticoagulant (LA). Of the 52 women with RPL who were positive for anti-β_2GPI/HLA–DR antibodies, 35 (67.3%) had no aPLs that met the diagnostic criteria for antiphospholipid syndrome. The 3 women with RPL who had both anti-β_2GPI/HLA–DR and aCL/β_2GPI antibodies were positive for IgG anti-β_2GPI and IgG aCLs, and 2 of these 3 women were also positive for lupus anticoagulant.

(≥20 units/ml), 2.6% for LAC (normalized ratio ≥1.3), and 1.3% for IgG aCL/β_2GPI (≥1.8 units/ml). The rate of positivity for anti-β_2GPI/HLA–DR antibodies was substantially higher (22.9%) than that for any of the aPLs that met the diagnostic criteria for APS. Of note, in 35 (67.3%) of the 52 women with RPL who were positive for anti-β_2GPI/HLA–DR antibodies, no aPLs were found (Figure 3). However, there was no significant difference in the serum levels of anti-β_2GPI/ HLA–DR antibodies between aPL-positive women with RPL (n = 45) and aPL-negative women with RPL (n = 182) (median 29.4 anti-β_2GPI/HLA–DR antibody units [range 0–927.5] versus median 20.4 anti-β_2GPI/HLA–DR antibody units [range 0–1,952.0]) (P = 0.12) (Supplementary Figure 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41410/abstract).

Additionally, all 3 women with RPL who had double or triple positivity for aPLs that met the APS diagnostic criteria were also positive for anti-β_2GPI/HLA–DR antibodies, and 2 women with triple positivity had anti-β_2GPI/HLA–DR antibody levels of >100 anti-β_2GPI/HLA–DR antibody units (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41410/abstract). One woman with triple positivity for aPLs and a high anti-β_2GPI/HLA–DR antibody serum level (927.5 anti-β_2GPI/HLA–DR antibody units) had a history of 2 miscarriages that occurred before 10 gestational weeks and termination of pregnancy due to life-threatening HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome at 14 gestational weeks. Another woman with triple positivity for aPLs and an anti-β_2GPI/HLA–DR antibody level of 330.7 anti-β_2GPI/HLA–DR antibody units had a history of thromboembolism with cerebral infarction and 2 fetal deaths occurring after 10 gestational weeks.

The association between anti-β_2GPI/HLA–DR antibodies and other commonly accepted risk factors for RPL may be reflected in the causes of and risk factors for RPL (Figure 4). Details of risk factors for 33 women with multiple RPL risk factors are shown in Supplementary Table 2, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41410/abstract. The incidence of risk factors for all 227 women with RPL were as follows: uterine malformation (8.4%), thyroid dysfunction (6.6%), chromosome karyotype abnormality (in male and female partners) (4.8%), aPL positivity (19.8%), and unexplained causes (53.3%). Anti-β_2GPI/HLA–DR antibodies were most frequently detected in women with RPL (22.9%). Further, proportions of women who were positive for anti-β_2GPI/HLA–DR antibodies by risk factor were as follows: uterine malformation (5 of 19, or 26.3%), thyroid dysfunction

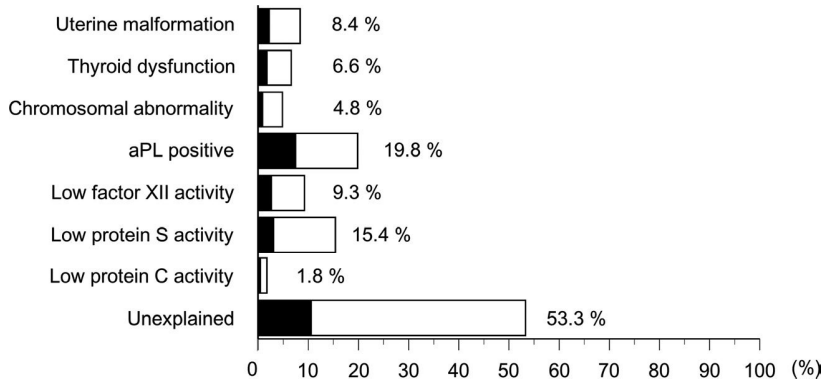


Figure 4. Risk factors for or causes of recurrent pregnancy loss (RPL) among 227 women with RPL. The risk factors for/causes of RPL observed included uterine malformations, thyroid dysfunction, chromosomal abnormalities, antiphospholipid antibodies (aPLs), low factor XII activity, low protein S activity, and low protein C activity. In addition, some of the women with RPL had multiple risk factors, and some had risk factors for or causes of RPL that were unexplained. Solid proportions of the bars indicate the frequencies of women with RPL who were also positive for anti–β_2-glycoprotein I/HLA–DR antibodies (n = 52).

(4 of 15, or 26.7%), chromosome karyotype abnormality in female and male partners (2 of 11, or 18.2%), aPL positivity (17 of 45, or 37.8%), low factor XII activity (6 of 21, or 28.6%), low PS activity (7 of 35, or 20%), and low PC activity (1 of 4, or 25%).

In total, 121 (53.3%) of 227 women with RPL had no risk factors, and 24 (19.8%) of these 121 women with unexplained RPL were positive for anti-β_2GPI/HLA–DR antibodies. There was no significant difference in the proportion of women who were positive for

Table 2. HLA–DRB1 allele frequencies in 208 control subjects (healthy women of childbearing potential) and 227 women with RPL and their partners*

		Women with RPL			Partners of women with RPL		
HLA–DR allele	Healthy controls (n = 208)	All (n = 227)	Anti-β_2GPI/ HLA–DR+ (n = 52)	Anti-β_2GPI/ HLA–DR– (n = 175)	All (n = 227)	Anti-β_2GPI/ HLA–DR+ (n = 52)	Anti-β_2GPI/ HLA–DR– (n = 175)
DR1							
01:01	4.7	6.5	3.9	7.2	5.3	6.7	4.9
01:02	0	0	0	0	0	0	0
DR3*03:01	0.2	0.4	0	0.6	0.5	1.0	0.4
DR4							
04:01	1.5	1.1	1.0	1.2	1.6	1.0	1.8
04:02	0	0	0	0	0	0	0
04:03	2.2	4.7	6.9	4.0	3.3	1.0	4.0
04:04	0.7	0.7	1.0	0.6	0	0	0
04:05	11.2	12.9	15.7	12.1	18.0	13.0	19.4
04:06	3.5	1.8	4.9	0.9	3.8	2.0	4.3
04:07	0	0	0	0	0.8	0	1.1
04:08	0	0	0	0	0	0	0
04:10	1.7	2.2	2.0	2.3	1.1	1.0	1.1
04:11	0	0	0	0	0	0	0
DR7*07:01	0.5	0.7	1.0	0.6	0.5	1.0	0.4
DR8							
08:02	3.7	3.6	5.9	2.9	2.8	5.0	2.5
08:03	6.5	6.0	6.9	5.8	8.7	8.7	8.9
08:09	0	0	0	0	0.5	1.0	0.4
08:23	0	0	0	0	0	0	0
DR9*09:01	16.4	15.6	14.7	15.9	14.7	24.0	12.0
DR10*10:01	1.5	0.7	1.0	0.6	1.4	2.0	1.4
DR11							
11:01	2.5	2.0	2.0	2.0	2.1	2.9	1.7
11:06	0	0	0	0	0	0	0
11:08	0	0	0	0	0	0	0
DR12							
12:01	3.5	4.2	2.0	4.9	2.1	2.9	1.7
12:02	1.7	2.0	1.0	2.3	1.8	1.0	2.0
12:05	0	0	0	0	0	0	0
DR13							
13:01	0.5	0.4	0	0.6	0.5	1.0	0.4
13:02	6.7	5.1	6.9	4.6	5.1	6.7	4.6
13:07	0	0	0	0	0	0	0
DR14							
14:54	3.2	3.8	4.9	3.5	2.1	1.0	2.3
14:02	0	0	0	0	0	0	0
14:03	2.2	0.7	0	0.9	1.2	0	1.5
14:04	0	0	0	0	0	0	0
14:05	2.0	2.5	3.9	2.0	1.9	1.0	2.5
14:06	1.7	2.5	0	3.2	0.9	1.0	1.1
14:07	0.2	0	0	0	0.2	0	0.4
14:12	0	0	0	0	0	0	0
14:29	0	0	0	0	0	0	0
DR15							
15:01	8.0	8.3	4.9	9.2	6.4	3.8	7.1
15:02	11.2	10.7	9.8	11.0	8.4	11.5	7.4
15:04	0	0	0	0	0	0	0
15:11	0	0	0	0	0	0	0
DR16*16:02	1.7	0.9	0	1.2	0.9	2.0	0.7

* Values are the %. RPL = recurrent pregnancy loss; anti-β_2GPI = anti–β_2-glycoprotein I.

anti-β_2GPI/HLA–DR antibodies among groups of each risk factor for RPL.

Allele frequencies in couples with RPL. To assess whether HLA–DR4, HLA–DR7, and HLA–DR13 susceptibility alleles for APS could also be susceptibility alleles for RPL (21–24), HLA–DR alleles in couples with RPL were prospectively typed. HLA–DRB1 allele frequencies in healthy control women and in couples with RPL are displayed in Table 2. Frequencies of HLA–DR4 in the 52 women with RPL who were positive for anti-β_2GPI/HLA–DR antibodies were significantly higher than in the 175 women with RPL who were negative for β_2GPI/HLA–DR antibodies (31.5% versus 21.1%) ($P < 0.05$), but frequencies of HLA–DR7 and HLA–DR13 were not significantly different between the 2 groups. Conversely, frequencies of HLA–DR4 among the 52 partners of women with RPL who were positive for anti-β_2GPI/HLA–DR antibodies were significantly lower than in the 175 partners of women with RPL who were negative for β_2GPI/HLA–DR antibodies (18.0% versus 31.7%) ($P < 0.05$). Allele frequencies of HLA–DR7 and HLA–DR13 were not significantly different between the 2 groups.

In comparing couples with differing genotypes, more women were found to be positive for anti-β_2GPI/HLA–DR antibodies in those couples in which the woman had HLA–DR4 and the partner did not (HLA–DR4+/HLA–DR4–) as compared to couples in which the woman lacked HLA–DR4 and the partner had HLA–DR4 (37.3% versus 15.5%; $P < 0.01$). In addition, more women in the HLA–DR4+/HLA–DR4– couples were positive for anti-β_2GPI/HLA–DR antibodies compared to women in couples in which both partners lacked HLA–DR4 (37.3% versus 19.4%; $P < 0.05$).

DISCUSSION

Several clinical factors are known to be involved in the pathophysiology of RPL. The known causes of/risk factors for RPL and their frequencies among pregnant women and their partners are as follows: uterine malformation (1.8–27.0%), thyroid dysfunction (1.7–9.5%), chromosome karyotype abnormality in male and female partners (2.6–7.7%), aPL positivity (8.7–20.3%), and unexplained causes (>50%) (18,19,25–27). In the present prospective, multicenter, cross-sectional study, frequencies of the causes of and risk factors for RPL were almost identical to those observed in previous reports, and among the 227 women with RPL who did not have commonly accepted risk factors for RPL, unknown etiologic causes of RPL were noted in 121 (53.3%). The present study demonstrated for the first time that 52 women with RPL (22.9%) were positive (≥52.6 anti-β_2GPI/HLA–DR antibody units) for autoantibodies against the β_2GPI/HLA–DR complex and suggested that the anti-β_2GPI/HLA–DR antibody may be the most frequently detected biomarker for RPL. Further, 24 (19.8%) of 121 women with unexplained RPL were positive for anti-β_2GPI/HLA–DR antibodies. Thus, anti-β_2GPI/HLA–DR antibodies may be involved in the pathogenesis of RPL and may also be assessed as a major risk factor for RPL independently of other risk factors.

Anti-β_2GPI/HLA–DR antibodies coexisted with aPLs that met the diagnostic criteria for APS as well as other risk factors. Findings from a previous study have suggested that anti-β_2GPI/HLA–DR antibodies may damage vascular endothelial cells of the placental decidua (13). Therefore, anti-β_2GPI/HLA–DR antibodies may promote RPL in combination with other risk factors, such as thrombogenesis caused by aPLs, low factor XII activity, low PS activity, and low PC activity, and impaired placentation due to thyroid dysfunction.

In the present study, 139 (61.2%) of 227 women with RPL had clinical manifestations of APS, including vascular thrombosis (n = 1), ≥3 unexplained miscarriages prior to 10 gestational weeks (n = 102), ≥1 unexplained fetal death after 10 gestational weeks (n = 34), and premature births before 34 gestational weeks due to hypertensive disorders of pregnancy, fetal growth restriction, or recognized features of placental insufficiency (n = 3). No aPLs that met the APS diagnostic criteria were found in 112 (80.6%) of 139 women with clinical manifestations of APS. However, 21 (18.8%) of the 112 women who lacked aPLs were found to have anti-β_2GPI/HLA–DR antibodies, suggesting that measurement of anti-β_2GPI/HLA–DR antibodies may be more sensitive than aPLs for diagnosing APS. There was no significant difference in the serum levels of anti-β_2GPI/ HLA–DR antibodies between women with RPL and aPLs and women without aPLs.

β_2GPI is the main phospholipid-binding molecule recognized by aPLs (5,6). Circular β_2GPI is converted to linear β_2GPI, which exposes the major epitope for aPLs when β_2GPI binds to anionic phospholipids (7). Therefore, aPLs are detected by ELISA in clinical settings using plates containing solid-phase negatively charged phospholipids, i.e., cardiolipin, and β_2GPI or negatively charged plates containing β_2GPI (8,9). However, β_2GPI/HLA–DR complexes express epitopes shared by β_2GPI/cardiolipin complexes as well as unique epitopes that are not present on these complexes (13). The present study found that 17 (32.7%) of 52 women with RPL who were positive for anti-β_2GPI/HLA–DR antibodies also had aPLs that met APS diagnostic criteria, but the remaining women (35 of 52, or 67.3%) did not. Therefore, measurement of anti-β_2GPI/HLA–DR antibodies may help improve the sensitivity of diagnostic testing for APS because of the recognition of unique epitopes that are not recognized by conventional aPLs.

The proportion of women with RPL in the presence of anti-β_2GPI/HLA–DR antibodies was higher among the 11 women who had ≥2 pregnancy losses at 10 gestational weeks or later than among the 185 women who only had pregnancy losses before 10 gestational weeks or the 31 women who experienced pregnancy losses both before 10 gestational weeks and at 10 gestational weeks or later. Anti-β_2GPI/HLA–DR antibodies might therefore be more closely associated with pregnancy losses at 10 or more

gestational weeks than those before 10 gestational weeks, similar to conventional aPLs (28).

The presence of multiple aPLs that meet APS diagnostic criteria and LAC positivity is strongly associated with the severity of thromboembolisms and pregnancy complications (29–34). In the present study, 4 of 6 women with RPL and LAC positivity were positive for anti-β_2GPI/HLA–DR antibodies. Additionally, all 3 women with RPL who had double or triple positivity for aPLs that met APS criteria had anti-β_2GPI/HLA–DR antibodies, and the 2 women with triple positivity for aPLs had very high anti-β_2GPI/HLA–DR antibody levels (927.5 anti-β_2GPI/HLA–DR antibody units and 330.7 anti-β_2GPI/HLA–DR antibody units), with the first woman, who was positive for IgG aCLs, IgG anti-β_2GPI, and LAC, experiencing early-onset HELLP syndrome at 14 weeks of gestation, and the second woman, who was positive for IgG aCLs, IgG anti-β_2GPI, and LAC, experiencing a thromboembolism with cerebral infarction (described in cases 1 and 5, respectively, in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at http://onlinelibrary.wiley.com/doi/10.1002/art.41410/abstract). Multiple positivity for aPLs that met APS diagnostic criteria may be associated with high levels of anti-β_2GPI/HLA–DR antibodies, and these conditions may be causally associated with the severity of APS manifestations.

Anti-β_2GPI/HLA–DR antibodies are likely involved in the pathogenesis of not only RPL, but also vascular thrombosis, which is another clinical manifestation of APS. A previous study demonstrated that autoantibodies against β_2GPI/HLA class II complexes mediated complement-dependent cytotoxicity for cells expressing the β_2GPI/HLA class II complexes on the vascular endothelial cells of placental decidual tissue obtained from women with APS (13). Thus, autoantibodies against β_2GPI/HLA class II complexes may cause spontaneous miscarriages and vascular thrombosis by damaging vascular endothelial cells of the placental decidua or systemic vessels expressing β_2GPI/HLA class II complexes in a complement-dependent manner.

Alleles of HLA–DR4, HLA–DR7, and HLA–DR13 carry susceptibility to APS (21–24). A previous study has demonstrated that the binding ability of HLA–DR to β_2GPI differed depending on HLA–DR alleles. The HLA–DR alleles DRA*01:01/DRB1*07:01, DRA*01:01/DRB1*04:02, DRA*01:01/DRB1*03:04, and DRA*01:01/DRB1*13:01 showed strong binding to β_2GPI, and antiphospholipid monoclonal antibodies (mAb) obtained from an APS patient (mAb EY2C9) also strongly bound to β_2GPI complexed with these alleles (13). These results indicated that a binding affinity of β_2GPI to each allele of HLA–DR is important for autoantibody recognition of β_2GPI/HLA–DR complexes. Conversely, the HLA–DR allele itself does not affect the autoantibody binding to β_2GPI/HLA–DR complexes, unlike T cell receptors. Because mAb EY2C9 was the most efficient at recognizing β_2GPI complexes, an assay system for detecting autoantibodies against the β_2GPI/HLA–DR7 complex can be a highly sensitive method for the diagnosis of APS. For this reason, in the present study, the β_2GPI/HLA–DR7 complex was used as an antigen to detect autoantibodies against the β_2GPI/HLA–DR complex in women with RPL.

Alleles of HLA–DR3, HLA–DR4, HLA–DR10, and HLA–DR15 carry susceptibility to RPL (35–37). A systematic review and meta-analysis revealed that HLA–DR4 significantly increases the risk for RPL (35). In the present study, the allele frequencies of HLA–DR4 among 52 women who were positive for anti-β_2GPI/HLA–DR antibodies were significantly higher than those among 175 women who were negative for anti-β_2GPI/HLA–DR antibodies (31.5% versus 21.1%) ($P < 0.05$). β_2GPI/HLA class II complexes are localized to vascular endothelial cells in the placental decidua derived from mothers with APS, which come into direct contact with the villi derived from fetuses (13). HLA–DR4 has a strong ability to bind to β_2GPI, and mAb EY2C9 also strongly binds to the β_2GPI/HLA–DR4 complex (13). If a woman carries the HLA–DR4 allele, her vascular endothelial cells of the placental decidua may express HLA–DR4. Inflammation caused by infection or physiologic stress may induce the formation of β_2GPI/HLA–DR4 complexes on vascular endothelial cells, and then anti-β_2GPI/HLA–DR antibodies may damage vascular endothelial cells expressing β_2GPI/HLA–DR4 complexes, ultimately leading to pregnancy losses. Women who carry the HLA–DR4 allele may have increased risk for RPL if they are also positive for anti-β_2GPI/HLA–DR antibodies.

In contrast, allele frequencies of HLA–DR4 among 52 partners of women who were positive for anti-β_2GPI/HLA–DR antibodies were significantly lower than those among 175 partners of women who were negative for anti-β_2GPI/HLA–DR antibodies (18.0% versus 31.7%) ($P < 0.05$). Couples that consist of female partners who carry the HLA–DR4 allele and male partners who do not carry the HLA–DR4 allele may have high susceptibility to RPL if the production of anti-β_2GPI/HLA–DR antibodies is also present in the female partner. This suggests that not anti–HLA–DR4 antibodies, but anti-β_2GPI/HLA–DR4 antibodies, cause RPL.

The standard treatment for pregnant women with APS is combination therapy with heparin and low-dose aspirin (15), and heparin/aspirin therapy may also be effective in the treatment of women with RPL and anti-β_2GPI/HLA–DR antibodies. Presently, a cohort study designed to assess the efficacy of antiplatelet and anticoagulant therapy for these women is already being undertaken.

The present study had some limitations. One limitation is that the strict specificity of anti-β_2GPI/HLA–DR antibodies for the diagnosis of APS cannot be determined. Therefore, prospective, observational case–control studies, in which individuals who tested positive for anti-β_2GPI/HLA–DR antibodies are classified as APS patients, are needed. Patient histories of vascular thrombosis and obstetric complications, including hypertensive disorders of pregnancy and fetal growth restriction, were not evaluated.

Future studies assessing whether anti-β_2GPI/HLA–DR antibodies are causally associated with thrombosis, hypertensive disorders of pregnancy, and fetal growth restriction are necessary

for the advancement of our understanding of the pathogenesis of obstetric APS, and such findings may be useful in providing new treatment strategies for women with obstetric APS.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Yamada had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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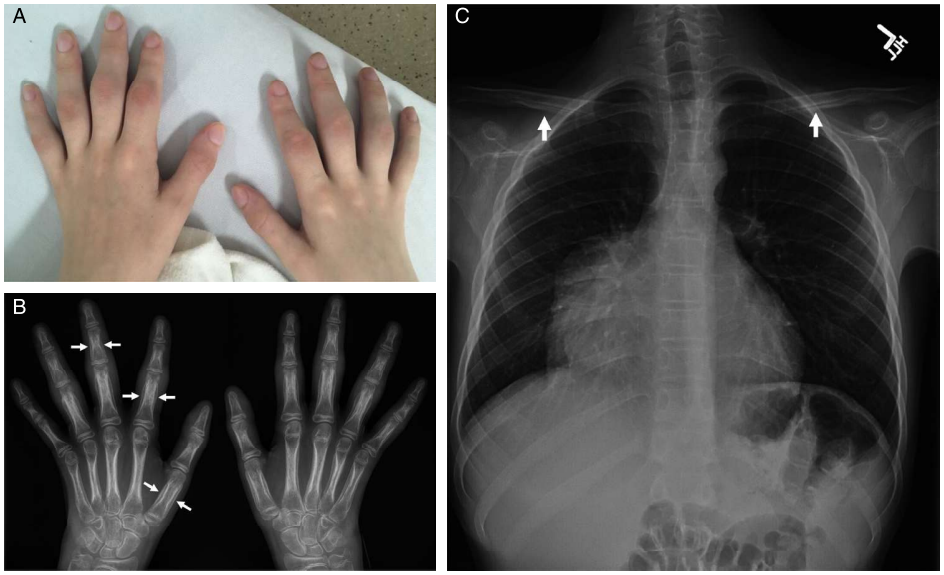
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Clinical Images: Secondary hypertrophic osteoarthropathy in pediatric Hodgkin lymphoma



The patient, a previously healthy 14-year-old boy, presented to the pediatric rheumatology clinic with a 6-month history of progressive swelling of the distal extremities, weakness, functional limitation, and a 12-kg weight loss. He had not experienced fevers or night sweats. Physical examination demonstrated symmetric distal swelling of all 4 limbs; clubbing in all digits; and polyarthritis involving the knees, ankles, and proximal interphalangeal joints of the hands (**A**). Laboratory testing revealed leukocytosis, anemia, thrombocytosis, and elevated levels of inflammation markers. Radiography showed diffuse and extensive periostitis in the long bones: phalanges and metacarpal bones (**arrows** in **B**), clavicles (**arrows** in **C**), and humeri, radii, ulnae, femurs, tibiae, and fibulae (results not shown). Radiography of the chest showed a mediastinal mass adjacent to the right heart border (**C**). Evaluation of the 8.9 cm × 6.9 cm × 8.5 cm nodular mass revealed stage IB Hodgkin lymphoma, nodular subtype. The patient was diagnosed as having secondary hypertrophic osteoarthropathy (HOA) due to Hodgkin lymphoma. Following chemotherapy, his musculoskeletal symptoms completely resolved. The proposed pathogenesis of HOA involves increased circulation to distal peripheries and the release of growth factors, leading to increased fibroblast and osteoblast activity. This results in the clinical triad of digital clubbing, periostitis, and arthritis—usually in a distal and symmetric distribution. Imaging features of periostitis include opaque lines around the cortex along the long bones (most commonly the tibia, fibula, radius, and ulna). Stages of periostitis can be rated by the number of affected bones, site of bony involvement (diaphysis, metaphysis, and epiphysis), and shape of the periosteal reaction (monolayer, lamellar, or irregular). Secondary HOA resulting from paraneoplastic processes is rarer in children compared with adults. Other pediatric secondary HOA etiologies include congenital cyanotic heart disease, cystic fibrosis, and inflammatory bowel disease. A high index of suspicion is warranted to investigate for underlying secondary causes of HOA in children.

Herman H. Y. Tam, MBBS, MSc, FRCPC
Ronald M. Laxer, MD, FRCPC
The Hospital for Sick Children and University of Toronto
Toronto, Ontario, Canada

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A semen-based stimulation method to analyze cytokine production by uterine CD56^{bright} natural killer cells in women with recurrent pregnancy loss

Ayako Taima [a,**], Atsushi Fukui [b,*], Ayano Yamaya [b], Megumi Yokota [a], Rie Fukuhara [a], Yoshihito Yokoyama [a]

[a] Department of Obstetrics and Gynecology, Graduate School of Medicine, Hirosaki University, Hirosaki, Aomori, Japan
[b] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

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ABSTRACT

Cytokine secretion by NK cells is abnormal in some women with recurrent pregnancy loss (RPL). Cytokine production is usually evaluated after stimulation with PMA and ionomycin. However, stimulation of uterine NK cells with semen corresponds more closely to physiological conditions at the time of conception. As seminal plasma has immunomodulatory properties, we aimed to elucidate compatibility between uterine NK cells and semen. Endometrial samples were stimulated with PMA/ionomycin, semen, seminal plasma, or spermatozoa. Thereafter, cytokine production by NK (CD56^{bright}) cells was evaluated using flow cytometry and compared between women with and without a history of RPL associated with abnormal NK cell distribution in the endometrium or unexplained RPL. The ratios (%) of NK cells producing IFN-γ and TNF-α (NK1 phenotype), IL-4 (NK1/NK2 phenotype), and IL-10 (NK1/NKr1 phenotype) were significantly lower after stimulation with semen than with PMA/ionomycin ($P < 0.01$). After exposure to semen, ratios (%) of NK cells producing IL-4 and IL-10 in patients with unexplained RPL were significantly lower ($P < 0.05$), whereas those of NK1/NK2 and NK1/NKr1 were significantly higher ($P < 0.01$) than those in controls. The shift of endometrial NK cells to the NK2 phenotype was more pronounced when stimulated by semen than by PMA/ionomycin. However, a semen-induced shift to NK1 in women with unexplained RPL could induce miscarriage. Couple-specific immunological compatibility tests through semen stimulation *in vitro* might provide important information to avoid RPL.

1. Introduction

Recurrent pregnancy loss (RPL) might be caused by the generation of antiphospholipid antibodies, uterine malformations, and endocrine dysfunctions (European Society of Human Reproduction and Embryology, 2017). However, the etiology has not been identified in over 50 % of patients with RPL, (Saito et al., 2011). Reproductive performance might be negatively influenced by immunologically dysfunctional NK cells, including impaired structure and dysregulated cytokine production (Fukui et al., 2008; Raghupathy et al., 2000; Seshadri and Sunkara, 2014).

The NK cells are classified as CD56^{dim} and CD56^{bright} cells according to the surface density of the CD56 antigen (Biron and Brossay, 2001; Lodoen and Lanier, 2006). CD56^{dim} cells are predominant in peripheral blood and are associated with cytotoxicity, whereas CD56^{bright} cells that are mainly located in the uterine endometrium and decidua, are associated with cytokine production (Caligiuri, 2008; Cooper et al., 2001; Fukui et al., 2017). Similar to T cells, NK cells are also subtyped according to their cytokine secretion profiles (Hoshino et al., 1999; Loza and Perussia, 2001; Peritt et al., 1998; Warren et al., 1995). The NK1 cells secrete type 1 cytokines, such as IFN-γ and TNF-α, which negatively affect the maintenance of pregnancy (Fukui et al., 2017; Higuma-Myojyo et al., 2005), NK2 cells produce the type 2 cytokines, IL-4, IL-5, and IL-13, and NKr1 cells produce transforming growth factor (TGF)-β and

Abbreviations: IVF-ET, *in vitro* fertilization-embryo transfer; PB, Pacific blue; PC5, Phycoerythrin-cyanine 5; PG, prostaglandin; PKC, protein kinase C; RPL, recurrent pregnancy loss.

* Corresponding author at: Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-Cho, Nishinomiya, Hyogo 663-8501, Japan.
** Corresponding author at: Department of Obstetrics and Gynecology, Graduate School of Medicine, 5 Zaifu-Cho, Hirosaki, Aomori 036-8562, Japan.
E-mail addresses: a_taima@hirosaki-u.ac.jp (A. Taima), at-fukui@hyo-med.ac.jp (A. Fukui).

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IL-10. These cytokines induce humoral immune responses that reportedly protect the fetus (Higuma-Myojyo et al., 2005; Mehrotra et al., 1998).

Cytokine production by immune cells is typically evaluated by flow cytometry after stimulation with PMA and ionomycin, which activate lymphocytes to trigger the protein kinase C (PKC) and Ca^{2+}-dependent signaling pathways (Gibson et al., 2016). However, such stimulation does not correspond with physiological conditions.

Physiological exposure to seminal fluids reportedly exerts immunomodulatory effects. Seminal plasma contains immunosuppressive factors, such as TGF-β and prostaglandin E (PGE) (Kelly, 1995; Kelly and Critchley, 1997; Tarter et al., 1986; Vallely et al., 1988). We previously showed that sexual intercourse before ovulation stimulates the recruitment of $CD56^{bright}/CD16^{-}$NK cells to the endometrium (Kimura et al., 2009), suggesting that semen might change the immunological environment in the uterus by stimulating NK cells to regulate cytokine production. Furthermore, some multiparous women experience repeated abortions coinciding with a change of partner, suggesting a degree of compatibility between uterine NK cells and semen. The present study aimed to develop a new physiological couple-specific method based on semen stimulation to determine the deregulation of cytokine production by endometrial NK cells in women with unexplained RPL.

2. Materials and methods

2.1. Study participants

Participants of this prospective study were recruited from the Department of Obstetrics and Gynecology, Hirosaki University Hospital, Hirosaki, Aomori, Japan, between May 1, 2015, and May 31, 2018. The study protocol was approved by the local Institutional Review Board and all participants provided written, informed consent. Women with endocrine, anatomical, infectious, or autoimmune disorders, including antiphospholipid antibody syndrome and a genetic predisposition towards RPL, were excluded from the study.

2.2. Preparation of lymphocytes from endometrial tissue

Endometrial specimens were collected from the women at the mid-secretory phase of the menstrual cycle using an EndoSuction cell sampler (Hakko, Nagano, Japan). Tissues were washed in saline to remove blood cells, placed in RPMI-1640 medium containing 10 % fetal bovine serum, 2 % L-glutamine, 1 % penicillin, and 1 % streptomycin, and mechanically disrupted using small ophthalmologic scissors and a gentleMACS Dissociator (Miltenyi Biotec K.K., Bergisch Gladbach, Germany) to obtain single-cell suspensions (Fuchinoue et al., 2016; Fukui et al., 1999; Kamoi et al., 2015; Yokota et al., 2013). The concentration of uterine endometrial lymphocytes was adjusted to 5×10^6 cells/mL.

2.3. Measurement of intracellular cytokine production by endometrial NK cells

Intracellular cytokine production by uterine NK cells was evaluated in 200 μL of endometrial cell suspensions that were stimulated or not with semen, spermatozoa, seminal plasma or PMA (25 ng/mL), ionomycin (1 μM), and Brefeldin-A (10 ng/mL) for 4 h.

Total sperm counts in normozoospermic semen samples from healthy volunteers or spouses of the endometrial donors were, $54.75 \pm 7.98 \times 10^6$ and $62.75 \pm 38.35 \times 10^6$, respectively, and the ratios of motile sperm were 78.20 % ± 4.45 % and 46.70 % ± 22.58 %, respectively. Semen was separated into spermatozoa and seminal plasma using PureCeption™ Sperm Wash kits (Origio Japan, Yokohama, Japan) as described by the manufacturer. Endometrial (EM) cell suspensions of leukocytes were incubated with spermatozoa, seminal plasma, or total semen at a 1:1 ratio (200 μL) overnight at 37 °C under a 5 % CO_2 humidified atmosphere. Brefeldin-A (10 ng/mL) was added for the last 4 h. The cells were washed in PBS, stained with anti-CD56-Phycoerythrin-Cyanine 5 (PC5) and anti-CD45-Pacific Blue (PB) antibodies (Beckman Coulter, Inc., Brea, CA, USA) for 20 min at room temperature, then washed and stabilized with Cytofix/Cytoperm (Beckton Dickinson, Franklin Lakes, NJ, USA) for 20 min. After washing with perm/wash buffer, the cells were stained with anti-TNF-α-fluorescein isothiocyanate (FITC), anti-IFN-γ-phycoerythrin (PE), and anti-IL-4-PE antibodies (all from Beckman Coulter, Inc.) or anti-IL-10-FITC antibody (Invitrogen, Carlsbad, CA, USA). Cells were washed twice with perm/wash buffer and resuspended in 250 μL PBS for flow cytometry.

2.4. Flow cytometry of endometrial NK cells

Endometrial NK killer cells were assessed as four fluorescent colors using a FACSCanto flow cytometer (Beckton Dickinson, San Jose, CA, USA). Full list-mode data acquisition, recovery, and analysis were conducted using FACSDiva software (Beckton Dickinson). The gating strategy was as follows. Endometrial lymphocytes were identified in singlet cells, isolated by CD45 positive events, and selected based on forward and side scatter gating. Appropriate isotype controls were used for each antibody. For each sample, 3×10^4 CD-45 positive cells were evaluated. The ratios (%) of $CD56^{bright}$ NK cells producing the cytokines, TNF-α^+, IFN-γ^+, IL-4^+, and IL-10^+, were determined by flow cytometry.

2.5. Statistical analysis

Data were evaluated using SPSS version 20.0 (IBM, Armonk, NY, USA). Age distribution and numbers of pregnancies, deliveries, and miscarriages in the study groups were analyzed by one-way ANOVA and Tukey tests. Results are shown as means ± standard deviation (SD). Ratios of $CD56^{bright}$ cells producing cytokines are shown as medians with interquartile ranges (IQR) and were compared using Mann–Whitney *U*-tests. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cytokine production by NK cells stimulated with PMA/ionomycin or semen

We evaluated the effects of various stimuli on cytokine production in uterine endometrial NK cells collected from 72 women, of whom 9 were unstimulated, (control), and 11, and 52 were stimulated with PMA/ionomycin and semen, respectively. Table 1 shows that age, and the number of pregnancies, deliveries, and miscarriages among the groups did not significantly differ.

The ratios of $CD56^{bright}$ cells producing IFN-γ were higher when stimulated with semen compared with controls ($P = 0.155$) but significantly lower than those stimulated with PMA/ionomycin ($P < 0.01$; Fig. 1a). The ratios of $CD56^{bright}$ cells producing TNF-α were significantly higher when stimulated with semen compared with controls ($P < 0.01$) but significantly lower than those of producing IFN-γ and stimulated with PMA/ionomycin ($P < 0.01$) (Fig. 1b). In contrast, the ratios of NK cells producing IL-10- or IL-4- were higher after stimulation with semen, than PMA/ionomycin (Fig. 1c and d). The trends for ratios of

Table 1
Age and obstetric and infertility histories of endometrium donors.

	Unstimulated (n = 9)	PMA/Ionomycin (n = 11)	Semen (n = 52)	*P*
Age(years)	35.78 ± 5.29	37.00 ± 3.41	35.15 ± 4.51	N.S
No. of pregnancies	0.89 ± 0.93	1.82 ± 2.56	1.12 ± 1.82	N.S
No. of deliveries	0.22 ± 0.44	0.27 ± 0.47	0.23 ± 0.43	N.S
No. of abortions	0.33 ± 0.71	1.00 ± 1.79	0.54 ± 1.28	N.S

The data are presented as the mean ± SD and analyzed by one-way ANOVA. NS, not significant.

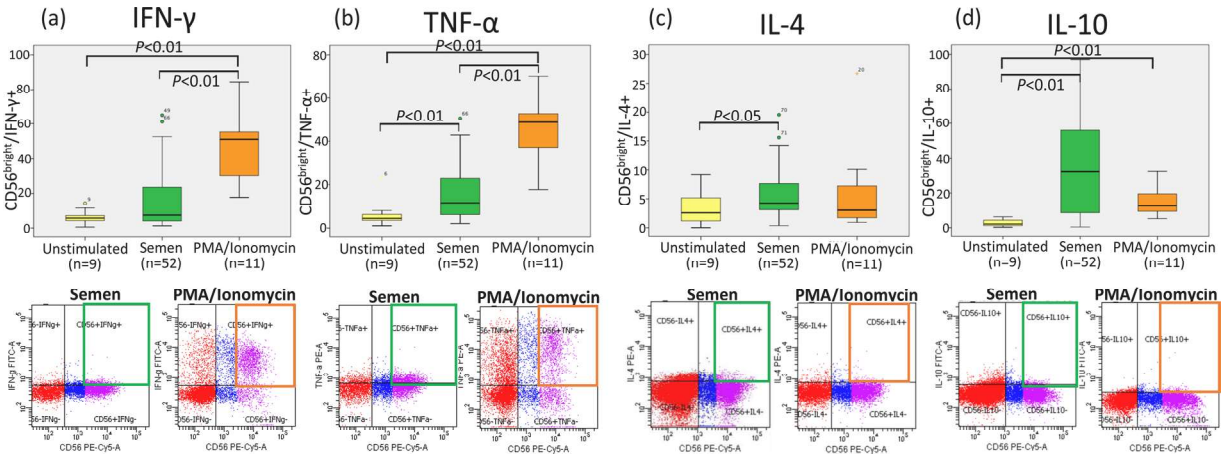


Fig. 1. Cytokine production by uterine NK cells stimulated with semen and PMA/ionomycin. Box-and-whisker plots show proportions of CD56^{bright} NK cells expressing (a) IFN-γ, (b) TNF-α, (c) IL-4, and (d) IL-10. Horizontal lines, median values;, boxes, interquartile range; whiskers, extreme values.

CD56^{dim} NK cells producing these cytokines were the same (data not shown). Moreover, the NK1/NK2 (IFN-γ or TNF-α/IL-4) and NK1/NKr1 (IFN-γ or TNF-α/ IL-10) ratios were significantly lower after stimulation with semen than PMA/ionomycin (Fig. 2).

3.2. Cytokine production by CD56^{bright} NK cells stimulated with semen, seminal plasma, or spermatozoa

To determine which semen components have the highest stimulatory potential, we collected uterine samples from 14 women (age, 36.29 ± 3.43 y; pregnancies, 3.36 ± 1.95; deliveries, 0.36 ± 0.75; miscarriages, 2.79 ± 1.81). Single-cell suspensions were stimulated with semen, spermatozoa, or seminal plasma. The ratios of CD56^{bright} NK cells producing IFN-γ were significantly higher in samples stimulated with semen or seminal plasma than sperm ($P < 0.05$), and those of CD56^{bright} NK cells producing TNF-α were significantly higher after stimulation with seminal plasma than with sperm ($P < 0.05$; Fig. 3). Stimulating CD56^{bright} NK cells producing IFN-γ or TNF-α with semen and seminal plasma did not result in significant differences (Fig. 3a). These results indicated that seminal plasma, rather than spermatozoa, is responsible for the semen-induced production of IFN-γ and TNF-α by endometrial CD56^{bright} NK cells.

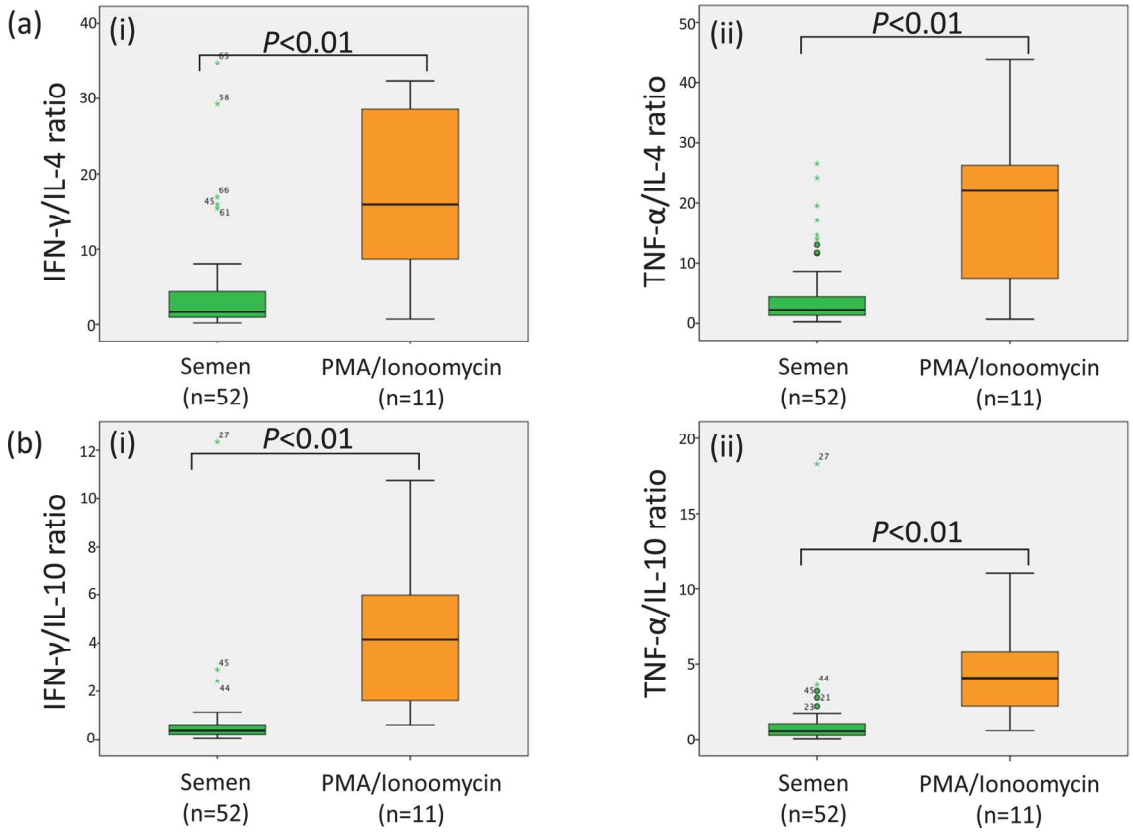


Fig. 2. NK1/NK2 and NK1/NKr1 cell ratios in endometrial samples after stimulation with semen and PMA/ionomycin. (a) NK1/NK2 ratios: IFN-γ/IL-4 (i) and TNF-α/IL-4 (ii). (b) NK1/NKr1 ratios: IFN-γ/IL-10 (i) and TNF-α/IL-10 (ii). Horizontal lines, median values; boxes, interquartile ranges; whiskers, extreme values.

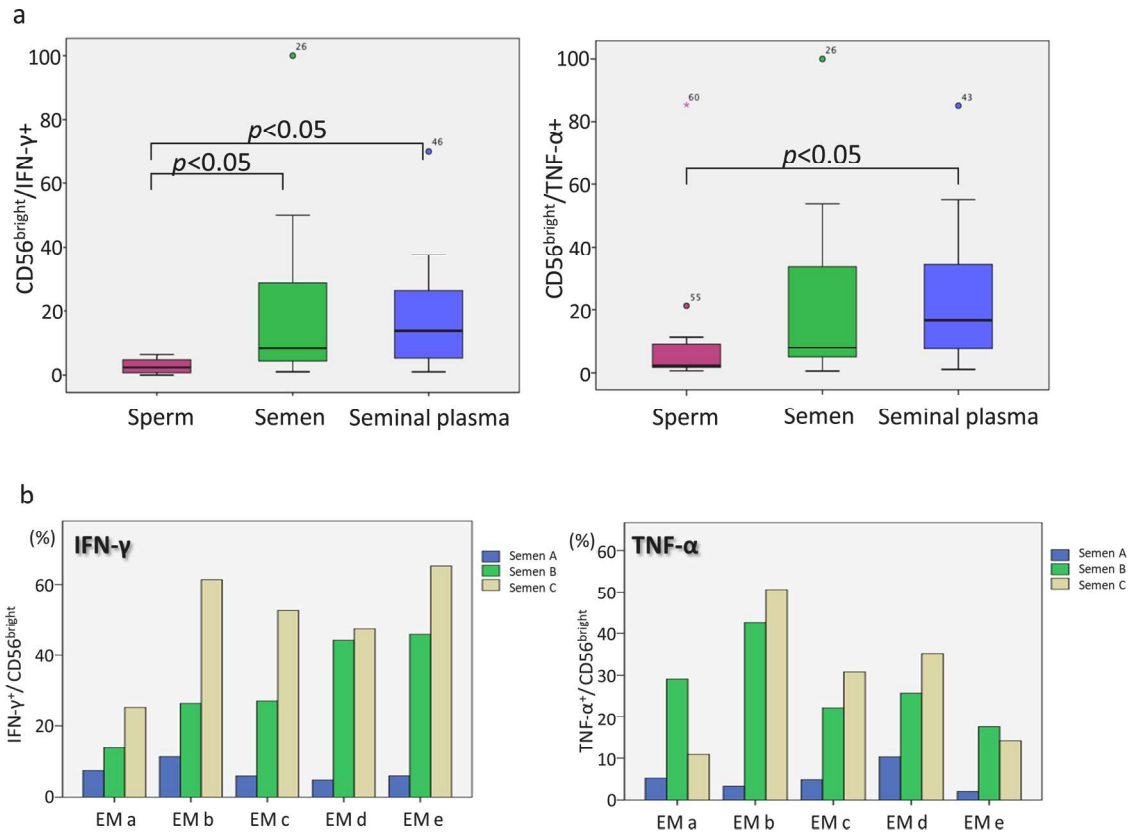


Fig. 3. IFN-γ and TNF-α production by stimulated uterine CD56^{bright} NK cells. (a) Cells stimulated by semen, spermatozoa, and seminal plasma. Horizontal lines, median values; boxes, interquartile range; whiskers, extreme values. (b) Proportions of CD56^{bright} NK cells producing IFN-γ and TNF-α determined by flow cytometry in endometrial cell samples (a–e from five women) stimulated with three semen samples (A–C).

3.3. Compatibility of uterine CD56^{bright} NK cells and semen

We tested the hypothesis that compatibility between uterine CD56^{bright} NK cells and semen is couple-specific. Uterine endometrial samples collected from five women (age, 36.40 ± 4.62 y; pregnancies, 0.60 ± 0.89; deliveries, 0.20 ± 0.45; miscarriages, 0), were stimulated with semen.

We determined the ratios of CD56^{bright} NK cells producing IFN-γ or TNF- α among the semen-stimulated NK cells in all endometrial samples (Fig. 3b). The ratios were the highest for those producing IFN-γ (EMa–EMd) after stimulation with Semen C and the lowest by Semen A. Semen samples B and C stimulated the most CD56^{bright} NK cells producing TNF-α in the EMa and EMe samples, and in the EMb, EMc, and EMd samples, respectively (Fig. 3b).

3.4. Cytokine production by semen-stimulated endometrial CD56^{bright} NK cells in women with RPL

We investigated whether cytokine production by uterine NK cells is deregulated in samples from 29 women, among whom, 12 were parous without a history of RPL (control), eight had unexplained RPL (uRPL), and nine had a history of RPL with immunological abnormalities (RPL-IA) such as aberrant NK cell distribution in the endometrium (NK activity > 40 % or CD56^{dim}/CD16^{+} NK cells > 18 %) (Fukui, 2011). Table 2 shows that age and numbers of pregnancies did not significantly differ among the three groups; however, the numbers of deliveries and miscarriages were significantly lower and higher, respectively ($P < 0.01$ for both) in the RPL groups compared with the control group. Semen samples were provided by patients' partners and the results of semen tests did not significantly differ among the three groups (data not shown) (Tables 3 and 4).

Table 2
Age and obstetric and infertility histories of women with and without RLP.

	Control (n = 12)	RPL-IA (n = 9)	uRPL (n = 8)	*P*
Age(years)	37.83 ± 4.06	36.44 ± 5.00	37.25 ± 5.17	N.S
No. of pregnancies	2.08 ± 1.17	3.00 ± 1.12	3.00 ± 1.07	N.S
No. of deliveries	1.25 ± 0.45	0	0	<0.01
No. of abortions	0.42 ± 0.67	2.67 ± 0.71	2.75 ± 0.71	<0.01

The data are presented as the mean ± SD and analyzed by one-way ANOVA. RPL-IA, Recurrent Pregnancy Loss with Immunological Abnormality; uRPL, Unexplained Recurrent Pregnancy Loss; NS, not significant.

Table 3
Results of semen test.

	Control (n = 12)	RPL IA (n = 9)	uRPL (n = 8)	*P*
Volume of semen (ml)	2.83 ± 0.90	3.13 ± 0.80	3.76 ± 0.76	N.S
Sperm concentration (10^6/mL)	62.17 ± 29.16	72.78 ± 39.59	63.08 ± 46.23	N.S
Sperm motility (Total) (%)	44.45 ± 24.42	34.69 ± 14.17	54.49 ± 22.30	N.S
Leukocytes (106/mL)	4.33 ± 3.11	5.56 ± 5.48	4.33 ± 3.11	N.S

These results were analyzed by one-way ANOVA. All date are mean ± standard deviation. NS, not significant; RPL IA, Recurrent Pregnancy Loss with Immunological Abnormality; uRPL, Unexplained Recurrent Pregnancy Loss.

After stimulation with PMA/ionomycin, the ratios of NK cells producing TNF-α were the highest in women with uRPL ($P < 0.05$ *vs.* RPL-IA). However, these ratios did not significantly differ among the groups

Table 4
Cytokine producing CD56^{bright} NK cells in each group stimulated by semen.

CD56^{bright}	Control (n = 12)		RPL IA (n = 9)		uRPL (n = 8)		P-value
	Median(%)	Interquartile range	Median(%)	Interquartile range	Median(%)	Interquartile range	
INF-γ	5.5	3.25–8.75	5	3.00–9.00	4	0.50–9.00	N.S
TNF-α	7	3.25–8.75	4.00†	3.00–5.00	9	4.00–22.25	<0.05
IL-4	4	2.25–6.75	2	1.00–6.50	2.00‡	1.00–2.75	<0.05
IL-10	7	1.5–10.75	6	2.00–11.00	1.50‡	1.00–2.75	<0.05

Values are expressed as median (Interquartile range). †P<0.05: Difference between control and RPA IA was analyzed by Mann-Whitney U-test. ‡P<0.05: Difference between control and uRPL was analyzed by Mann-Whitney U-test; NS, not significant; RPL IA, Recurrent Pregnancy Loss with Immunological Abnormality; uRPL, Unexplained Recurrent Pregnancy Loss.

after stimulation with semen (Fig. 4a). Moreover, the ratios of CD56^{bright} NK cells producing IL-4 and IL-10 were the lowest in women with uRPL after semen stimulation (both $P < 0.05$ *vs*. control) but did not significantly differ among the three groups after stimulation with PMA/ionomycin (Fig. 4a). Furthermore, the ratios of NK1/NK2 cells and NK1/NKr1 cells were highest in the women with uRPL after stimulation with semen (all $P < 0.05$) but did not significantly differ from the others after PMA/ionomycin stimulation (Fig. 4b).

4. Discussion

Our findings indicated that cytokine secretion profiles of endometrial NK cells differed between stimulation with PMA/ionomycin and semen, and that seminal plasma, rather than spermatozoa, is the activating component in the semen. These results are consistent with previous findings of the immunomodulatory properties of seminal plasma. Polyamine and 19-OH-PGE, and PGE_2 in human seminal plasma suppress NK cell activity *in vitro*. Furthermore, seminal TGF-β recruits inflammatory cells into the uterus (Tremellen et al., 1998) and is a causative factor of immunosuppression caused by seminal plasma depleted of PG and polyamine (Ochsenkühn et al., 2006). Immunomodulators in seminal fluid such as TGF-β and activin might influence cervical immune functions after coitus (Sharkey et al., 2015, 2012). However, stimulating endometrial NK cells with semen *in vitro* has not been reported. Uterine NK cells reside in endometrial tissues, not on the surface. These cells are less likely to directly contact seminal plasma and sperm because seminal plasma cannot enter the uterine cavity. Our stimulation methods with semen, seminal plasma and sperm do not accurately reflect physiological conditions. Therefore, assessing stimulation status *in vivo* or reproducing it *in vitro* is considered a study limitation. Nonetheless, we aimed

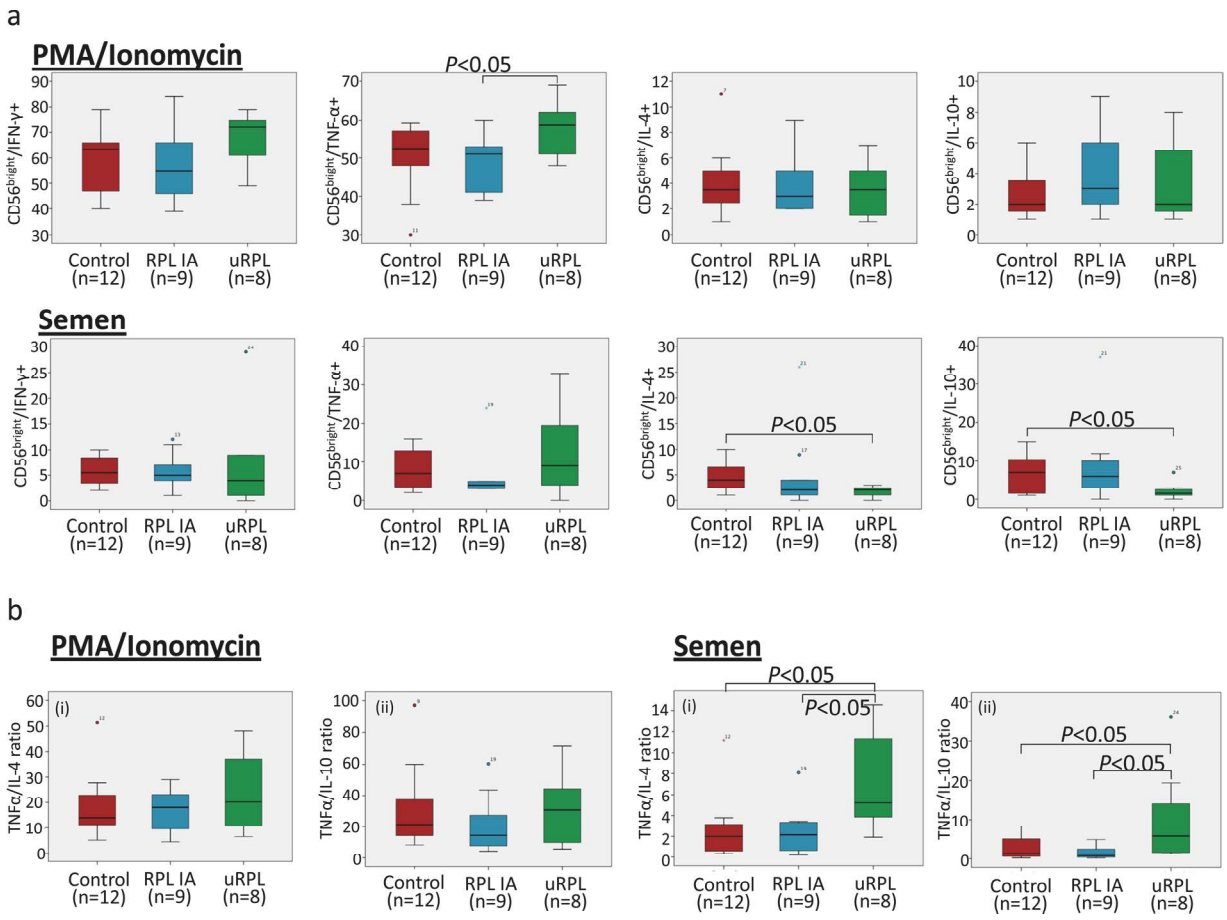


Fig. 4. Cytokine production by uterine CD56^{bright} NK cells and ratios of NK1/NK2 and NK1/NKr1 cells derived from women with and without RPL. a. Cytokine production by uterine CD56^{bright} NK cells. (b) Ratios of NK1/NK2 cells and NK1/NKr1 cells and (i) TNF-α/IL-4 (ii) TNF-α/IL-10 in endometrial samples after stimulation with PMA/ionomycin or semen. Horizontal lines, medians; boxes, interquartile ranges; whiskers, extreme values. Control, no RPL. RPL, recurrent pregnancy loss; RPL-IA, recurrent pregnancy loss with immunological abnormalities; uRPL, unexplained recurrent pregnancy loss.

to develop a physiological method of stimulation using semen rather than PMA/ionomycin.

We previously showed that exposure to semen *in vivo* increases the proportion of $CD56^{bright}$ NK cells in the endometrium (Kimura et al., 2009), suggesting that semen functions in the recruitment of NK cells into the uterus. However, only the NK cell surface markers, CD16 and CD56 were analyzed in that study. Here, we further investigated the effects of semen on the activation of endometrial NK cells *in vitro*, specifically focusing on their cytokine production, which has not been previously examined.

Our results indicated that semen could stimulate uterine NK cells to produce cytokines. Major histocompatibility complex molecules are expressed by ejaculated spermatozoa and seminal plasma (Koelman et al., 2000; Schaller et al., 2009), and endometrial lymphocytes respond to alloantigens in semen to generate an immune response. Furthermore, immunosuppressive factors in seminal plasma such as PG and TGF-β are thought to modulate cytokines. This transient inflammation might have a priming effect, preparing the uterine environment for pregnancy. However, we did not evaluate cytokine levels in semen, which might be difficult. This is also a limitation of the present study.

Compared with PMA/ionomycin, semen caused significantly weaker upregulation of the type 1 cytokines IFN-γ and TNF-α, indicating that semen induced a shift to the NK2 phenotype more efficiently than PMA/ionomycin. Therefore, we compared the NK1/NK2 and NK1/NKr1 cell ratios by flow and found significantly lower ratios in samples stimulated with semen than with PMA/ionomycin, confirming that semen shifted the NK cell balance to the NK2 phenotype. Furthermore, these results indicated that stimulating NK cells with PMA/ionomycin, which is commonly used *in vitro* (Fukui et al., 2008; Higuma-Myojyo et al., 2005), might not faithfully reproduce the physiological situation.

To determine the seminal component responsible for NK cell activation, we separated semen into sperm and seminal plasma. The significantly higher ratios of NK1 cells producing IFN-γ and TNF-α when stimulated with seminal plasma than with spermatozoa, indicated that seminal plasma contains immunoactive agents that upregulate cytokine expression in uterine NK cells. Although the endometrium and seminal plasma do not directly contact during sexual intercourse, high concentrations of immunomodulatory molecules in seminal plasma, such as macrophage colony-stimulating factor (M-CSF) and TGF-β, have been detected in cervical mucus (Fujii et al., 2006). Semen-derived factors in the vagina and cervical canal might extend their functional activity to the uterus (Tremellen et al., 1998).

Furthermore, TGF-β derived from seminal plasma has been detected in post-acrosomal regions of spermatozoa and at a higher density at the acidic pH typical of the vaginal environment (Chu et al., 1996), suggesting that spermatozoa can transport immunosuppressive factors after exposure to acidic conditions during intercourse. Thus, our results indicate that stimulation with semen is closer to the actual physiological situation than PMA/ionomycin for evaluating cytokine production by endometrial immune cells.

We also found that cytokine production by uterine NK cells could depend on individual semen samples, because semen from the same men produced distinct IFN-γ/TNF-α expression profiles in different women. These findings suggest compatibility profiles between partners in the activation of endometrial immune cells, regardless of the concentration of specific semen-derived immunomodulatory factors.

The comparison of cytokine production profiles in women with and without RPL provided further evidence to support this notion. Production of the type 2 cytokines, IL-4 and IL-10 by endometrial NK cells did not significantly differ among the groups examined. However, the proportion of NK cells producing type 2 cytokines was significantly lower in the uRPL, than the control group after stimulation with semen, resulting in higher NK1/NK2 cell ratios. Exposure to semen induces a shift to the NK2 phenotype (low NK1/NK2 cell ratio) (Kimura et al., 2009; Robertson et al., 1997; Tremellen et al., 1998) during normal pregnancy, and an NK1 shift (high NK1/NK2 cell ratio) in situations of infertility or implantation failure (Fukui et al., 2008). Our results suggested that miscarriage can occur *via* a semen-induced NK1 shift, even in some women even without immunological abnormalities, supporting the concept of immunological compatibility between individuals. Therefore, couples with fertility problems should undergo tests *in vitro* to evaluate the effect of semen on cytokine production in the endometrium. *In vitro* fertilization-embryo transfer (IVF-ET) that does not involve exposure to seminal plasma should be considered as the treatment of choice for patients with infertility or RPL and immunological abnormalities. Future studies should confirm the effects of immunological compatibility on conception and pregnancy maintenance.

Shima et al. found notably increased Treg cells in pregnant mice and that seminal plasma plays an important role in this process (Shima et al., 2015). In addition, Johansson et al. found increased T cells and activated NK cells in mice after sexual intercourse (Johansson et al., 2004). Moreover, Lin et al. found that $CD25^{+}$ NK cells suppress the induction of cytotoxic T cells in the uteri of pregnant mice (Lin et al., 2009). Thus, further studies are needed to evaluate the role of T cells in the regulation of pregnancy, in synergy with NK cells.

5. Conclusion

Our results showed that cytokine production by endometrial NK cells can be regulated by exposure to semen, which contains immunosuppressive factors, suggesting immunological compatibility between semen and the endometrium. The phenotype of NK cells shifts to NK1 in women with RPL and immunological abnormalities. However, a phenotype shift might be induced after semen stimulation in women with unexplained RPL. Therefore, couple-specific immunological compatibility should be tested with semen stimulation *in vitro*, which is more physiological than PMA/ionomycin stimulation and could provide useful clinical information for the treatment of RPL.

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Declaration of Competing Interest

The authors report no declarations of interest.

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EMBRYO BIOLOGY

Mitochondrial oxygen consumption rate of human embryos declines with maternal age

Naoharu Morimoto[1,2,3] · Shu Hashimoto[2] · Masaya Yamanaka[1] · Tatsuya Nakano[1] · Manabu Satoh[1] · Yoshiharu Nakaoka[1] · Hisataka Iwata[4] · Atsushi Fukui[3] · Yoshiharu Morimoto[5] · Hiroaki Shibahara[3]

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Abstract

Purpose The fertility of women decreases with age because of factors such as an increased incidence of aneuploidies and—possibly—decreased mitochondrial activity in oocytes. However, the relationship between maternal aging and mitochondrial function of their embryos remains unknown. Here, we assessed the relationship between maternal age and mitochondrial functions in their oocytes and embryos

Methods The relationships between maternal age and oxygen consumption rates (OCRs), mitochondrial DNA (mtDNA) copy numbers, or blastocyst development was investigated using 81 embryos donated from 63 infertility couples. The developmental rates from morulae to blastocysts were retrospectively analyzed using data of 105 patients.

Results The OCRs of morulae decreased with maternal age ($r^2 = 0.48$, $P < 0.05$) although there were no relationships between maternal age and mtDNA copy number in any stages. The more oxygen consumed at the morula stage, the shorter time was required for embryo development to the mid-stage blastocyst ($r^2 = 0.236$, $P < 0.05$). According to the clinical data analysis, the developmental rate from morulae to blastocysts decreased with maternal age ($P < 0.05$, < 37 years, 81.1%, vs. ≥ 37 years, 64.1%).

Conclusions The data of the present study revealed that mitochondrial function at the morula stage of human embryos decreased with their maternal age and a decrease of mitochondrial function led to slow-paced development and impaired developmental rate from morulae to blastocysts.

Keywords Maternal age · Mitochondrial function · Oxygen consumption rate

Introduction

The fertility of women decreases with age [1]. One of the main reasons for the poor development of embryos obtained from older women is an increased rate of chromosomal aberrations [2] caused by premature bivalent separation into univalents during meiosis [3]. Additionally, a decline in mitochondrial function in oocytes with advancing age has also been proposed as a reason for poor embryo developmental competence [4–7]. The adverse effects of maternal aging on oocyte mitochondria have been reported as morphological abnormalities such as mitochondrial swelling, vacuolization and alterations to cristae [8, 9], and a possible decrease in mitochondrial DNA (mtDNA) copy number [10, 11]. However, the effects of maternal aging on mitochondrial function, such as oxygen consumption rate (OCR) in embryos, are not well understood.

It has been shown that the mitochondrial function of preimplantation embryos increases as their development advances toward the blastocyst stage, especially after embryonic (zygotic) gene activation [12–15]. In mammalian cells, adenosine triphosphate (ATP) is mainly generated by the mitochondrial electron transport system. Thus, the measurement of the OCR of developing embryos could be used as an effective tool to assess mitochondrial function for evaluation of

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✉ Shu Hashimoto
hashimoto@ivfnamba.com

[1] IVF Namba Clinic, Osaka 550-0015, Japan

[2] Reproductive Science, Graduate School of Medicine, Osaka City University, Osaka 545-8585, Japan

[3] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan

[4] Department of Animal Science, Tokyo University of Agriculture, Kanagawa 243-0034, Japan

[5] HORAC Grand Front Osaka Clinic, Osaka 530-0011, Japan

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their normality and developmental potential [12–18], which may help to evaluate embryo development potential prior to implantation.

Here we aimed to explore the relationship between maternal age and mitochondrial function of developing embryos.

Materials and methods

To assess the relationship between maternal aging and mitochondrial function of their embryos, vitrified–warmed cleavage stage embryos on day 3 (68–70 h after ICSI) and blastocysts on day 5 (116–118 h after ICSI), donated from 53 couples, were measured for OCRs and mtDNA copy numbers (Supplementary Fig. 1). All the embryos were received after obtaining written informed consent.

To determine the relationship between OCRs of morulae and their developmental competence, we assessed the time required from morulae to the mid-stage blastocyst after the measurement of their OCRs. Time-lapse images were captured by light-emitting diode illumination six times per hour over 2 days using a Primovision system (Vitrolife Japan Corporation, Tokyo, Japan) as described [19]. Embryos were cultured individually in potassium simplex optimized medium containing amino acids [20] (KSOMaa) and 5% (v/v) synthetic serum substitute (SSS, 99193; Irvine Scientific, St. Ana, CA, USA) at 37 °C under 5% CO_2, 5% O_2, and 90% N_2 with high humidity. Eighteen embryos donated from 10 couples were examined. Only embryos donated from women aged 34–36 years at OPU were used to avoid female age effects.

In a retrospective analysis of clinical data regarding developmental competence from the morula to blastocyst stages, OPU was performed on 105 patients between February 2014 and July 2015 (Tables 1 and 2). Embryos were cultured in a single-step medium (SAGE 1-Step™ 67010010, CooperSurgical/Origio, Målov Denmark or Continuous Single Culture®, 90164, Fujifilm, Tokyo, Japan). Blastocyst morphology was evaluated at 120 and 144 h after insemination according to Gardner and Lane [21]. Blastocysts that were scored at least 3AA, but not grade C, were regarded as morphologically good. The developmental rates were compared between two age groups of women (< 37 years vs. ≥ 37 years).

Ovarian stimulation and insemination

Patients were subjected to controlled ovarian stimulation according to their medical history as described [22]. Ovulation was induced by hCG administration when at least one leading follicle reached 18 mm in diameter. Transvaginal follicle aspiration was carried out 36 h after the hCG injection. Insemination was carried out by ICSI or coculture with their partner's spermatozoa for conventional in vitro fertilization 40 h after hCG administration.

Table 1 Maternal characteristics in retrospective study

	< 37 years	≥ 37 years
No. of patient	49	56
Maternal age (years)	33.1	39
Body mass index (kg/m^2)	20.3	20.6
Number of previous OPU	0.16	0.29
Stimulation protocol		
Agonist	31	30
Antagonist	18	26
Current smoker	0	0
Ex-smoker	3	2
Cause of infertility		
Tubal	8	7
PCO	2	4
Endometriosis	9	9
Male	11	12
Amount of FSH dose (IU)		
FSH	1091	1304
HMG	880	928
Number of ova		
Retrieved	18	15
Mature	16	13
Fertilized	13	10
Fertilization rate	81.3	76.9
Number of		
cIVF	20	24
ICSI	29	32
Proportion of ICSI	59	57

OCR measurements

Embryos were frozen by vitrification [23] on days 3 or 5 after ICSI. After warming, day 3 embryos were cultured in KSOMaa medium at 37 °C under 5% CO_2, 5% O_2, and 90% N_2 with high humidity for 17–22 h. Day 5 blastocysts were cultured for 8 h, and then their OCR was assessed because vitrified-warmed embryos recovered their OCR to the same level before vitrification 8 h after warming [17]. Morulae on day 4 that reached the compaction stage from 17 to 22 h after warming were included in the analysis. Blastocysts on day 5 that reached the expanded stage with a tightly packed inner cell mass (ICM) and trophectoderm (TE) consisting of cohesive epithelium were included in the analysis. The OCR of samples was measured using scanning electrochemical microscopy (CRAS-1.0; Clino Ltd., Miyagi, Japan) as described [15, 17] at 37 °C under air. Briefly, each

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Table 2 Retrospective analysis of rates of development from morulae to blastocysts

	Sage		CSC	
	< 37 years	> 37 years	< 37 years	> 37 years
Number of patients				
	35*	29*	16*	29*
Number of cultured				
	251	207	145	186
Number of morulae				
	174	117	106	134
Rate (%)†	69.3[a]	56.5[a]	73.1	72.0
Number of blastocysts on day 5				
	135	75	92	86
Rate (%)‡	77.6[a]	64.1[a]	86.8[b]	64.2[b]
Number of morphologically good blastocysts on day 5				
	54	24	32	31
Rate (%)‡	31	20.5	30.2	23.1
Number of blastocysts on day 6				
	160	100	97	111
Rate (%)‡	92.2[a]	85.5[a]	91.5[b]	82.8[b]
Number of morphologically good blastocysts on day 6				
	65	35	42	34
Rate (%)‡	37.4	29.9	39.6[b]	25.4[b]

Embryos were cultured in SAGE 1-Step™ medium (Sage) or Continuous Single Culture® medium (CSC) as described in the Methods. *Embryos obtained from 3 patients were randomly divided and cultured in two media. †The rate of morulae per cultured fertilized ova, ‡The rate of blastocysts per morulae. [ab] Values with same superscripts within each column differ ($P < 0.05$)

oocyte or embryo was transferred into a cone-shaped microwell filled with 5-mL human tubal fluid (HTF) medium buffered with 21-mM HEPES containing 2.7-mM glucose, 0.33-mM pyruvate, and 5% SSS (mHTF), where it sank to the bottom and remained at the lowest point. A platinum microdisk electrode was loaded with 5 mL of mHTF, and its tip potential was maintained at − 0.6 V versus the Ag/AgCl electrode with a potentiostat to monitor the local oxygen concentration. The microelectrode scanned along the z-axis from the edge of the sample, and the OCR was calculated with custom software based on the spherical diffusion theory [24]. OCR was measured at three points for each embryo and at the ICM and both TE sides of blastocysts. The mean value was shown as its OCR read because there was no difference in the OCR value between ICM and TE sides [17]. The OCR was also measured in the presence of mitochondrial toxins (mitotoxins) [15, 18]. Stock solutions of 1-mM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP; C2920, Merck Millipore Co., Darmstadt, Germany) and 1-M sodium cyanide (380970, Sigma-Aldrich) were prepared in ethanol and water, respectively. During the recording of OCR by embryos in mHTF medium, either FCCP or cyanide was added to give final concentrations of 1 μM and 1 mM, respectively. Cyanide at 1 mM normally completely inhibits mitochondrial cytochrome c oxidase activity. The mitochondrial OCRs (mtOCRs) were calculated by subtracting the value obtained in the presence of cyanide from those obtained without any mitochondrial toxins. The maximum mitochondrial OCRs (maxOCRs) value was calculated by subtracting the values obtained in the presence of cyanide from those obtained in the presence of FCCP.

Mitochondrial DNA measurements

All specimens were placed in 2-μL DNase-free water droplet individually immediately after OCR measurement and cryopreserved at − 70 °C until assay [15]. Each sample was lysed in 4-μL aliquots of lysis buffer (20-mM Tris (# 252859, Merck Millipore Co.), 0.4 mg/ml proteinase K (# P2308, Merck Millipore Co.), 0.9% Nonidet P-40 (# 21-3277-2, Merck Millipore Co.), and 0.9% Tween 20 (# P1379, Merck Millipore Co.)) at 55 °C for 30 min, followed by heating at 95 °C for 5 min; the lysate was then diluted in DNase-free water to a final volume of 40 μL. A 4-μL aliquot of the lysate was added to 12.5-μL Quantifact SYBR Green master mix (Qiagen, Venlo, Limburg, the Netherlands), 8.5-μl DNase-free water, and 1 μM of each prime polymerase chain reaction (PCR) amplifications were run in triplicate in a Rotorgene Q thermal cycler (Qiagen) according to the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 5 s, and 60 °C for 10 s. SYBR green fluorescence was measured at the end of each cycle. The melting curve was analyzed to check the specificity of the PCR products. A standard curve was generated for each run using 10fold serial dilutions representing copies of the external standard (10^3 to 10^6 copies). The external standard used in the present study was the PCR product of the corresponding gene cloned into a vector using the Zero Blunt TOPO PCR cloning kit (Thermo Fisher Scientific, Waltham, MA, USA), and the product was confirmed by sequencing before use. The primers were designed using Primer3Plus software (http://sourceforge.net/projects/primer3/) and targeted the NADH-ubiquinone oxidoreductase chain 1 (ND1; 126 bp region from 3485 to 3610) and ND6 gene sequences (163 bp region from 14,407 to 14,549) of human mitochondria (NC012920.1).

Study approval

The study was performed in accordance with Declaration of Helsinki protocols 7th version. Prior to inclusion in the study, all donating couples gave their written informed consent. This study was approved by the local ethics Institutional Review

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Board of IVF Namba Clinic, the institutional review board of Hyogo College of Medicine, and the Japan Society of Obstetrics and Gynecology (Registry Nos 135 and 138).

Statistics

The relationship between maternal age and mtDNA/OCRs of embryos was assessed using Pearson's correlation coefficient test after the confirmation of normal distribution by a Chi-square goodness-of-fit test. Data obtained from a retrospective clinical analysis regarding developmental competence was compared using Chi-square tests. $P < 0.05$ was considered statistically significant.

Results

Effect of maternal age on OCRs and mtDNA copy number

To assess the relationship between maternal age and mitochondrial function of their embryos, 30 morulae on day 4 after ICSI and 34 expanded blastocysts on day 5, donated from 24 and 29 couples, respectively, were used for study. All embryos used in this study were inseminated by ICSI to avoid the contamination of mtDNA from spermatozoa attached to the zona pellucida. Women whose infertility causes were reported as being endometriosis, premature ovarian insufficiency, or hormone imbalance were excluded.

The mtOCRs and maxOCRs of morulae on day 4 after ICSI decreased with maternal age (mtOCRs, $P < 0.05$, $r^2 = 0.4834$; maxOCRs, $P < 0.05$, $r^2 = 0.5081$, Fig. 1a, b). However, there were no relationships between maternal age and mtOCRs or maxOCRs of their blastocysts on day 5 (Fig. 1d, e). Similarly, mtDNA copy numbers in different stages of developing embryos did not show any correlation with maternal age (Fig. 1c, f).

Relationship between OCRs of morulae and their development: do morulae with higher OCRs have higher developmental competence?

To address this, we captured time-lapse images from the morula to blastocyst stages following the OCR measurement of morulae without using mitotoxins. To avoid the effect of

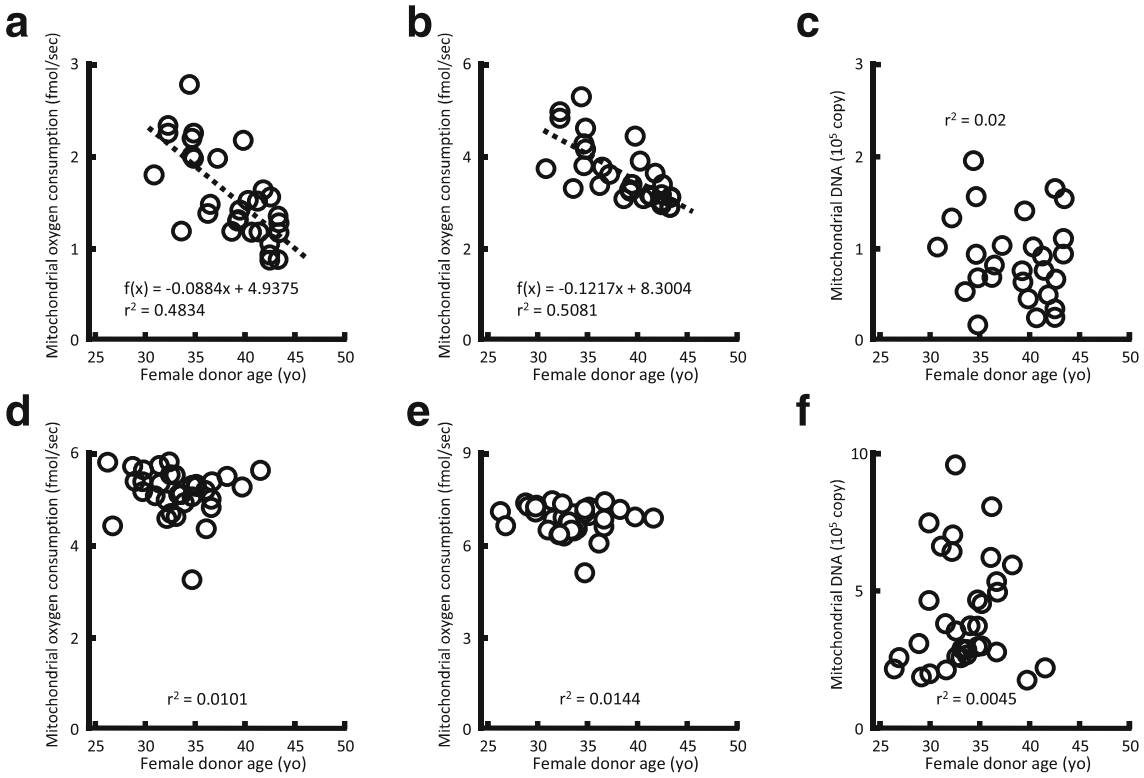


Fig. 1 Relationship between maternal age and mitochondrial oxygen consumption rates (mtOCRs), maximum mitochondrial OCRs (maxOCRs), or mitochondrial DNA (mtDNA) numbers of embryos. (**a, b**) Relationships between maternal age and mtOCRs or maxOCRs of morulae on day 4 ($n = 30$) and (**d, e**) expanded blastocysts on day 5 ($n = 34$). (**c**) Relationships between maternal age and mtDNA copy number of their morulae on day 4 ($n = 28$) and (**f**) expanded blastocysts on day 5 ($n = 34$) are shown. The mtOCRs and maxOCRs of morulae on day 4 after ICSI decreased with maternal age (mtOCRs, $P < 0.05$, $r^2 = 0.4834$; maxOCRs, $P < 0.05$, $r^2 = 0.5081$) (**c, g**). Only embryos inseminated by ICSI were used to avoid contamination with mtDNA from spermatozoa on the zona pellucida

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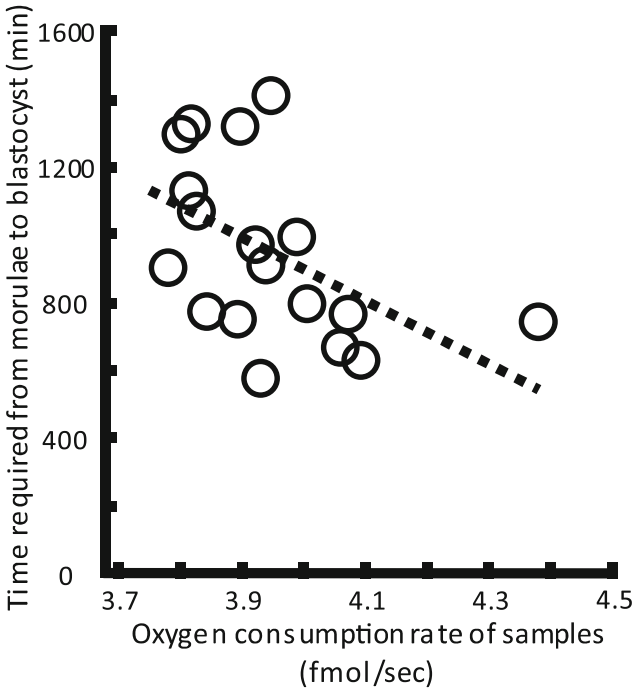


Fig. 2 Relationship between OCRs of morulae and duration required to develop from morulae to mid-stage blastocysts. Morphological changes from morulae to blastocysts following measurement of OCRs of morulae cultured without mitotoxins were recorded every 10 min. To avoid the effect of maternal age, 6–8-cell stage embryos on day 3 were used from women aged 34–36 years at OPU. Eighteen embryos were donated from 10 couples. The more oxygen morulae consumed, the shorter was the duration required for embryo development between OCR measurement and mid-stage blastocysts ($r^2 = 0.236$, $P < 0.05$). Mid-stage was defined as when the blastocoel reached half the size of the blastocyst

maternal age, we used eighteen embryos at 6–8 cell stage on day 3 donated from 10 couples among whom the woman's age ranged from 34.0 to 35.9 years at oocyte pick-up. At the morula stage, the more oxygen consumed, the shorter time was required for embryo development to the mid-stage blastocyst ($r^2 = 0.236$, $P < 0.05$; Fig. 2). Mid-stage was defined as when the blastocoel size reached half that of the blastocyst.

Retrospective analysis of developmental competence from morulae to blastocysts

To determine how maternal age might affect morphological changes in embryos, the developmental rates to the blastocyst stage and the rate of formation of morphologically good blastocysts from morulae were compared retrospectively between two age groups of women (< 37 years $n = 280$ vs. ≥ 37 years $n = 251$). Retrospective analysis revealed that these development rates on day 5 after insemination decreased significantly with maternal age, being a blastulation rate of 81.1% in the younger vs. 64.1% in the older group ($P < 0.01$), and a 30.7% morphologically good blastocyst formation rate in the younger vs. 21.9% in the older group ($P < 0.05$; Fig. 3a, b). At day 6, the rates of development to the blastocyst and morphologically good blastocyst stages also significantly decreased with maternal age, being a 92.1% blastulation rate in the younger vs. 84.1% in the older group ($P < 0.01$) and a 38.2% morphologically good blastocyst formation rate in the younger vs. 27.5% in the older group ($P < 0.01$). These embryos were cultured in two commercially available media. The effects of maternal age were separately assessed in each medium. Irrespective of the culture medium, the adverse effect of maternal age was still observed in the morphologically good blastocyst formation rate (Table 2).

Discussion

Here we found that the mitochondrial function of human morulae decreased with maternal age. Maternal age had no link

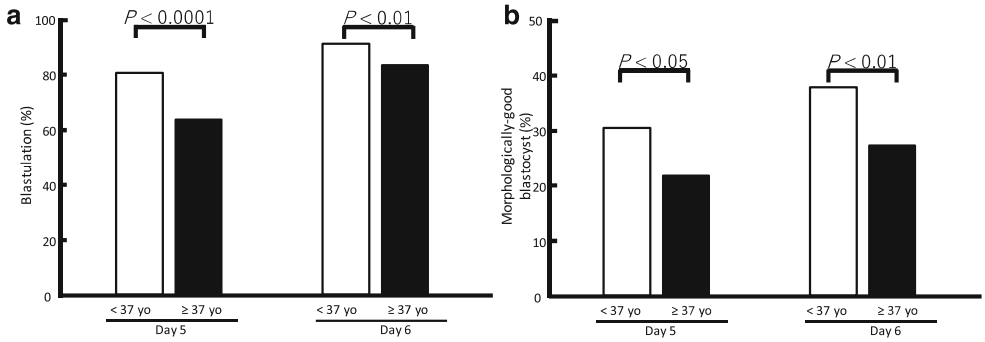


Fig. 3 Retrospective analysis of developmental competence from morulae to blastocysts in the clinical data set. The rates of development from morulae to blastocysts (**a**) and to form morphologically good blastocysts (**b**) were compared retrospectively between two maternal age groups (< 37 years; $n = 280$; vs. ≥ 37 years, $n = 251$). Retrospective analysis revealed that the rates of development from morulae to blastocysts and to morphologically good blastocysts on day 5 after insemination decreased with maternal age. The rates of development from morulae to blastocysts and to morphologically good blastocysts on day 6 also decreased with maternal age. The data were for embryos cultured in two different commercially-available media (see Methods). Similar adverse effects of maternal age were observed in both culture media (Supplementary Figure)

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with the copy number of mtDNA at both stages of embryo development or mtOCRs of blastocyst stage. The mtOCRs remain low until 8-cell stage in humans [15] and mice [14]. These data confirmed previous findings that mitochondrial biogenesis in humans is normally activated after embryonic gene activation at the 8-cell [15] or blastocyst stages [9, 25]. Moreover, the OCRs of human embryos rise dramatically following the 8-cell stage [15]. Accordingly, some degree of mitochondrial dysfunction in human embryos along with maternal aging could emerge after the 8-cell stage. Moreover, formation of the blastocoel cavity depends on cellular Na/K-ATPase activity and requires high levels of ATP [26]. These findings imply that morulae with reduced mitochondrial function might not form a blastocoel or have a delayed start as shown in Fig. 2. Subsequently, expanded blastocysts on day 5 had high mitochondrial function irrespective of maternal age (Fig. 1d, e) because embryos on day 4 without sufficient mitochondrial function could not reach the expanded blastocyst stage on the next day.

It has been generally believed that higher mtDNA content in TE cells correlates with a stressed state, such as aneuploidy and advanced maternal age [27–29]. This idea is supported by "quiet embryo hypothesis" which proposed that viable embryos have lower metabolism [30]. However, this hypothesis was based on metabolism before the start of blastulation which requires more energy [26]. Moreover, it has been shown that there was no correlation of mtDNA content in human blastocysts with their maternal age using an accurate quantitation of mtDNA copy number [31] consistent with our data using whole embryo. Therefore, time and further studies will tell whether the maternal age affect mtDNA content in their blastocysts.

To avoid the effect of cause of infertility on mtDNA content and OCRs, we excluded from the analysis women whose infertility causes were reported as being endometriosis, premature ovarian insufficiency, or hormone imbalance. However, embryos obtained from two different stimulation protocols (agonist and antagonist cycles) were included in the analysis. There were no differences in the OCR and mtDMA content of blastocysts between two different protocols (data not shown). Further studies should be required whether the patients' background except for maternal age or stimulation protocol affects the OCR an mtDNA content of their embryos.

The data of the present study revealed that mitochondrial function at the morula stage of human embryos decreased with their maternal age and a decrease of mitochondrial function led to slow-paced development and impaired developmental rate from morulae to blastocysts.

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Authors' roles N.M. and S.H. designed the experiment, interpreted the results, and wrote the manuscript with help from all authors. M.Y. measured OCRs. T.N., M.S., and Y.N. were involved in the analysis of clinical data. H.I. designed the qPCR procedures. A.F., H.S., and Y.M. supervised the project.

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Compliance with ethical standards

Conflict of interest The authors declare that they no conflict of interest exists.

Ethical approval The study was performed in accordance with Declaration of Helsinki protocols 7th version. This study was approved by the local ethics Institutional Review Board of IVF Namba Clinic, the institutional review board of Hyogo College of Medicine, and the Japan Society of Obstetrics and Gynecology (Registry Nos 135 and 138).

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Review article

COVID-19 and immunomodulation treatment for women with reproductive failures

Joanne Kwak-Kim[a,*], Kuniaki Ota[b], Nayoung Sung[a], Changsheng Huang[a,c], Lujain Alsubki[a,d], Sungki Lee[e], Jae Won Han[e], Aera Han[f], Xiuhua Yang[g], Wael Saab[h], Youssef Derbala[i], Wen-Juan Wang[j], Qiaohua He[k], Aihua Liao[l], Toshifumi Takahashi[b], Marcelo Borges Cavalcante[m,n], Ricardo Barini[o], Shihua Bao[p], Atsushi Fukui[q], Nathalie Lédée[r,s], Carolyn Coulam[t]

[a] *Reproductive Medicine and Immunology, Obstetrics and Gynecology, Clinical Sciences Department, Center for Cancer Cell Biology, Immunology and Infection, Chicago Medical School, Rosalind Franklin University of Medicine and Science, 830 West End Court, Vernon Hills, IL, 60061, USA*
[b] *Fukushima Medical Center for Children and Women, Fukushima Medical University, 1 Hikarigaoka, Fukushima City, Fukushima 960-1295, Japan*
[c] *Traditional Chinese Medicine Department of Rheumatism, Huazhong University of Science and Technology Union Shenzhen Hospital, China*
[d] *Department of Obstetrics and Gynecology, King Faisal Specialist Hospital and Research Center, Riyadh. Saudi Arabia*
[e] *Department of Obstetrics and Gynecology, Myuonggok Medical Research Center, Konyang University College of Medicine, Daejeon, South Korea*
[f] *Department of Obstetrics and Gynecology, MizMedi Hospital, Seoul, South Korea*
[g] *Department of Obstetrics, The First Hospital of China Medical University, Shenyang, China*
[h] *The Centre for Reproductive & Genetic Health, 230-232 Great Portland St, Fitzrovia, London W1W 5QS, UK*
[i] *Obstetrics and Gynecology, Beaumont Hospital, Dearborn, Grosse Pointe, MI, USA*
[j] *Reproduction Medical Center, The Affiliated Yantai Yuhuangding Hospital of Qingdao University, Yantai, 264000, China*
[k] *Department of Reproductive Medicine Center, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, Henan Provincial People's Hospital of Henan University, Zhengzhou, Henan, 450003, China*
[l] *Institute of Reproductive Health, Center for Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China*
[m] *Department of Obstetrics and Gynecology, Fortaleza University (UNIFOR), Fortaleza, CE, Brazil*
[n] *CONCEPTUS – Reproductive Medicine, Fortaleza, CE, Brazil*
[o] *Department of Obstetrics and Gynecology, Campinas University (UNICAMP), Campinas, SP, Brazil*
[p] *Department of Reproductive Immunology, Shanghai First Maternity and Infant Hospital, Tongji University School of Medicine, Shanghai, 201204, China*
[q] *Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan*
[r] *MatriceLAB Innove, Pépinière Paris Santé Cochin, Hôpital Cochin, 29 rue du faubourg St Jacques, 75014 Paris, France*
[s] *Centre d'assistance médicale à la procréation, Hôpital des Bluets, 4 rue Lasson, 75012, Paris, France*
[t] *Clinical Immunology Laboratory, Rosalind Franklin University of Medicine and Science, North Chicago, 60064, IL, USA*

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ABSTRACT

COVID-19 pandemic is affecting various areas of health care, including human reproduction. Many women with reproductive failures, during the peri-implantation period and pregnancy, are on the immunotherapy using immune modulators and immunosuppressant due to underlying autoimmune diseases, cellular immune dysfunction, and rheumatic conditions. Many questions have been raised for women with immunotherapy during the COVID-19 pandemic, including infection susceptibility, how to manage women with an increased risk of and active COVID-19 infection. SARS-CoV-2 is a novel virus, and not enough information exists. Yet, we aim to review the data from previous coronavirus outbreaks and current COVID-19 and provide interim guidelines for immunotherapy in women with reproductive failures.

* Corresponding author at: Reproductive Medicine and Immunology Obstetrics and Gynecology, Clinical Sciences Department Center for Cancer Cell Biology, Immunology and Infection, Chicago Medical School, Rosalind Franklin University of Medicine and Science, 830 West End Court, Suite 400, Vernin Hills, IL, 60061 USA.
E-mail address: Joanne.KwakKim@rosalindfranklin.edu (J. Kwak-Kim).

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1. Introduction

With the Coronavirus Disease 2019 (COVID-19) pandemic, patient care has been significantly challenged not only for the COVID-19 cases but for the others, including pregnant women with a history of reproductive failures (RF), such as recurrent pregnancy losses (RPL), and repeated implantation failures (RIF), with immune etiologies including autoimmune diseases, cellular immune dysfunction, and rheumatic conditions. These RF women with immune etiology (RFI) may have been on various immunosuppressants, immunomodulators, or anticoagulants. Whether these treatment modalities increase the risk for COVID-19 infection and aggravate COVID-19, have been a major concern for already vulnerable pregnant women with RFI.

SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus), which caused the SARS outbreak in 2003, infects macrophages and T cells (Perlman and Dandekar, 2005) and induces various cytokines, such as type I IFN, TNF-α, IL-1, etc., and B cell-related antibodies (Prompetchara et al., 2020). However, it is unclear if SARS-CoV-2 infects the same kinds of immune effectors. SARS-CoV-2 has been speculated to induce the influx of neutrophils and monocytes/macrophages at the infection site, which results in hyperproduction of proinflammatory cytokines. Specific T helper (Th) 1 and Th17 cells may be activated and contribute to exacerbating inflammatory responses. B cells and plasma cells produce SARS-CoV-2 specific antibodies that may neutralize viral particles (Prompetchara et al., 2020). B cell reduction was reported in the early phase of the COVID-19, which in turn, affects antibody production (Lin et al., 2020), and severe lymphopenia was often manifested in severe COVID-19 cases (Zhu et al., 2020b). In hospitalized severe COVID-19 patients, high plasma levels of various cytokines, including IL-2, IL-7, IL-10, G-CSF, inducible protein-10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1A (MIP-1A), and TNF-α, were observed (Huang et al., 2020). The primary cause of mortality in severe cases was cytokine storm, and these findings were in line with SARS and middle east respiratory syndrome (MERS) (Price et al., 2020)

The novel SARS-CoV-2 uses the angiotensin-converting enzyme 2 (ACE2) for the cellular entry like SARS-CoV and mainly spreads through the respiratory tract (Guo et al., 2020). The ACE2 mRNA is abundantly expressed in endometrial epithelial cells in the secretory phase (Vaz-Silva et al., 2009), ovaries, and testes (Honorato-Sampaio et al., 2012). Renin-angiotensin system is involved in female reproductive processes, including follicular development (Barreta et al., 2015), steroid hormone production, oocyte maturation, and ovulation (Reis et al., 2011). Considering the reported relationship between ACE2 and viral pneumonia (Xu et al., 2020), COVID-19 may attack the follicular membrane and granulosa cells of the ovary, affecting folliculogenesis and the quality of oocytes, and causing female infertility and pregnancy losses. Additionally, it may damage endometrial epithelial cells and affect early embryo implantation, although no studies suggested that COVID-19 has any specific effect on the female reproductive system.

The specific effect on pregnancy has not been reported in COVID-19 cases, although studies are limited. However, previous studies have confirmed that the viral pandemic, such as the 2003 SARS pandemic, 2009 H1N1 influenza, or 2013 MERS, was associated with an increased incidence of maternal and perinatal complications, such as spontaneous abortion, premature delivery, and intrauterine growth restriction (Assiri et al., 2016). SARS-CoV and MERS-CoV, however, did not show any vertical transmission (Assiri et al., 2016). A recent analysis of 38 pregnant women with COVID-19 showed no intrauterine or transplacental transmission of SARS-CoV-2 from mother to fetus (Schwartz, 2020), although there is a concern for vertical transmission.

Women with a history of RFI already have an increased risk of obstetrical complications. Therefore, the continuation of immunotherapy is critical for these women. In this review, we aim to deliver the interim guidelines for current immunotherapy for women with RFI, concerning the COVID-19 pandemic. This article was developed by the international collaboration of experts who have been working in the field of reproductive immunology.

2. Immunotherapy

2.1. Prednisone

Prednisone is a synthetic corticosteroid that mainly acts as an immunosuppressant. For its potent and broad-spectrum anti-inflammatory and immune-suppressive properties, prednisone has been utilized to treat autoimmune and chronic inflammatory diseases, and often the first line immunotherapeutic agent prescribed in the painful context of repeated implantation failure (RIF) or RPL of immune etiologies (Rhen and Cidlowski, 2005). Prednisone also plays an important role in mediating proper folliculogenesis, increasing production of growth factors, suppressing androgenic hormones, and suppressing NK cell activity and Th1/Th2 cell ratios (Keay et al., 2001). In early reports (Quenby et al., 2003), corticosteroid therapy has been reported to be beneficial in women with consecutive miscarriages since it reduces endometrial NK cells in women with RPL (Quenby et al., 2005). However, in routine in-vitro fertilization (IVF) cycles, the Cochrane review did not demonstrate the benefit of peri-implantation corticosteroid administration (Boomsma et al., 2012). On the other hand, prednisolone, which has a relatively mild effect, was reported to improve the embryo implantation rate after IVF and protect against miscarriage when administered from embryo implantation through the early placentation phase (Robertson et al., 2016). It is reported that less than half of RIF patients with immune deregulation may be prednisone responders and would benefit from its administration (Lédée et al., 2018a).

Low-dose prednisolone treatment was suggested to have a beneficial effect on COVID-19 and have been widely used to treat COVID-19 induced lung injury or septic shock (Russell et al., 2020). However, current interim guidance from WHO advises against the routine use of corticosteroids for the management of suspected and confirmed COVID-19 cases (Clinical management of severe acute respiratory infection SARI when COVID-19 disease is suspected, interim guidance). Currently, no data is available for women in early gestation with COVID-19 infection, although previously, 57.1 % of miscarriage rate was reported in women infected with SARS-CoV in the early pregnancy (Wong et al., 2004). Therefore, pregnant women with RFI and infected with COVID-19 may have an increased risk of early pregnancy loss. Overall, no specific evidence exists to demonstrate whether women infected with COVID-19 in the early pregnancy might get benefit from corticosteroids to prevent miscarriage. Therefore, prednisone is not recommended for women with early pregnancy and COVID-19 infection unless otherwise indicated.

In pregnant women with RFI, low dose prednisone treatment can be continued if the COVID-19 infection chance is low. If the patient has an increased risk of COVID-19 infection or on high dose prednisone treatment, tapering off or decreasing the dose is recommended to reduce the susceptibility to viral infection. If women have COVID-19 infection, tapering off the prednisone treatment is recommended to avoid worsening the disease, and switching into another treatment modality should be considered for the maintenance of pregnancy.

2.2. Heparin treatment

Women with RFI are often accompanied by thrombophilia, such as antiphospholipid syndrome and inherited thrombophilia. Anticoagulants are commonly used to treat the thrombotic features of RFI during pregnancy or assisted reproductive technology (ART). With the pandemic spread of SARS-CoV-2, many reports and guidelines concerning pregnancy are being updated, but there are only a few reports about heparin use in RFI patients. So, it is necessary to address some recommendations regarding anticoagulation treatment in RFI

women with COVID-19.

Heparin is primarily used for anticoagulation in women with RFI, and low molecular weight heparin (LMWH) is the drug of choice due to its numerous advantages over unfractionated heparin (uFH). These drugs are safe in pregnancy, and in addition to its antithrombotic effect, heparin provides a favorable environment for implantation and placental development (Nelson and Greer, 2008). By adhering to selectin, heparin initiates the implantation process and downregulates E-cadherin expression in the decidua, which promotes trophoblast invasion. Additionally, heparin enhances the heparin-binding epidermal growth factor, which is important in preventing the apoptosis of trophoblast (Nelson and Greer, 2008). LMWH has been reported to have various immunological effects, including inhibition of TNF-α and IL-6 in placental villi (Mousavi et al., 2015; Zenerino et al., 2017).

Moreover, heparin binds to certain proteins that exhibit an antiviral effect. Many different viruses, including herpes simplex virus (HSV)-1 and -2, attach to heparan sulfate on the host cell surface at the beginning of infection (Trybala et al., 2000). Because of the similarity in structure between heparan sulfate and heparin, heparin interacts with viral proteins in HSV, human immunodeficiency virus (HIV), and dengue virus, and inhibits viral entry into the cells (Nelson and Greer, 2008). The antiviral effects of heparin were found in some other viral infection including human cytomegalovirus, human papillomavirus, poxvirus, vaccinia virus, enterovirus 71, hepatitis C virus, orf virus, and respiratory syncytial virus in vitro (Krusat and Streckert, 1997; Scagliarini et al., 2004; Basu et al., 2007; Pourianfar et al., 2014; Khanna et al., 2017; Gonzalez et al., 2018; Liu et al., 2018). Furthermore, replication of SARS-CoV Nsp 15 protein was also inhibited by heparin in vitro (Bhardwaj et al., 2004).

From the Chinese experience of COVID-19, heparin was recommended to rescue severe cases with coagulopathy induced by SARS-CoV-2 infection. A research group suggested the use of 40−60 mg enoxaparin/day or uFH, 10,000–15,000 IU/day, which decreased the mortality of severe COVID-19 patients with sepsis-induced coagulopathy (Tang et al., 2020). Similarly, a review study proposed to inject LMWH 100 IU/Kg, every 12 h for 3–4 days, if the level of D-dimer becomes four times higher than the upper normal limit, unless contraindicated (Lin et al., 2020). From the above, even though data are not enough, heparin, including uFH and LMWH, may play a role in limiting COVID-19 infection via both improvement of tissue circulation and antiviral effect. Thus, it is likely that at least there is no harm to use heparin for anticoagulation, even in pregnant RFI women with thrombophilia. Taken together, when COVID-19 is suspicious or confirmed, there is no need to stop heparin administration in pregnant women with RFI whose heparin is indicated. In the earliest stages of the COVID-19 pandemic, numerous reproductive societies, including ASRM (American Society for Reproductive Medicine), ESHRE (European Society of Human Reproduction and Embryology), HFEA (Human Fertilization and embryology Authority), etc., recommended postponing the assisted reproductive treatment and pregnancy during COVID-19 pandemic (American Society for Reproductive Medicine ASRM patient management and clinical recommendations during the coronavirus COVID-19 pandemic). Recently, with the additional data and successful mitigation strategies, ASRM and ESHRE sanctioned gradual and judicious resumption of delivery of full reproductive care (COVID-19 and Human Reproduction Joint Statement: ASRM/ESHRE/IFFS). In brief, for non-pregnant and pregnant RFI women with thrombophilia, heparin treatment can be continued even with COVID-19 infection.

2.3. Intravenous immunoglobulin G and COVID-19

Intravenous Immunoglobulin G (IVIg) is a blood product, which comprises pooled IgG from the serum of thousands of donors. IVIg is primarily used as a replacement treatment for immunodeficiencies but also indicated for autoimmune and inflammatory disorders. Studies have reported potential benefits of IVIg when applied for patients with RFI (Kwak et al., 1996), such as increased NK cell levels and cytotoxicity, elevated Th1/Th2 cell ratios, and antiphospholipid antibody syndrome (Ruiz et al., 1996; Kwak-Kim et al., 2003).

The immunomodulatory effects of IVIg are mediated through two functional domains: $F(ab')_2$, antigen-binding fragment, and Fc, crystallizable fragment. The $F(ab')_2$ fragment plays a role in neutralizing cytokine and autoantibody, scavenging of complements, killing of target cells by antibody-dependent cytotoxicity, and blocking cell-cell interactions mediated by cell-surface receptors. The Fc fragment modulates by activating and inhibiting Fcγ receptor (FcR) expression on immune cells (Schwab and Nimmerjahn, 2013). IVIg has shown efficacy in the treatment of patients with influenza (Liu et al., 2016a) and SARS (Ho et al., 2004), including SARS cases with leukopenia and thrombocytopenia (Wang et al., 2004). It is speculated that massive IVIg treatment (300～500 mg/kg/day, 5 days) may become an effective treatment to interrupt cytokine storm for severe COVID-19 cases and prevent lung injury by blocking FcR in COVID-19 with pneumonia (Fu et al., 2020; Lin et al., 2020). IVIg clinical trial (The Efficacy of Intravenous Immunoglobulin Therapy for Severe 2019-nCoV Infected Pneumonia) is on-going now for the treatment of severe COVID-19. Recently, three COVID-19 cases were reported to be treated with high-dose IVIg (25 g per day for five days) at the early stage of clinical deterioration (Cao et al., 2020). IVIg, either conventional or created from recovered patients after COVID-19 were suggested as a treatment option combined with antiviral drugs to neutralized COVID-19 (Jawhara, 2020).

IVIg is a plasma protein therapy, which might be of concern for the risk of virus contamination. During the manufacturing process, viral particles are inactivated and removed by solvent–detergent, low pH incubation, nanofiltration, or others process (Dichtelmller et al., 2009, 2011; Caballero et al., 2014). The Plasma Protein Therapeutics Associations (PPTA) has issued that the SARS-CoV-2 is not a concern for the safety of plasma protein therapies, including immunoglobulin, manufactured by PPTA member companies (New coronavirus SARS-CoV-2 and the safety margins of plasma protein therapies). With the currently available data, it is unlikely that the use of IVIg in patients with RFI will impact the chances of contracting the disease or negatively affect the clinical course in women with COVID-19 infection during pregnancy. Hence, IVIg treatment can be continued for these women with RFI during the COVID-19 pandemic.

2.4. Lymphocyte immunotherapy (LIT) during COVID-19 pandemic

LIT has been proposed for the treatment of couples with a history of RPL of unknown etiology since 1981 (Taylor and Faulk, 1981). However, in 2002, its effectiveness in treating pregnancy losses and safety got challenged, and the Food and Drug Administration (FDA), USA limited its use to a research protocol (Wong et al., 2014). Due to the controversies in its effectiveness and the outbreak of Zika virus infection, the Federal Council of Medicine in Brazil limited the use of LIT to a research project in 2014 (Cavalcante et al., 2018). Currently, LIT is utilized in China and other countries, and an updated meta-analysis with a larger number of participants have demonstrated a beneficial effect of LIT over placebo, which reinitiated the debate in its usage (Liu et al., 2016b; Cavalcante et al., 2017).

Briefly, LIT is a lymphocyte concentrate prepared from the fresh peripheral whole blood of the partner or third party. Most protocols inject the lymphocyte concentrates intradermally before and during the first trimester of pregnancy (Cavalcante et al., 2017). Therefore, there is a potential risk of infectious disease transmission with LIT. Early studies investigated infectious complications of LIT demonstrated that women submitted to LIT had a lower infection rate compared to controls (Anon, 1994; Kling et al., 2006). However, recently, HIV transmission was reported in five Chinese women who received LIT, due to multiple failures in the laboratory process, such as delay in the diagnosis of HIV in blood donors and the reuse of blood tubes during vaccine processing

(Pan et al., 2018).

The dynamics of SARS-CoV-2 during the active phase of the disease is still poorly understood. It is known that the main form of contagion of COVID-19 is through respiratory droplets, but other forms of transmission are possible. Studies evaluating biodistribution of SARS-CoV-2 revealed the highest viral detection rate in bronchoalveolar lavage fluid (14/15; 93 %), followed by sputum (72/104; 72 %), nasal swabs (5/8; 63 %), fiber bronchoscope brush biopsy (6/13; 46 %), pharyngeal swabs (126/398; 32 %), feces (44/153; 29 %), and blood (3/307; 1%) (Wang et al., 2020b), while another study detected SARS-CoV-2 in 40 % (6/15) of blood specimen (Zhu et al., 2020b). Due to possible transmission of SARS-CoV-2 via lymphocyte donors' blood and a lack of knowledge about the impact of LIT on the immune response in COVID-19 cases, we suggest the suspension of LIT for women with RFI during COVID-19 pandemic period.

2.5. Intralipid treatment

Intralipid® is a fat emulsion containing soy bean oil, glycerin and egg phospholipids. The classical use of Intralipid® infusion is parenteral nutrition in patients unable to tolerate an oral diet. Influence of soybeanoil emulsion on the immune system has been reported (Furukawa et al., 2002). Intralipid has been reported to be an effective treatment for women experiencing RFI who display elevated uterine NK cells (Coulam and Acacio, 2012). However, the level of evidence is Level III or IV because of significant heterogeneity across the studies using various methods to quantify NK cells. Measurement of the number of NK cells does not address the more significant issue of the biological activity of these cells (Roussev et al., 2007). Tests defining the function of uterine NK cells are needed. Despite these limitations, the results of published studies suggest that Intralipid® can be used successfully as a therapeutic option for the treatment of women experiencing RFI (Coulam and Acacio, 2012).

In a RIF or RPL context, Intralipid® infusion are administrated at low dose (slow perfusion of 100 ml diluted Intralipid®) when compared with the classical dose used in parental nutrition (500 ml) and once a month and not daily as it is used in parental nutrition. Immune effects of Intralipid® appear to be highly dependent on the dose (Kagawa et al., 2013). In healthy volunteers receiving soybean oil emulsion as a single dose of 500 ml, it promoted lymphocyte and neutrophil death that might enhance the susceptibility to infections (Cury-Boaventura, 2006). However, Kagawa et al., reported that Intralipid® infusion (0.5g/kg/day) did not affect plasma cytokines (IL-6, MCP) and ex vivo T cell proliferation of severely stressed patients who need hyperalimentation (Kagawa, 2013). Patients with the essential fatty acid deficiency have a susceptibility to infection (Fell et al., 2015), and intralipid® infusion is indicated for these patients. In women with RFI, Intralipid® infusion (low dose and monthly administration) has been reported to suppress immune properties of circulating NK cells and decrease the hyper-activation of uterine NK cells through the regulation of NK cell recruitment and the downregulation of endometrial IL-18 and IL-15 (Lédée et al., 2018b). There is no evidence that Intralipid® infusion increases infection susceptibility. However, in patients with Intralipid® infusion, contamination of the intravenous catheter may result in sepsis or thrombophlebitis when hypertonic solutions are concurrently infused. Based on these findings, in pregnant women with RFI and on-going Intralipid® infusion treatment, Intralipid infusion® can be continued if patients do not have an increased risk for viral exposure or infection. In pregnant women with RFI and COVID-19, Intralipid® infusion should be curtailed due to a possible immunosuppressive function of intralipid infusion (Roussev et al., 2007) unless otherwise indicated. In severe COVID-19 cases, intralipid infusion has been utilized for parenteral nutritional supplementation (Caccialanza et al., 2020).

2.6. Hydroxychloroquine

Hydroxychloroquine, a multi-purpose drug, was initially used as an antimalarial agent in 1955 (Wallace, 1996). Subsequently, anti-inflammatory and immunomodulatory properties of hydroxychloroquine have been reported (Sciascia et al., 2016). Hydroxychloroquine has been utilized for autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, antiphospholipid antibody syndrome, and RFI (Sciascia et al., 2016; Ghasemnejad-Berenji et al., 2018). It exerts its role in the immune system via multiple mechanisms. One of the mechanisms is inhibiting the Toll-like receptors (TLR-3, 7, and 9). It binds to nucleic acid in the maternal circulation, released from trophoblast as a response of placental hypoxemia, thus inhibiting an inflammatory immune response (Scharfe-Nugent et al., 2012). Another mechanism is inhibiting the release of Th1 cytokines, such as TNF-α and IFN-γ (Weber and Levitz, 2000), leading to a switch toward a Th2 predominance which promotes maternal-fetal tolerance (Wegmann et al., 1993).

Over the past few decades, hydroxychloroquine has been proposed to be utilized for certain viral infections since it inhibits cytokine production by T cells, including IL-1, IL-2, IL-6, IL-18, TNF-α, IFN-γ, and Th17 related cytokines, reduces chemokines, such as CCL2 (MCP-1) and CXCL10 (IP-10), inhibits micro-RNA expression, and decreases the synthesis of DNA, RNA, and proteins in thymocytes (Al-Bari, 2015). Since its function is mainly immune-modulatory, infection risk has been reported not to be increased in pregnant patients with hydroxychloroquine treatment, even though pregnancy and autoimmunity are associated with increased susceptibility and severity of infections (Maddur et al., 2010; Sappenfield et al., 2013), unless patients have autoimmune flare-ups or on the other types of immunosuppressant, like prednisone or cyclophosphamide (Danza and Ruiz-Irastorza, 2013).

Hydroxychloroquine is a chloroquine analog. Both drugs have been recommended by the Centers for Disease Control and FDA to treat hospitalized COVID-19 patients (Centers for Disease Control and Prevention, 2020; Colson et al., 2020; Food and Drug Administration, 2020), and recently, recommended against the use of both drugs for the treatment of COVID-19 except in a clinical trial. In vitro study has shown that both drugs inhibit SARS-CoV, SARS-CoV-2, and other coronaviruses, while the efficacy of hydroxychloroquine is relatively higher than chloroquine (Wang et al., 2020a; Yao et al., 2020).). There are on-going studies regarding the role of hydroxychloroquine in the treatment and prevention of COVID-19.

In light of the available data, urgent cessation or tapering off hydroxychloroquine and chloroquine are not necessary for pregnant women with RFI, autoimmune diseases, or rheumatic conditions due to the COVID-19 pandemic. In COVID-19 cases, the continuation of hydroxychloroquine and chloroquine treatment should be further discussed with primary care physicians since the contradictory data have been reported in regards to COVID-19.

2.7. Anti-TNF drug

TNF-α is one of the most important mediators for acute and chronic systemic inflammatory responses. It promotes the production of other cytokines and chemokines and plays an important role in severe inflammatory conditions such as septic shock (Chu, 2013). In patients with SARS, plasma TNF-α levels are moderately upregulated (Yoshikawa et al., 2009), although an in vitro study has suggested that TNF-α induction may be mediated by the shedding of ACE2, thus allowing cellular entry to coronavirus (Haga et al., 2008). In patients with COVID-19, the levels of certain cytokines, including TNF-α, IL-6, and IL-10, correlate with disease severity (Huang et al., 2020). TNF-α hyperproduction in the serum of patients with COVID-19 was a phenomenon that was not observed in patients with SARS. Therefore, it is possible that anti-TNF-α drugs, which are widely used in the clinical practice of rheumatology, may be effective in patients with COVID-19

(Fu et al., 2020). A randomized controlled trial of adalimumab (Humira®), a human monoclonal TNF-α antibody, on COVID-19 is currently underway in China (ChiCTR2000030089, http://www.chictr.org.cn/showprojen.aspx?proj=49889).

RPL has been reported to be associated with dysregulation of Th1 and Th 2 immunity to trophoblasts and excessive local TNF-α production, which prevents implantation of the embryo and triggers immunological pregnancy losses (Reid et al., 2001). Furthermore, TNF-α inhibitors such as Humira® counteract the increased production of Th1 cytokines and consequently improve the IVF and embryo transfer success rate by modulating high TNF-α levels (Winger et al., 2009). Pregnant women in the very early stages of pregnancy may benefit from the fertility-promoting effects of TNF-α inhibitor treatment. However, there are not enough clinical data to support anti-TNF treatment for RPL and RIF.

Currently, no clear evidence is present if TNF-α blocker is harmful to patients with COVID-19 (Russell et al., 2020). TNF-α inhibitors, as immunomodulators, it is reported that TNF-α inhibitors can be continued if viral symptoms by COVID-19 are mild; however, if viral symptoms worsen or high fever develops, TNF-α inhibitors should be stopped (Price et al., 2020). Therefore, TNF-α inhibitors may be continued in pregnant women with autoimmune and rheumatic conditions. In pregnant women with COVID-19, TNF-α inhibitors may be continued unless viral symptoms worsen and febrile.

2.8. Tacrolimus

Tacrolimus is a macrolide immunosuppressant, which is widely used in solid organ transplantation, especially the liver, kidney, and heart transplantation. It is a calcineurin inhibitor that suppresses the production of IL-2 and thus, inhibits the development and proliferation of T cells (Kay et al., 1989). It has been reported that tacrolimus regulates T cell subsets in patients with RIF and shifted Th1/Th2 cell ratios. Consequently, it promotes the development of a pregnancy and improving the pregnancy outcome of these patients (Nakagawa et al.). Recently, the effectiveness of immunosuppressants, such as tacrolimus in women with RFI, has been reported (Nakagawa et al., 2015; Nakagawa et al., 2017).

Recent studies have found that IL-2, IL-6, IL-7, IP-10, G-CSF, TNF-α, and IL-10 were all elevated in most severe COVID-19 patients (Huang et al., 2020), suggesting that cytokine storm may be associated with disease severity. Furthermore, regulatory T (Treg) cells were decreased in patients with severe COVID-19 (Chen et al., 2020). Regulating immune balance and reducing non-specific inflammatory responses by immunosuppressants may be beneficial for COVID-19 patients with severe inflammation (Mehta et al., 2020). Tacrolimus has inhibitory effects on Th1 immunity, which may play a critical role in patients with severe COVID-19. However, currently, there is a lack of relevant reports on the use of tacrolimus in COVID-19, but in severe patients, the use of related cytokine inhibitors and corticosteroid hormones has achieved some results (Russell et al., 2020).

Theoretically, immunosuppressants used in the post-transplant population may lead to lower immune function, and infection with the virus may cause more severe symptoms. However, an Italian study showed no increased risk of severe complications in immunosuppressed patients compared to the general population (children and adults) (D'antiga, 2020). It is speculated that due to the preexisting hyperactivated immune responses, the application of immunosuppressive agents may not worsen the outcomes. For patients with severe COVID-19 pneumonia, tacrolimus may be an attempted method (Russell et al., 2020). However, for mild patients, the use of immunosuppressive agents is not conducive to fight against viral infection. Renal transplant recipients diagnosed with non-critical COVID-19 pneumonia were successfully cured by reducing the use of immunosuppressants, including tacrolimus, mycophenolate, and prednisone and a low-dose methylprednisolone-based treatment regimen (Zhu et al., 2020a).

In pregnant women with RIF, tacrolimus can be continued with caution to avoid a viral exposure, but stopping the medication should be considered when viral symptoms present, especially with known or potential exposure for COVID-19 (Price et al., 2020).

2.9. Vitamin D

Vitamin D suppresses viral replication while it has immune regulatory effects on various immune effectors (Wu et al., 2019; Jakovac, 2020). It reduces the incidence of influenza infections and acute respiratory tract infections (Cannell et al., 2006; Martineau et al., 2017). Women with RPL have an increased prevalence of vitamin D deficiency, and vitamin D plays a major role in auto- and cellular immune responses (Ota et al., 2013, 2015). Hence, women with RPL and vitamin D deficiency may have increased susceptibility to COVID-19 infection.

Both SARS and the current COVID-19 became epidemic in wintertime when vitamin D levels were decreased since UV-B exposure for vitamin D synthesis in the skin is low (Yin and Wunderink, 2018; Zhu et al., 2020b). The mortality rate of COVID-19 was lower in countries south of latitude 35 degrees of northern hemisphere (Rhodes et al., 2020), suggesting the role of vitamin D in COVID-19. Since no specific treatment is present for COVID-19, vitamin D supplementation as adjuvant therapy for pregnant women with RFI and COVID-19 infection can be considered.

3. Conclusion

At different stages of pregnancy, the immune responses and hormonal status vary, which is closely associated with the outcome of the COVID-19 infection (Jiao, 2020). Therefore, when considering immunosuppressive and immunomodulatory treatment for pregnant women with RFI, pharmacokinetics, and pharmacodynamics of intended drugs, gestational age, and their current immune profiles should be considered not only for avoiding infection with SARS-CoV-2 but not to exacerbate COVID-19. So far, low dose prednisone, IVIg, tacrolimus, and heparin may have some beneficial effect on COVID-19. There is no clear evidence for limiting the application of anti-TNF-α agents and Intralipid® in non-infected women with RFI. Most immune therapy, except LIT can still be used with caution on a case-by-case, and the information is evolving rapidly. The majority of the current clinical data are based on the observation, and further validation is needed.

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GAMETE BIOLOGY

Improving the maturation rate of human oocytes collected ex vivo during the cryopreservation of ovarian tissue

Dmitry Nikiforov[1,2] · Cheng Junping[1,3] · Jesus Cadenas[1] · Vallari Shukla[4] · Robert Blanshard[5,6] · Susanne Elisabeth Pors[1] · Stine Gry Kristensen[1] · Kirsten Tryde Macklon[7] · Lotte Colmorn[7] · Erik Ernst[8,9] · Anne-Mette Bay-Bjørn[8] · Zeinab Ghezelayagh[1] · Yu Wakimoto[1,10] · Marie Louise Grøndahl[11] · Eva Hoffmann[4] · Claus Yding Andersen[1]

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Abstract

Purpose The aim of the present study was to improve the in vitro maturation (IVM) procedure using oocytes from surplus ovarian tissue after fertility preservation.

Methods Twenty-five patients aged 17–37 years were included in the study. Maturation was compared between oocytes collected in HEPES-buffered medium or saline, and we determined whether transport on ice prior to oocyte collection affected maturation. Two different IVM media were used that were supplemented with and without recombinant human midkine. Mature oocytes were assessed for aneuploidy using next-generation sequencing (NGS).

Results On average, 36 immature oocytes were collected from each patient (range 7–90, $N = 895$). Oocytes recovered from HEPES-buffered medium matured at a higher rate than oocytes recovered from saline (36% vs 26%, $p < 0.01$). Ovarian transportation on ice prior to the procedure negatively affected maturation compared with non-transported samples (42% vs 27%, $p < 0.01$). The addition of midkine improved maturation rate (34% vs 27%, $p < 0.05$). On average, 11 MII oocytes were obtained per patient (range 1–30). NGS of 53 MII oocytes and their first polar bodies indicated that 64% were euploid.

Conclusions The study demonstrated unexpectedly high number of immature oocytes collected from surplus ovarian tissue without any stimulation. The overall MII rate was one in three, resulting in a total number of MII oocytes that was similar to the number obtained after ovarian stimulation. If these MII oocytes prove suitable for IVF, they will provide a substantial improvement in fertility preservation for patients and advance IVM as an interesting platform for further improvements in assisted reproduction.

Keywords Human immature oocytes · Fertility preservation · Ovarian cryopreservation · In vitro maturation · Oocyte diameter

Introduction

Immature human oocytes are not widely used to treat infertility, particularly the immature oocytes collected from small antral follicles with diameters less than 6 mm [1]. In many fertility clinics, non-MII oocytes are discarded, while at some centres, immature oocytes might be employed in different in vitro maturation (IVM) protocols. Researchers have previously attempted to define IVM in terms of the follicular diameter from which oocytes are derived and other conditions, such as necessity of triggering and gonadotropin use prior to oocyte collection [2, 3]. In the present study, IVM was defined as advancement of the nuclear maturation from the germinal vesicle stage to the MII stage with the extrusion of a first polar body during culture.

Since 1991, when the first pregnancy was obtained after in vitro fertilization (IVF) using in vitro matured oocytes, the clinical use of IVM has increased, but at a slow pace. This situation might reflect the technical challenges related to the procedure and the necessity for a different timing compared with normal IVF. The introduction of fertility-preserving

Dmitry Nikiforov and Cheng Junping contributed equally to this work.

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✉ Dmitry Nikiforov
dmitry.nikiforov@regionh.dk

Extended author information available on the last page of the article

techniques in which ovarian tissue is cryopreserved, however, provided a possibility to develop a new IVM model based on oocytes obtained from very small antral follicles (SAF) [4]. In connection with ovarian tissue cryopreservation (OTC), one whole ovary is often surgically excised and the cortical tissue is frozen. The remaining medulla tissue is normally discarded, since it contains the majority of growing follicles, which are too large and their architecture is too complicated to sustain freezing. Importantly, immature oocytes collected from surplus medulla tissue can be matured in vitro without previous hormonal stimulation of the patient. This option is particularly interesting in prepubertal girls and for patients with cancer in whom ovarian stimulation is contraindicated or chemotherapy cannot be delayed [5]. Immature oocytes have been aspirated ex vivo from SAF, which normally exceed a diameter of 3–5 mm and are visible on the ovarian surface [6–8]. These oocytes may be able to increase the fertility potential in addition to the cryopreserved cortex. Three pregnancies have been reported from embryos obtained from IVM of these immature oocytes [9–11].

Small antral follicles in the medulla may span diameters ranging from less than 1 mm to approximately 10 mm, and several studies have attempted to collect all immature oocytes released from SAF during the preparation of ovarian cortical tissue [12–14]. The meiotic and developmental competence of an oocyte is normally considered associated with the diameter of the corresponding follicle [15, 16]. Therefore, oocytes from SAF, with a diameter of only a few millimetres, are likely to display reduced competence compared with follicles with diameters exceeding 10 mm [17, 18]. Nonetheless, OTC now provides new opportunities for optimizing IVM culture conditions and developing fertility treatments based on oocytes obtained from SAF [10]. Different factors might affect the maturation rates of ex vivo collected oocytes, including whether they were collected from ovaries transported on ice prior to the dissection of the tissue, the type of medium from which oocytes were collected after the dissection of ovarian tissue and the addition of chemical substances/growth factors capable of enhancing maturation rates. In a previous study, we collected an average of 11 immature oocytes from the surplus medulla of one ovary from each patient, with an average MII rate of approximately 30% [14]. Importantly, the aneuploidy rate of in vitro matured MII oocytes obtained from SAF is currently unknown.

The aim of the present study was to increase the number of immature oocytes collected from surplus tissue. Furthermore, we focused on increasing the nuclear maturation rate of oocytes from SAF, which was performed by optimizing the oocyte collection procedure and increasing their maturation rates by adding midkine—a growth factor belonging to the heparin-binding growth factor family that was reported to improve IVM in cattle [19]. The importance of midkine has been also confirmed in mice, where it appeared to be a crucial factor for follicular maturation [20]. Although the detailed mechanism of action and signalling pathways activated by midkine are currently unknown, midkine acts indirectly on cumulus cells and triggers the secretion of factors acting on the oocyte [21]. In addition, the morphometric characteristics of oocytes, including the oolemma diameter and zona pellucida thickness, were evaluated. The importance of cumulus cells surrounding the oocyte for sustaining maturation and the aneuploidy rate of the in vitro matured MII oocytes were assessed as well.

Materials and methods

Study design and patients

In a prospective cohort study conducted from December 2017 to March 2018, the ovarian cortex of 26 consecutive patients (mean age 28 years, range 17–37) was cryopreserved at the Laboratory of Reproductive Biology, University Hospital of Copenhagen, Denmark. None of the patients had received any type of exogenous gonadotropins or chemo-/radiotherapy prior to the procedure, and patients' characteristics are presented in supplementary Table 1. Petri dishes in which the cortical tissue was prepared and containing the remaining ovarian medulla were examined for the presence of immature oocytes. Twenty-five patients were included in the study based on following inclusion criteria: (1) two ovaries were present, and (2) OTC was indicated. One patient was excluded prior to the collection of oocytes due to a diagnosis of imminent premature ovarian insufficiency and thereby having a priori fewer oocytes than an average patient.

The ethical committee of the municipalities of Capital Region of Denmark approved the project (H-2-2011-044), and all the patients signed an informed consent form prior to the surgical procedure, in which they consented to donate their surplus ovarian tissue for research. The assessment of aneuploidy and General Data Protection Regulation (GDPR) were approved by the ethics committee and data protection agency.

Collection of immature oocytes

One entire ovary from each patient was excised surgically through a unilateral oophorectomy and delivered to the laboratory from the local or collaborating hospitals. Ovaries were delivered in either α-MEM (Invitrogen, UK) at 37 °C within 10 min (when the surgical procedure was performed at the local hospital) or in IVF flushing medium (Origio A/S, Denmark) on ice within 2–5 h (when the surgical procedure performed at collaborating hospitals) [22]. Upon arrival, each ovary was cut into two halves and the cortex was isolated using sterile scalpels and forceps, as previously described [23, 24].

The entire procedure for cortex preparation was performed in a flow hood at room temperature using dissection medium at ambient temperature or pre-cooled (4 °C), which corresponded to the temperature during transportation. Since the beginning of the OTC program at our facility, saline was used as the medium to prepare cortical tissue prior to freezing. We compared saline with HEPES-buffered HTF medium (Invitrogen, UK) to test whether results of IVM may be improved by using a medium with an optimal pH during collection.

Immediately after tissue preparation, all petri dishes containing surplus tissue were meticulously examined for immature oocytes. Pieces of medulla tissue were finely dissected once with a tissue chopper (McIlwain Tissue Chopper, Campden Instruments Ltd., UK) to optimize the harvest of immature eggs. Settings for the chopper ensured that tissue was cut into pieces of approximately 2 mm^3. Thereafter, pieces of the tissue were rinsed with saline or the HEPES-buffered HTF medium and were also examined for the presence of oocytes. Collection was performed within a period of up to 1 h by two operators simultaneously using a stereomicroscope (Leica MZ12, Germany) on a 37 °C heating table in a flow hood, and petri dishes were evaluated twice by both operators. Immature oocytes were collected in the holding medium consisting of McCoy's 5α-medium containing 25 mM HEPES (Invitrogen, UK) and supplemented with 5 mg/ml human serum albumin (HSA–CSL Behring 20%, Germany), 2 mM Glutamax (Invitrogen, UK), 0.05 mg/ml penicillin/streptomycin (Invitrogen, UK), 10 μg/ml insulin, 5.5 μg/ml transferrin and 6.7 ng/ml selenium (ITS, Invitrogen, UK) [13, 14]. The immature oocytes (Fig. 1a) were grouped as either cumulus-oocyte complexes (COCs) or naked oocytes (NOs) (Fig. 1b). COCs were further grouped according to the size of the surrounding cumulus mass: small COCs with 3–10 layers of cumulus cells (Fig. 1c) and large COSs with more than 10 layers of cells (Fig. 1d). NOs were either surrounded by small clumps of cumulus cells or completely naked. Oocytes or COCs were classified as having a healthy appearance if they had the following characteristics: an absence of dark coloured cumulus cells, a spherically shaped cytoplasm and integral oolemma. Only oocytes with a healthy appearance were selected for further culture, which corresponded to approximately 95% of all collected oocytes, while oocytes with dark cumulus cells, a dark/irregular ooplasm or mechanical damage were excluded.

In vitro maturation and evaluation parameters

The MediCult IVM system (Origio A/S, Denmark) was used as previously described [13, 14], with modifications. Briefly, IVM medium was supplemented with 75 mIU/ml rhFSH (Rekovelle, Ferring, Denmark), 100 mIU/ml rhLH (Luveris, Merck Serono, Germany) and 10 mg/ml HSA. After the collection and subdivision of oocytes into the three groups as described above, oocytes from each group were equally divided between the control medium (IVM medium) or experimental group (IVM medium supplemented with 1 μg/ml recombinant human midkine (SRP3114, Sigma, Germany)). Immature oocytes were washed with the respective IVM culture medium, placed individually into 25 μl drops of media and covered with liquid paraffin (Origio A/S, Denmark). The culture dishes were pre-equilibrated overnight prior to oocyte collection in an atmosphere containing 5% CO_2 and ambient O_2 at 37 °C, conditions that were maintained for the subsequent 44–48 h culture period. The expansion of the cumulus in large and small cumulus complexes was evaluated after 24 and 44–48 h under the stereomicroscope. After 44–48 h, all oocytes were denudated by repeated gentle aspiration using denudation glass pipettes with a diameter of 130–133 μm (Vitrolife, Sweden) and data were recorded for each oocyte separately. Denuded oocytes were examined under inverted microscope (Carl Zeiss Axiovert 135, Germany) equipped with Hoffman interference optics with × 20 objective lenses and classified into the germinal vesicle (GV) stage, metaphase-I (MI) or metaphase-II (MII). GV stage oocytes were identified based on their distinct germinal vesicle, refractive nucleolus, darkened centre and granular ooplasm (Fig. 1g). MI oocytes were identified as cells without either a GV or polar body (Fig. 1f), and MII oocytes were identified by the presence of the first polar body (Fig. 1e). Oocytes that remained immature (GV or MI) after 44–48 h were regarded as incapable of maturation.

Photos of all oocytes were captured with the clear oolemma in focus using the Carl Zeiss microscope and the AxioVision software (SE64 Rel.4.9.1). Data were stored for subsequent analyses, which included measurements of the oocyte diameter (ooplasm diameter) and zona pellucida thickness. The values were obtained by calculating the average values of the parameters measured twice perpendicularly for each specific oocyte (Fig. 2).

Oocyte vitrification

All MII oocytes were vitrified after the IVM procedure in accordance with the protocol described by Lucena et al. [25], with modifications. Briefly, oocytes were washed with three drops of basic solution (BS), which contained 199 medium supplemented with 10% HSA. Then, the last third drop of BS was merged with the first drop of equilibration solution (ES) containing 7.5% (v/v) of dimethyl sulfoxide (DMSO, Sigma, Germany) and 7.5% (v/v) of ethylene glycol (EG, Merck, Germany) in the BS for 3 min. Then, the previous drop was merged with the second ES drop for another 3 min, and finally oocytes were moved into the third ES drop for 6 min. During this process, three drops of vitrification solution (VS) were prepared that contained 15% (v/v) EG, 15% (v/v) DMSO, 0.5 M sucrose (Sigma, Germany) and 10% HSA. After equilibration, oocytes were transferred to

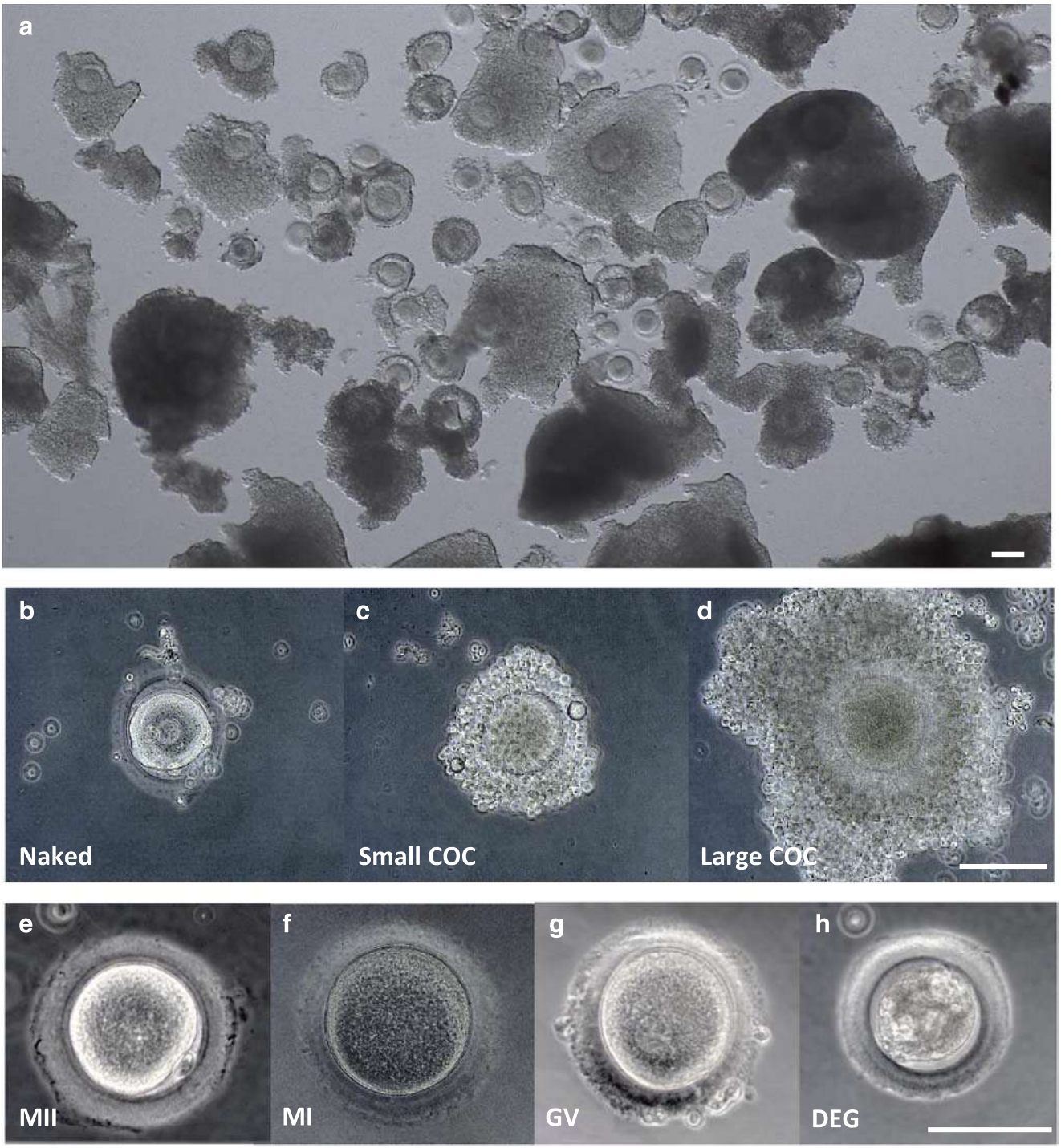


Fig. 1 **a** Immature oocytes collected from surplus medulla during the preparation of ovarian tissue for cryopreservation. Scale bar = 100 μm. **b**–**d** Different categories of immature oocytes: naked immature oocyte (**b**), small COC (**c**) and large COC (**d**). Scale bar = 100 μm. **e**–**h** Different stages of oocytes after 2 days of in vitro maturation; MII (**e**), MI (**f**), GV (**g**) and degenerated oocyte (**h**). Scale bar = 100 μm. DEG degenerated, COC cumulus-oocyte complex

each of the three VS drops for 20 s, loaded on the Cryolock carrier device (Biotech, USA), and then plunged into liquid nitrogen. The total time in VS was up to 1 min. The volume of all drops was 30 μl and the procedure was performed at room temperature.

Warming of oocytes after vitrification

Some vitrified oocytes were selected for warming with a subsequent first polar body biopsy and genetic analysis, as described below. Because we were unable to perform this

procedure for all vitrified oocytes, we selected oocytes from patients of different ages to cover the range of women included in the study.

One millilitre of thawing solution (TS, which is BS supplemented with 1 M sucrose) was pre-warmed in a central well dish at 37 °C [25]. When the carrier was removed from the liquid nitrogen, the tip of the carrier was quickly immersed in TS for 1 min. Then, oocytes were transferred to 1 ml of dilution solution (DS) containing 0.5 M sucrose in the BS for 3 min and finally transferred to 1 ml of BS for 5 min (the last step was repeated twice). DS and BS were pre-warmed at room temperature for 30 min, and the entire procedure was performed at room temperature.

First polar body biopsy and tubing

Vitrified/warmed oocytes were maintained in a cleavage medium (Origio A/S, Denmark) in individual drops under oil in an incubator with an atmosphere of 5% CO_2 and ambient O_2 at 37 °C for at least 1 h prior to biopsy. Individual oocytes were fixed in a micro-manipulation system (Narishige, Japan) with a holding pipet. A laser (Research Instruments, UK) was employed to dissect the zona pellucida. The polar body was aspirated such that it did not entirely enter the biopsy pipet (Origio A/S, Denmark) and was released in the same drop as the oocyte. Then, the zona pellucida was removed with the further assistance of the laser and biopsy pipet. The biopsied first polar body and the oocyte were placed in separate DNA/RNA-free test tubes with the stripper pipet (Origio A/S, Denmark) in in a volume of approximately 5 µl. Immediately thereafter, test tubes were flash-frozen in liquid nitrogen and stored at − 80 °C prior to further procedures.

Whole genome amplification and next-generation sequencing

To amplify the DNA from the single MII oocyte, we used the SureMDA (Illumina Inc.) according to the manufacturer's instructions (User guide: 15052710, Revision B; Illumina Inc.) [26]. Briefly working in a pre-PCR area, we centrifuged the tube at 300×*g* for 3 min., immediately followed by lysis. After collecting the lysis buffer and single oocyte sample, we centrifuged the sample for 5 s at 300×*g*, followed by the addition of the stop solution and amplification reagents. The DNA was amplified at 30 °C using a reduced amplification time of 2 h. We assessed the quality of the SureMDA product using gel electrophoresis and generated sequencing libraries using a custom Nextera®-based library preparation workflow (Illumina Inc.). MDA products were quantified (Quant-IT™ dsDNA High-Sensitivity Assay Kit, Thermo Fisher Scientific Inc.), and 100 ng were tagmented according to the Nextera® DNA Library Prep Reference Guide (Part: 15027987 v01; Illumina Inc.). Tagmented DNA was cleaned with 1.8× Solid Phase Reversible Immobilization (SPRI) (Part: 15041032; Illumina Inc.) prior to a limited number of cycles of PCR amplification for the addition of indexed barcodes using the following modified program: 72 °C for 3 min; 98 °C for 3 min; 10 cycles of 98 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s; 72 °C for 5 min; and a 10 °C hold. Amplified libraries were cleaned with 1× SPRI and normalized according to the

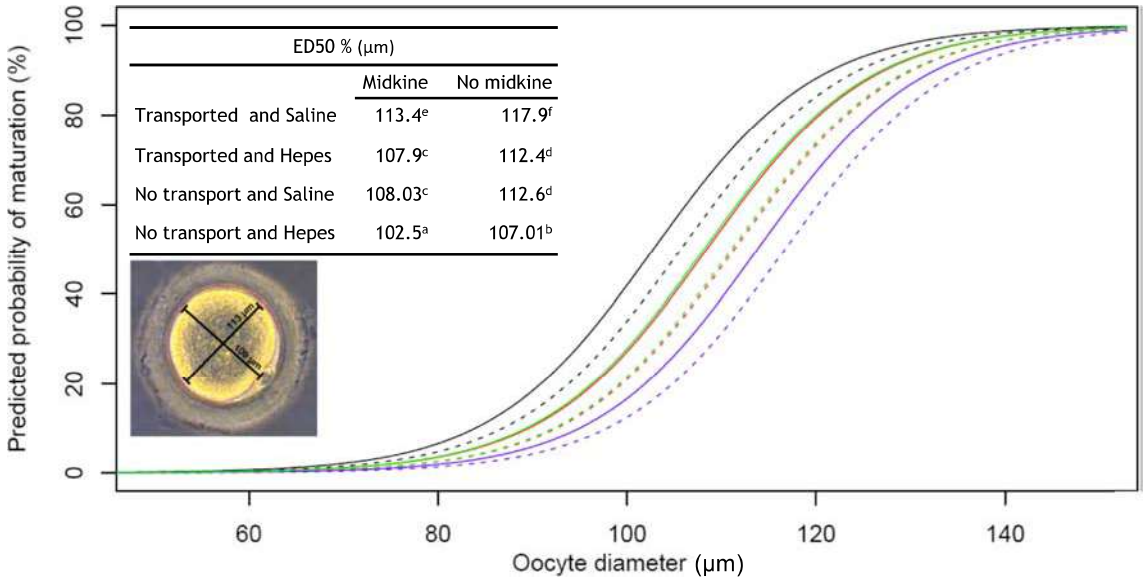


Fig. 2 Predicted probability of maturation based on oocyte diameter for combinations of handling and treatments. Punctuated lines: oocytes cultured without midkine. Full lines: oocytes cultured with midkine. Black, HEPES/no transport. Red, saline/no transport. Green, HEPES/transport. Blue, saline/transport. Left bellow, illustration for the measurement of oocyte diameter. Left top, equivalent diameter of which 50% (ED50%) of oocytes collected from surplus ovarian tissue will mature to MII stage. Values with different letters are significantly different ($p < 0.05$)

VeriSeq® PGS Library Prep Reference Guide (Part: 15052877 v03; Illumina Inc.). Normalized libraries were pooled for 24-plex sequencing using a Miseq System using Reagent Kit V3 consumables (2 × 36 bp; Part: MS-102-3001; Illumina Inc.). The data were analysed for copy number variations by extracting the .bam files and importing them into BlueFuse Multi® software (Illumina Inc.) with a custom, MDA-specific reference database. The analysis pipeline was validated using single cells and genomic DNA from four cell lines obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research, NJ, USA, two of which contained structural abnormalities: GM00526 (47, XY, + 13), GM04927 (47, XY, + 21), GM50121 (46, XY, − 18p 15.5 Mb, + 18q 59.3 Mb) and GM10985 (46, XX, − 3p 10.3 Mb). Cell lines were cultured according to the supplier's recommendations, and single cells were isolated manually using a previously described method [26]. The concordance per chromosome was greater than 99% for single cells ($n = 2208$).

Study design

We planned to include 25 consecutive ovaries surgically excised for OTC to obtain an average estimate of the number of oocytes that is possible to collect from surplus medulla tissue from one entire ovary. This approach prevented an equal distribution of ovaries that were delivered from the local hospital and ovaries that were transported on ice, because we were unable to control for the admission of patients. The oocytes collected from each ovary were allocated in equal numbers representing the three different groups of COCs to either one of the two media tested for IVM (i.e. with and without the addition of midkine). In order to not prolong the period of preparation for the cortical tissue, we decided to test the effect of the preparation medium on one ovary at a time and evaluated 12 and 13 ovaries, respectively in the two types of media. An overview of the patient's material for IVM of oocytes obtained from surplus medulla tissue after the dissection of ovarian tissue is shown in Fig. 3. Aneuploidy studies were performed on a randomly selected group of oocytes that sustained the MII transition and remained intact after warming.

Statistical analysis

The frequency of maturation of immature oocytes to the MII stage was analysed using a single multivariate mixed logistic regression model with maturation to MII (yes/no) as the outcome and transport (yes/no), collection medium (saline/HEPES-buffer), culture medium (with midkine/without midkine), COC size (naked oocytes, small COCs and large COCs) and oocyte diameter (linear effect) as explanatory variables. The patient effect was included as a random intercept. A cut-off value for the oocyte diameter where a 50% maturation rate was achieved (MII = yes) was determined using the predicted values obtained from the mixed logistic regression analysis. The distribution of COCs between treatments was analysed using the chi-square test. Diameters were analysed with one-way analysis of variance (ANOVA) in which the patient effect was included as a random intercept. All analyses were performed using R version 3.4.3. All p values less than 0.05 were regarded as significant.

Results

The main outcome measure was the number and frequency of MII oocytes obtained after IVM. Secondary outcome measures included whether the maturation rate was affected by the following parameters: (1) the originating hospital (transported vs non-transported ovaries), (2) the type of medium used for the dissection of ovarian tissue (saline vs HEPES-buffered medium) and (3) the addition of midkine to the culture medium (IVM medium supplemented with or without midkine). Furthermore, euploidy/aneuploidy rates were calculated for IVM MII oocytes after the biopsy of the first polar body.

Immature oocytes

Eight hundred ninety-five immature oocytes from 25 ovaries (25 patients) were collected from the surplus tissue that remained after the isolation of the ovarian cortex for cryopreservation (Fig. 1). Seven ovaries were obtained from the local hospital with no transportation on ice and 18 ovaries were delivered from collaborating hospitals on ice (Fig. 3). The average number of oocytes collected per patient was 36 ± 5 (mean ± SEM, range 7–90). No significant correlation was observed between the number of oocytes collected and age of the patient ($R^2 = 0.03$) or the number of oocytes collected and ovarian volume ($R^2 = 0.26$). The distribution of oocytes among groups with different experimental conditions is presented in Table 1.

Maturation rate

The overall IVM rate was 31% (274 MII oocytes of 895 total oocytes), but a considerable variability was observed (range 6–55%, supplementary Table 1). The IVM rate for patients aged less than 20 years ($n = 2$, 40%) did not differ from patients aged 20–30 years ($n = 14$, 29%) and greater than 30 years ($n = 9$, 31%; $p > 0.1$).

The size of the cumulus cell mass was significantly associated with the maturation rate: oocytes surrounded by a large cumulus cell mass were more likely to mature (52%) than oocytes enclosed in small cumulus complexes

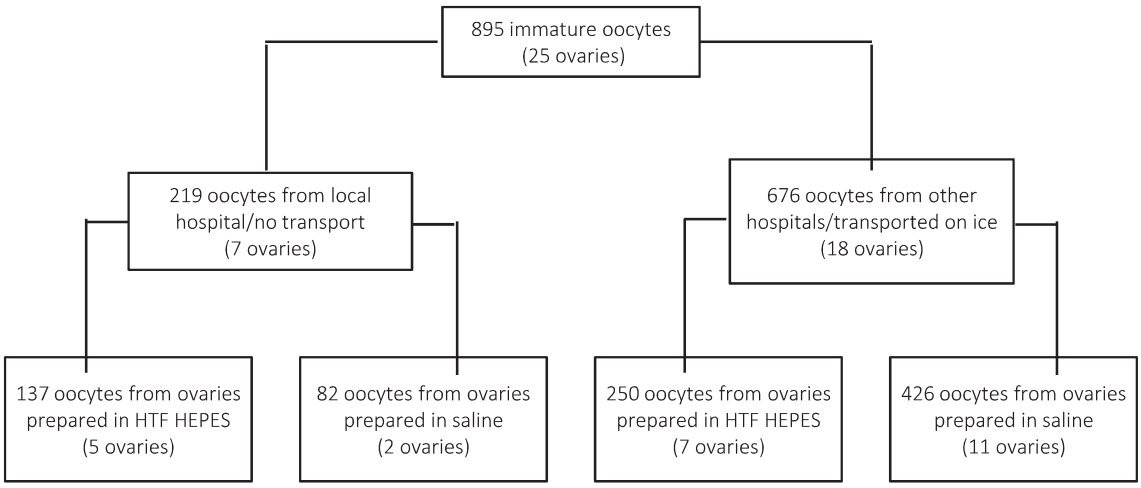


Fig. 3 Overview of patients material for in vitro maturation of oocytes obtained from surplus medulla after the dissection of ovarian tissue. A total of 25 ovaries from 25 patients were included in the study. Seven patients were operated at the local hospital (no transport of ovaries on ice was needed), while other 18 patients were operated at collaborating hospitals and their ovaries were delivered on ice. For the first 13 consecutive patients, saline was used as a medium for dissection and preparation of cortex, while for remaining 12 patients, the HEPES buffered HTF medium was used

(26%) or NOs (12%) ($p < 0.001$, Table 2). The number of large COCs was similar, regardless of whether the ovaries were transported, and the maturation rate did not differ (33% vs 40%, $p > 0.05$). Furthermore, for large COCs, the handling medium did not affect the MII rate (34% vs 35%, $p > 0.05$).

Effects of transportation, media and midkine supplementation on the oocyte maturation rate

Oocytes collected from ovaries originating from patients who received an operation at the local hospital (oocytes = 219, ovaries = 7) matured at a significantly higher rate (42%) than oocytes collected from ovaries transported on ice (oocytes = 676, ovaries = 18) prior to dissection (27%; $p = 0.0011$, Table 3).

The maturation rate for oocytes collected from ovaries dissected in HEPES-buffered medium (oocytes = 387, ovaries = 12) was significantly higher (36%) than oocytes collected from saline (oocytes = 508, ovaries = 13), which matured at a rate of 26% ($p = 0.0001$) (Table 3).

The addition of midkine to the culture media significantly increased the maturation rate. Oocytes cultured in the presence of midkine (oocytes = 438) matured at a rate of 34%, whereas the control group (oocytes = 457) matured at a rate of 27% (Table 3, $p = 0.04$).

Consequently, oocytes collected in HEPES-buffered medium from the local hospital that were cultured in the presence of midkine matured at a significantly higher rate (49%) than oocytes collected from transported ovaries in saline and cultured without midkine (23%) ($p < 0.001$) (Table 1).

Oocyte diameter

The average diameter was 110 ± 7 μm for of MII oocytes (n = 274), 105 ± 9 μm for MI stage cells (n = 223), 96 ± 12 μm for GV stage cells (n = 117) and 88 ± 19 μm for degenerated oocytes (n = 281). The differences in diameters between different maturation stages were all significant ($p < 0.001$). In contrast, the thickness of the zona pellucida was not significantly different among the groups of oocytes after 44–48 h of IVM culture (20 ± 4, 21 ± 5, 20 ± 5 and 21 ± 4 μm, respectively) ($p > 0.1$) (Table 4).

We developed a linear model to understand the effects of factors such as the collection site, collection medium and culture medium on the diameter. The equivalent diameter of the 50% MII transition (ED50% MII transition value, i.e. the diameter at which 50% of the oocytes will mature to the MII stage) under different conditions was calculated based on a linear model of diameters for all oocytes included in the study (Fig. 2). Clearly, oocytes that originated from ovaries delivered from the local hospital, dissected in HEPES-buffered medium and cultured with midkine showed the lowest ED50% value (102.5 μm) compared with any other group (Fig. 2). In contrast, the oocytes that originated from ovaries that were transported, dissected in saline and cultured without midkine had the highest ED50% value (117.9 μm). The diameter of the oocytes did not change during the culture period (data not shown).

Chromosomal error rates among MII oocytes after IVM of GV oocytes obtained from SAF

Next-generation sequencing was used to assess the aneuploidy rate of the mature MII oocytes after IVM (Fig. 4). For this

Table 1 The distribution of oocytes, number of mature oocytes and maturation rate for all different experimental groups: oocytes originated from transported (TRANSPORT) and non-transported (LOCAL) ovaries, collected from saline or HEPES buffered HTF medium, oocytes matured in the medium with (MK) and without (No MK) addition of midkine

	Saline						HEPES					
	No MK			MK			No MK			MK		
	Total (*n*)	MII (*n*)	MII rate (%)	Total (*n*)	MII (*n*)	MII rate (%)	Total (*n*)	MII (*n*)	MII rate (%)	Total (*n*)	MII (*n*)	MII rate (%)
Local	42	9	21	40	16	40	70	34	49	67	33	49
Transport	217	51	23	209	58	28	128	21	24	122	42	34

experiment, we removed the first polar body and isolated the MII oocyte, which was subjected to whole-genome amplification. Seventy oocytes from 13 patients were warmed for analysis. After warming, 53 oocytes were identified as alive and of good quality based on their morphology (survival rate of 76%) and were subsequently biopsied. An analysis of the shallow next-generation sequencing profiles (0.01×) revealed that 36% ± 3.3% of the 53 oocytes sampled from 13 patients aged 19 to 33 years were aneuploid (Fig. 4b and c). Thus, we estimated that approximately two-thirds of the MII oocytes obtained after IVM of GV oocytes from SAF of women aged less than 35 years were euploid with a normal chromosomal constellation. The distribution of the oocytes among the different parameters is presented in supplementary Table 2.

Discussion

To our knowledge, the present study is the largest to examine immature oocytes isolated from surplus medulla tissue and thus from SAF. This is a new source of human oocytes deriving from antral follicles with a very small diameter that has not been studied in great detail. However, this material provides for the first time an opportunity to explore how and if such oocytes can be matured in vitro employing new growth factors and culture conditions to potentially form a basis for a new platform for IVM and clinical applications in the future. The main finding was that a large number of immature oocytes was obtained from the surplus ovarian medulla tissue of each patient, which is the tissue that previously was discarded. Thus, this source of human oocytes has not previously been studied in great detail. On average, 36 immature oocytes were obtained from one ovary of an unselected group of patients, with a few women providing approximately 100 immature oocytes each, thereby increasing the average number. The IVM procedure yielded an average of 11 MII oocytes, with one patient producing 30. The ability to obtain such a high number of oocytes from a single ovary is a result of a meticulous search and recovery process. This procedure mainly included oocytes from SAF with a small diameter, which are rarely visible with the naked eye and most probably having diameters well below 2–3 mm. In previous IVM studies performed in our laboratory [13, 14], the average number of COCs collected per patient was 10 to 11. In the present study, we have tripled the average number of immature oocytes collected to 36 per patient. This improvement was possible partially due to the preparation of the surplus tissue, which was cut into small (approximately 2 mm^3) pieces with a tissue chopper prior to oocyte collection. During this procedure, more oocytes are released from SAF compared with previous studies where the tissue was minced into many small fragments with a scalpel by hand. In the present study, two experienced operators collected the oocytes and double checked one another, while only one operator performed the collection procedure in the previous studies.

The genomic analysis of MII oocytes revealed that 64% were euploid, which is 9% lower than estimates for MII oocytes retrieved from large and mature follicles after ovarian

Table 2 Maturation and cumulus expansion for immature oocytes in relation to the initial morphology

Category of oocyte	No.	No. MII oocytes (day 2)	Maturation rate (%)	Day 1		Day 2	
				No. COCs expandedcumulus	Cumulus expansion rate (%)	No. COCs expandedcumulus	Cumulus expansionrate (%)
Naked oocytes	212	25	12	–	–	–	–
Small COCs	399	102	26	54	14	72	18
Large COCs	284	147	52	56	20	155	55
Total	895	274	31	110	16	227	33

Table 3 Maturation rate in relation to transport, collection and culture media (mean ± SEM (range)). All *p* values present the difference in maturation rate estimated in a multivariate model using the parameters: transport, collection media, culture media, COC size and diameter as explanatory variables. Odds ratio from the model used to predict maturation rate shown in Fig. 2 for the different treatments

Group		Number of oocytes	Oocytes/ patient	MII oocytes/patient	Maturation rate (%)	*p* value	Odds ratio (95% CI)
Transport	Transported	676	38 ± 6 (9–90)	10 ± ±2 (1–27)	27 ± 2 (6–38)	0.0011	0.5 (0.4–0.8)
	Local	219	31 ± 7 (7–56)	13 ± 4 (2–30)	42 ± 5 (23–55)		1 (ref)
Collection media	HEPES	387	33 ± 8 (7–90)	12 ± 3 (2–30)	36 ± 4 (13–55)	0.0001	1 (ref)
	Saline	508	39 ± 5 (13–88)	10 ± 2 (1–27)	26 ± 3 (6–39)		0.5 (0.4–0.7)
Culture media	Midkine	438	20 ± 3 (3–65)	7 ± 1 (1–23)	34 ± 3 (11–58)	0.04	1.4 (1.0–2.0)
	Control	457	18 ± 4 (4–90)	6 ± 1 (1–24)	27 ± 3 (0–55)		1 (ref)
Total		895	36 ± 5 (7–90)	11 ± 2 (1–30)	31 ± 2 (6–55)		

Ref reference value for the odds-ratio

stimulation [27]. However, the wide range of aneuploidy rates reported for women aged less than 35 years prevents a ready comparison with our data, although the aneuploidy rate appears to be increased [28, 29]. Despite the slight increase in the aneuploidy rate, the large number of oocytes retrieved from the ovarian tissue provides MII oocytes in numbers that are comparable with 1 cycle of ovarian stimulation. On average, eight euploid MII oocytes were recovered and potentially used for fertility treatment. The potential for the embryonic development of oocytes matured in vitro is unknown but likely to be lower than oocytes matured in vivo during stimulated cycles, and the efficiency of embryo formation and further development remains to be determined. Currently, the Danish health authorities consider IVM an experimental procedure and additional ethical approval is needed before MII oocytes obtained after IVM are fertilized and the chromosomal constitution of embryos is assessed locally. This requirement for additional approval is an obvious limitation of the present study since the MII oocytes originating from SAF are likely to possess an attenuated developmental competence. Furthermore, the aneuploidy rate in the group of oocytes exposed to midkine was similar to oocytes that were not exposed to midkine, while the MII rate was significantly increased. Therefore, this study is the first to confirm that oocytes obtained from small follicles mature in vitro to the MII stage, most of them are euploid and the addition of human recombinant midkine to the culture medium significantly increases the maturation rate of these oocytes.

The isolation of immature oocytes from surplus tissue in connection with fertility preservation has previously been performed with highly variable recovery of oocytes ranging from 1 to 15 cells [7, 9, 10, 11, 12, 30, 31]. Some of these studies excluded naked oocytes, which may partially explain the lower numbers of immature oocytes collected than in the present study. Some studies did not mention specific details on whether naked oocytes were included for IVM. Interestingly, preliminary NGS results suggest that MII oocytes derived from naked oocytes at collection are likely to be euploid, despite the limited number of cells included. In our study, 3 of 3 oocytes derived from naked cells were normal (supplementary Table 2). Therefore, despite the low maturation rate of naked oocytes (12%), we considered every opportunity to improve the reproductive outcome for the patient.

IVM studies of prepubertal girls [4, 7, 32] revealed an attenuation of the average maturation rate compared with adults [8, 30, 33]. The maturation rate in our study was similar to or lower than other studies including large groups of patients [4, 10, 12, 30, 32, 34, 35], but we included oocytes from

Table 4 Oocyte diameter and zona pellucida thickness in relation to the maturation stage after 48 h of IVM. Data are mean ± SEM (range)

Oocyte stage	No. oocytes	Diameter of oocyte (μm) (mean ±SEM)	ZP thickness (μm) (mean ±SEM))
MII	274	110 ± 7 (83–148)	20 ± 4 (10–33)
MI	223	105 ± 9 (75–153)	21 ± 5 (10–51)
GV	117	96 ± 12 (49–118)	20 ± 5 (8–32)
DEG	281	88 ± 19 (37–142)	21 ± 4 (8–38)

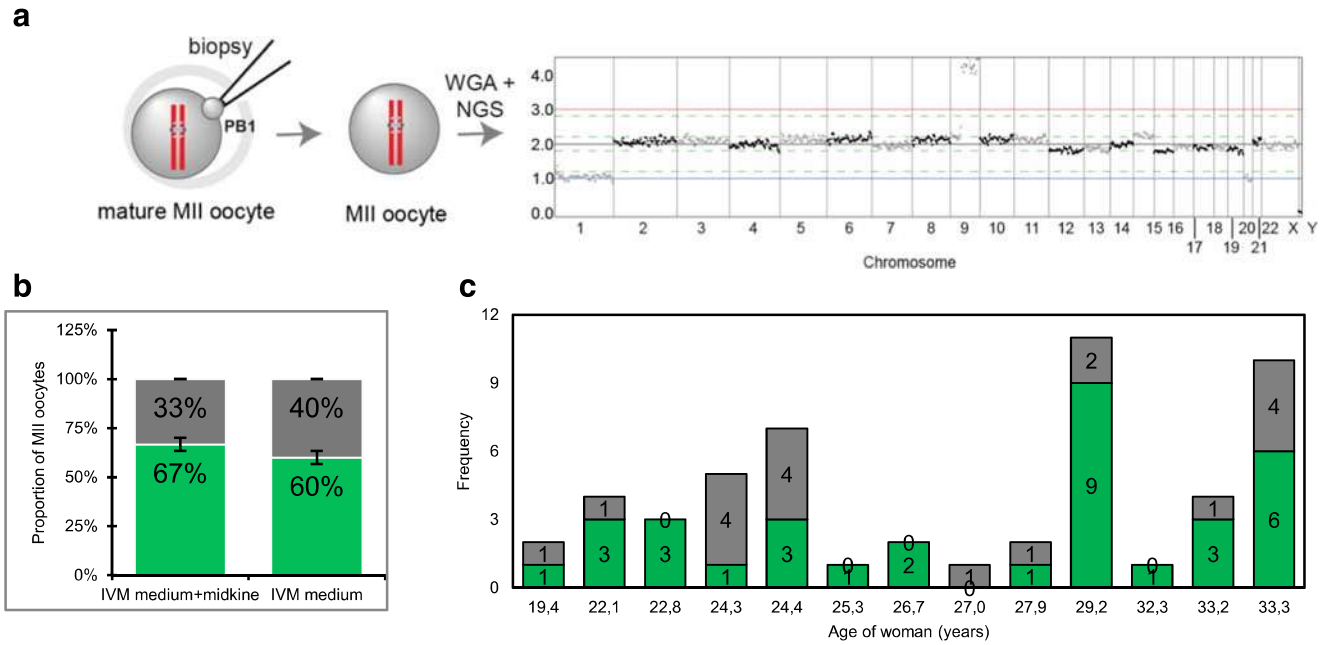


Fig. 4 Aneuploidy rates in oocytes from small antral follicles after in vitro maturation (IVM). **a** The first polar body was removed and the MII oocyte was used as template for single-cell wholegenome amplification (WGA) and shallow next-generation sequencing (NGS, 0.01×). **b** Aneuploidy rates in oocytes matured in the IVM protocol with or without midkine 1 mg/ml. The fraction of MII oocytes that were euploid shown in green, whereas the fraction that were aneuploidy is represented by grey. Error bars represent standard error. In total, 53 MII oocytes were assessed after IVM: 33 oocytes in the IVM medium + midkine group and 20 oocytes in the IVM medium group. It was found that 22 and 12 oocytes in these groups respectively were euploid. **c** The frequencies of euploid (green) and aneuploid (grey) MII oocytes per participant, ordered according to chronological age. The number on the green or grey part of the bar is the number of oocytes analysed

all diameters of follicles and the number of MII oocytes obtained per patient after IVM is the important measure. Previous studies [4, 10, 12, 30, 32, 34, 35] reported as maximum of 5 MII oocytes per patient, and the value more than doubled in the present study to an average of 11 MII oocytes.

Based on our findings, optimizing the collection procedure and using a more appropriate HTF HEPES-buffered media during the preparation of cortical tissue improves the resumption of meiosis. Immature oocytes that were cooled to approximately zero degrees during transportation for 2–5 h showed a significant reduction in their capacity to resume meiosis compared with oocytes obtained from the local hospital. We were unable to determine whether the cause is a reduced temperature or increased apoptosis in the follicles. Nevertheless, the number of oocytes recovered justifies their collection and putative use for fertility preservation. Some collected oocytes were present in very large COCs with many layers of somatic cells, which is a characteristic that is seldom observed in normal IVF. Surprisingly, the category of large COCs displayed the highest maturation rate (52%). We hypothesise that many cumulus cells are needed to sustain oocyte development, but we are currently unable to pinpoint the specific follicle diameter from which these large COCs derive. Collectively, immature oocytes obtained from the surplus medulla tissue may increase fertility opportunities for women receiving OTC to a larger extent than previously assumed.

The presence of the growth factor midkine significantly increased the maturation rate. Midkine was previously reported to affect maturation of immature bovine oocytes [19] by acting indirectly via granulosa cells and/or cumulus cells, as no effect was observed on naked oocytes [21]. An effect on somatic cells surrounding oocytes is supported by the findings from the present study, where a beneficial effect was only observed on large and small COCs. Furthermore, midkine has been shown to reduce the apoptosis of bovine cumulus cells during IVM [21]. Midkine is present in human granulosa and theca cells of the preovulatory follicles [36]. Therefore, based on our results and published data, we postulate that midkine is suitable for use in human IVM, but further studies are needed to verify this hypothesis. Taken together, the present study supports previous investigations of the ability of midkine to promote IVM and showed for the first time that recombinant human midkine significantly increased meiotic progression to the MII stage of human immature oocytes during a 44–48 h culture period. We recommend the potential inclusion of midkine in IVM medium in routine practice to increase the maturation rate of human oocytes.

The classification of human oocytes is not yet well defined, as oocytes will often merely be classified either as mature (metaphase II stage) or immature (germinal vesicle or metaphase I stage) cells [37]. However, immature oocytes now require further classification, as they are collected from

follicles with a range of diameters and their meiotic competence varies. The average diameter of IVM MII oocytes from SAF (110 ± 7 μm) is similar to the mean diameter of oocytes retrieved at IVF clinics after ovarian stimulation (112 ± 3 μm) [38]. At the same time, the diameter of oocytes originating from SAF appears to reflect their meiotic capacity: the larger the diameter, the better chance of supporting the MII transition. This observation confirms and extends an earlier study that obtained a similar result: 75% of 49 oocytes collected from ovaries excised from six women underwent the MII transition when the diameter exceeded 105 μm, while only 25% matured when the diameter was smaller [12]. Different factors have been employed to improve the conditions for oocyte collection and maturation, as reflected in the equivalent diameter of the 50% MII transition. The ED50% value is significantly reduced when ovaries from a local hospital were compared with ovaries transported at zero degrees. The same result was obtained when we compared HEPES-buffered media with saline and when midkine was added to the culture medium. Collectively, the diameter of immature oocytes from SAF reflects their meiotic capacity, and the ED50% MII transition is a useful parameter that can be employed to optimize conditions for human immature oocytes, including collection and culture. According to a previous study by Sánchez et al. [39], oocytes with a diameter less than 109 μm have a higher frequency of dispersed chromatin. Consistent with these results, the oocyte diameter is important as a first parameter to subdivide or categorize the large heterogeneous group of human immature oocytes from follicles with different diameters. In this context, the MII rate of oocytes with a diameter of less than 100 μm was as modest as 3% (9 MII oocytes of 274 oocytes). We, therefore, recommend that immature oocytes should be distinguished based on the diameter and propose two categories based on their developmental competence: immature human oocytes with diameters less than or greater than 100 μm. However, further investigations of the developmental competence based on these findings are warranted.

Although the collection of immature oocytes was intended to increase the potential fertility of women who had one ovary excised for OTC, the unexpectedly high number of immature oocytes collected may provide an opportunity to justify the procedure itself. Women in whom the transplantation of ovarian tissue is not recommended because of a risk of reintroducing malignant cells may benefit from this procedure, including women with ovarian cancer, borderline ovarian cancer, granulosa cell tumours or cancers with a high risk of infiltration of malignant cells into the ovarian tissue, such as leukaemia or colon cancer. OTC is currently not offered to many women with ovarian cancer because of the risk of reintroducing the malignancy during transplantation. However, the collection of immature oocytes would eliminate this risk since the oocytes will not include malignant cells. Therefore, this procedure is new and may provide a group of women who previously had no opportunity to preserve fertility with a chance of having their genetically own children for the first time.

The present study has weaknesses, and most pronounced limitation is the inability to follow the subsequent developmental competence of fertilized MII oocytes. Furthermore, an evaluation of the effect of the collection of oocytes in the two types of media was not able to be performed in a strictly randomized manner for practical reasons. Regardless of these limitations, the present study advances the use of immature oocytes collected from surplus medulla tissue, mainly due to the large number of oocytes included. The MII maturation rate was evaluated after a culture period of 44–48 h. The oocytes were not destined for use in the clinic, and for convenience, the evaluation was not performed at 36 h, which would be used in a clinical setting.

The longer-term perspective of the present findings is potentially the dawn of fertility treatment without stimulation with exogenous hormones in certain groups of women. Up to 100 immature oocytes may be harvested from one ovary of women with, for instance, PCOS. The collection of oocytes from SAF is envisioned to be optimized by developing new procedures for the aspiration of very small follicles. In women with PCOS and two ovaries, enough immature oocytes may be collected without ovarian stimulation to create equal or even higher numbers of MII oocytes for treatment than conventional ovarian stimulation. If necessary, repeated aspirations of oocytes may be performed with stockpiling of frozen oocytes between retrieval sessions. Prior to the use of immature oocytes from SAF on a larger scale, safety studies are required, and one aspect to evaluate will be the epigenetic status. To the best of our knowledge, the methylation status of human IVM oocytes collected ex vivo from SAF has not been evaluated. As shown for human IVM oocytes collected in vivo after hormonal stimulation [40–42], the IVM procedure may modify the methylation pattern under some conditions. However, an analysis of IVM-induced epigenetic changes in 11 new-borns after IVM in stimulated cycles [43] revealed relatively few changes, if any.

Conclusions

As shown in the present study, an unexpectedly high number of immature oocytes was obtained from surplus medulla tissue during cortex preparation and fertility preservation. Although many of these oocytes were derived from antral follicles with a diameter of only a few millimetres, the meiotic competence in terms of the MII transition was maintained at an overall rate of approximately one in three MII oocytes. The euploidy rate was acceptable, although the majority of ovaries were transported on ice with a significantly reduced MII rate than ovaries obtained from the local hospital.

Using this new source of human oocytes from very small antral follicles for IVM is currently an experimental exercise to form a platform for improving the IVM procedure, especially because the true clinical potential cannot be evaluated in our studies. An important component of this improvement is the identification of midkine as a growth factor that significantly increased the maturation rate when it was present in concentrations comparable with the preovulatory follicular fluid [32]. We hypothesised that oocytes from these small follicles undergo an accelerated meiotic competence after quick physical handling and placing them in an environment with high levels of FSH, whereby they avoid degenerative changes that they might have encountered in vivo. The study suggests a sub-division of the large ill-defined category of immature human oocytes based on an oocyte diameter, and a diameter of 100 μm separates oocytes that possess the capacity to undergo the MII transition from oocytes that are unable to sustain this transition. The maturation rate of 31% described in the present study results in the generation of an average of 11 MII oocytes, of which approximately two-thirds are euploid, similar to the results obtained with most ovarian stimulation regimes.

Authors' contributions CYA, DN, CJ, SEP and SGK planned the study. DN, CJ, VS, RB, SEP, SGK, MLG performed the experiments and collected the data. DN, CJ, SEP, SGK, JC, VS, RB, ZG, YW and EH analysed the data and produced figures. KTM, LC, EE and AMBB recruited patients and collected the tissues. DN, SEP, SGK and CYA wrote the manuscript. All authors approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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Affiliations

Dmitry Nikiforov[1,2] · Cheng Junping[1,3] · Jesus Cadenas[1] · Vallari Shukla[4] · Robert Blanshard[5,6] · Susanne Elisabeth Pors[1] · Stine Gry Kristensen[1] · Kirsten Tryde Macklon[7] · Lotte Colmorn[7] · Erik Ernst[8,9] · Anne-Mette Bay-Bjørn[8] · Zeinab Ghezelayagh[1] · Yu Wakimoto[1,10] · Marie Louise Grøndahl[11] · Eva Hoffmann[4] · Claus Yding Andersen[1]

[1] Laboratory of Reproductive Biology, The Juliane Marie Centre for Women, Children and Reproduction, Copenhagen University Hospital and Faculty of Health and Medical Sciences, University of Copenhagen, 2100 Copenhagen, Denmark

[2] Università degli studi di Teramo, 64100 Teramo, Italy

[3] People's Hospital of Guangxi Autonomous Region, Nanning 530000, China

[4] DNRF Center for Chromosome Stability, Department of Cellular and Molecular Medicine, Faculty of Health Sciences, University of Copenhagen, 2200 Copenhagen, Denmark

[5] Clinical Genomics Group, Illumina UK Ltd., Fulbourn, UK

[6] Genome Damage and Stability Centre, School of Life Sciences, University of Sussex, Brighton BN1 9RQ, UK

[7] The Fertility Clinic, Rigshospitalet, Copenhagen University Hospital, 2100 Copenhagen, Denmark

[8] The Fertility Clinic, Aarhus University Hospital, Skejby, 8200 Aarhus, Denmark

[9] The Fertility Clinic, Horsens Regional Hospital, 8700 Horsens, Denmark

[10] Department of Obstetrics and Gynecology, Hyogo College of Medicine, 663-8501 Nishi-nomiya, Hyogo, Japan

[11] The Fertility Clinic, Herlev Gentofte Hospital, 2740 Herlev, Denmark

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Association between spontaneous ovulation and serum anti-Müllerian hormone levels in a premature ovarian insufficiency patient after a multimodal treatment for breast cancer

Yu Wakimoto, Atushi Fukui, Goh Wakimoto, Yuji Ikezawa, Michie Matsuoka, Maya Omote, Yukiko Sugiyama, Yuji Ukita, Toru Kato and Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Abstract

The high toxicity of chemotherapy can damage a patient's gonadal function, leading to premature ovarian insufficiency (POI). Here, we report the case of a patient suffering from POI after chemotherapy for breast cancer, who 3 years later ovulated spontaneously and became pregnant. The patient, a 31-year-old infertile women, nulligravida, was diagnosed with breast cancer. The Anti-Müllerian Hormone (AMH) level in her serum was 1.85 ng/mL before multimodal treatment for cancer. She later visited our hospital for amenorrhea and 2 years after cancer treatment, she was diagnosed with POI. Her AMH level at that point was less than 0.1 ng/mL. One year after the diagnosis of POI, the patient's AMH level increased slightly to 0.14 ng/mL and she ovulated spontaneously. The patient later became pregnant using Assisted Reproductive Technology on the fourth attempt.During the course of treatment for infertility at our hospital, the AMH levels in her serum changed along with the recovery of ovarian function. These findings suggest the possibility that ovulation and pregnancy could be predicted by the chronological changes of the AMH levels in the patient's serum.

Key words: anti-Müllerian hormone, breast cancer survivor, fertility preservation, ovarian reserve, premature ovarian insufficiency.

Introduction

It is common during the different types of cancer treatments that female adolescents and young adult (AYA) patients with cancer experience premature ovarian insufficiency (POI) due to the high toxicity of chemotherapy damaging the patient's gonadal function.[1,2] However, there are several case reports in the literature involving POI patients who conceived during long-term follow-ups.[3,4] Here, we report the case of an AYA patient suffering from POI after multimodal breast cancer treatment.

Three years after treatment, the patient ovulated spontaneously and became pregnant. Upon further investigation, we found that follicle development had restarted in the patient despite undergoing chemotherapy only several years prior. During and after the patient's cancer treatment, serum Anti-Müllerian Hormone (AMH) levels were measured and it was found that the patient's AMH levels increased right before she became pregnant. We therefore found it reasonable to believe that AMH could be a possible predictor of a woman's ability to conceive after receiving chemotherapy. This case report is, as far as we know, the first to present the correlation between recovering

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Correspondence: Dr Yu Wakimoto, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan.
Email: yuuwakimoto@gmail.com

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AMH levels and pregnancy after treatment with Cyclophosphamide (CPA) and Tamoxifen for breast cancer.

Case Report

In 2010, at 28 years of age, the patient got married. After 1 year of marriage, the couple underwent infertility treatment for male factor infertility at a neighborhood clinic. Semen analysis was performed and revealed that the patient's husband had asthenozoospermia. The husband was referred to an urologist where he received varicocele surgery. During this time, AMH level in the patient's serum, which was measured by using a commercial assay kit (AMH GenII ELISA; Beckman Coulter Company) from SRL Inc, was 1.85 ng/mL. Once the husband recovered from surgery, the couple resumed infertility treatment using timed intercourse, but this was unsuccessful. In 2013, at 31 years of age, the patient presented with a palpable mass in her left breast and was diagnosed with breast cancer (stageI, T1N0M0 infiltrating ductal carcinoma, ER+, PR+, HER2-, KI67: 48.6%). At that time, a regional oncofertility network in Japan for providing information related to oncofertility counseling had not yet been established. Therefore, the oncologist who treated the patient's cancer did not provide the patient with adequate information about fertility preservation and the patient did not use ovary cryopreservation before chemotherapy.

The patient's cancer treatment included a left breast conserving mastectomy and chemotherapy using Docetaxel and CPA, both of which are known for being particularly damaging to a woman's gonads. Following this, the patient was simultaneously treated with Tamoxifen and radiation therapy (50Gy/25Fr) for 2 years and was subsequently treated with a gonadotropin-releasing hormone agonist for 3 months. The standard duration of hormonal therapy is 5 years; however, the patient decided to interrupt the hormone treatment because she felt her age along with chemotherapy greatly reduced her chances of becoming pregnant.

At 33 years of age, the patient went into remission and she was referred to our hospital with the desire to become pregnant. Once the patient was admitted, she was diagnosed with POI due to amenorrhea with raised gonadotrophins and low estradiol. At this point, her serum AMH level was found to be less than 0.1 ng/mL. We also tested the patient's serum for follicle stimulating hormone (FSH), luteinizing hormone

Table 1 Fertility treatment at our hospital

	iCOS	Gn or CC	Total of Gn (IU)	E2 level (pg/ml)	Day of E2 measurement	OPU	ICSI	ET	Pregnancy	Frozen
2016. 2	Short	450 IU × 18 day	8100	<10	Day 8, Day 11 Day14, Day17	Cancel				
5	CC	150 mg × 5 days		<10	Day 23, Day 33	Cancel				
7	CC	150 mg × 5 days		<10	Day 21, Day 28	Cancel				
8–10		(No visit to our hospital, BBT was bipase at this point: Ovarian function was recovered)								
11	Natural			241	Day 16	(IUI)			Failed	
12	CC			342	Day13 (2 days before OPU)	1	1/1	6 cell (III)	Failed	
2017. 2	CC	150 mg × 5 day		333	Day7 (2 days before OPU)	1	0/1			
4	Short	450 IU × 3 + 300 IU × 11	4650	1278	Day16 (2 days before OPU)	4	3/4	8 cell (II)	Failed	1 (4BB) (preserved)
6	Short	450 IU × 3 + 300 IU × 9 + retrozol 5 mg/day	4050	125	Day 14 (2 days before OPU)	2	1/2	4 cell (I)	Succeed	

CC, clomiphene citrate; ET, embryo transfer; Gn, gonadotropin; iCOS, individualized controlled ovarian stimulation; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; OPU, oocyte pick-up.

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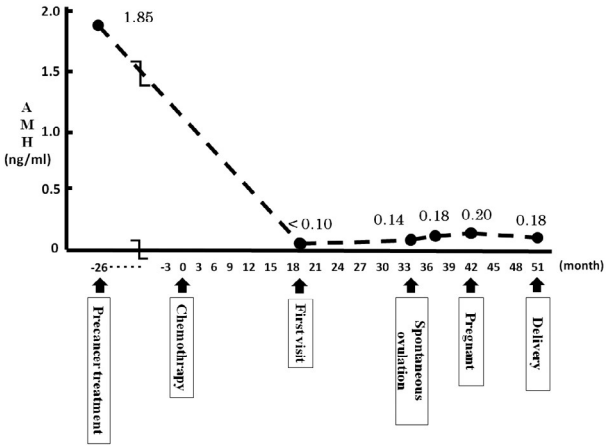


Figure 1 The change in the levels of Anti-Müllerian Hormone (AMH) during the course of the treatment. AMH in the patient's serum was 1.85 ng/mL before chemotherapy. When the patient first visited our hospital, serum AMH level was found to be less than 0.1 ng/mL. When we confirmed a spontaneous recovery of ovulation, AMH level was found to be 0.14 ng/mL. During pregnancy, AMH level in the patient's serum increased to 0.2 ng/mL and dropped slightly to 0.18 ng/mL a month after delivery.

and E2 and found their levels to be 18.83 IU/L, 8.95 IU/L and less than 10 pg/mL, respectively. We treated her with various treatment regimens, which are outlined in Table 1. However, despite our efforts, there was no recovery of an ovulatory cycle. The patient continued infertility treatment, and a year after she was diagnosed with POI. Her basal body temperature and estradiol level confirmed the spontaneous recovery of ovulation. We evaluated her serum AMH level and found that it increased to 0.14 ng/mL. At this point, we began infertility treatment using Assisted Reproductive Technology. After two unsuccessful embryo transfer attempts, the patient became pregnant on the third attempt using a four cell embryo. The stimulation protocol used short gonadotropin-releasing hormone agonist with 4050 IU of FSH plus retrozol. When we found the patient to be pregnant, we retested her serum AMH levels and found that it increased to 0.18 ng/mL. During her pregnancy, we continued to monitor her serum AMH level. It remained mostly stable until about a month after her Cesarean delivery (due to a breech presentation), which at that point, her serum AMH level dropped slightly to 0.18 ng/mL. Figure 1 shows the change of AMH levels in relation to breast cancer treatment, pregnancy and delivery.

Discussion

AMH is produced by the granulosa cells of the preantral and small antral follicles during the early stages of follicular recruitment. The number of these follicles is associated with the size of the primordial follicle pool. Thus, AMH represents a marker of ovarian reserve and the ovarian response.[2] Furthermore, it is reported that serum AMH concentration could be a clinically useful marker for the size of the remaining primordial follicle pool after cancer therapy.[2] However, AMH level immediately after the cancer therapy was reported to be inappropriate for the evaluation of the ovarian reserve.[4] Brougham *et al*.[2] reported that AMH decreased progressively during chemotherapy becoming undetectable in 50% of the patients in recovery in the low to medium risk groups after completion of the treatment. With patients in the high-risk group, AMH became undetectable and they showed no recovery.[2] In the current case, the patient received CPA for her chemotherapy, which is classified as an intermediate risk medicine for patients developing permanent amenorrhea.[5] This was the case with our patient as the high toxicity of CPA damaged her gonadal function leading to POI. As a result, AMH level in the patient's serum was found to be less than 0.1 ng/mL 2 years after chemotherapy.

Three years after chemotherapy, AMH level in the patient's serum increased to 0.14 ng/mL and the patient ovulated spontaneously. It is reported that CPA administration induces more severe oocyte damage and follicular closure to the developing follicle than to the primordial follicle.[6] It suggests that remaining primordial follicles began developing once the patient went into remission and the developing follicle produced AMH. The recovery in AMH after chemotherapy suggests the recovery of the pool of small growing follicles, which in turn reflects the size of the non-growing primordial pool.[2] Folliculogenesis is the process in which a recruited primordial follicle grows into a mature ovarian follicle ready to either ovulate its egg into the oviduct for fertilization or to atresia. This process is long, requiring almost 1 year for a primordial follicle to grow to the ovulatory stage.[7] However, the dynamics and regulatory mechanisms of follicular development in the ovary are not fully understood.[8] In this case, 3 years might have been the required time for a primordial follicle to restart follicle development, moving from a primordial follicle after chemotherapy to the development of the ovulatory stage.

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It is well known that many women become amenorrheic during chemotherapy, but some show resumption of menses.[9] Morarji *et al.*[1] reported that young breast cancer survivors often show significant damage to the ovarian function in spite of having normal menstrual cycles after cancer treatment. The number of ovarian follicles decreases with age, and therefore, age is a surrogate for the number of pretreatment follicles. It was reported that serum AMH in cancer survivors was roughly equivalent to that measured in control patients 12 years older.[9] Along with this, Anderson and Cameron reported that the women with a pretreatment AMH serum level of less than 1.9 ng/mL became amenorrheic after chemotherapy.[9] Therefore, AMH levels before administration of chemotherapy can predict later amenorrhea. In the current case, AMH level in the patient's serum before chemotherapy was 1.85 ng/mL. Interestingly, a study was done about the association between AMH levels and the probability of pregnancy in systemic lupus erythematosus patients exposed to cyclophosphamide. The authors found that the likelihood of becoming pregnant was not predicted by AMH levels, but by cyclophosphamide exposure and age. However, AMH levels decreased significantly with increasing age and cyclophosphamide exposure.[10]

AMH in the patient's serum was mostly stable during the course of her pregnancy. However, a previous study found that the AMH levels normally decrease during the course of pregnancy since folliculogenesis is inhibited.[11] Most of the primordial follicles are in a resting state and hence, a down-regulation in AMH production is observed. On the other hand, according to another report, a significant change was not observed in the AMH levels during pregnancy.[12] In our case, AMH levels in the patient's serum were beyond the healthy limits reported by recent studies both before and during pregnancy. Taken together, it is clear that more research needs to be done about AMH levels during pregnancy before a conclusion can be stated.

In our case report, fertility preservation of the patient's oocytes or ovaries was not performed before cancer therapy. Cryopreservation of sperm, embryos, unfertilized mature oocytes and ovarian tissues is performed for fertility preservation before AYA patients with cancer irreversibly lose fertility due to cancer therapy.[13,14] Guidelines from the American Society of Clinical Oncology state that the possibility of cancer treatment-induced infertility should be discussed with all cancer patients of reproductive age before adjuvant chemotherapy.[5,15] Thus, all healthcare providers should pay attention to fertility preservation guidelines to make it possible for AYA patients to conceive in the future before they are treated with chemotherapy, radiation therapy, or surgery.[16] In order to provide information related to onco-fertility counseling, as of December 2017, a regional onco-fertility network was established in Gifu, Shiga, Saitama and 18 other prefectures in Japan.[3,13] This network makes it easier for oncologists and reproduction specialists to exchange information about fertility preservation and cancer treatment in Japan.

This case study has some limitations. First, the levels of AMH were measured on random days during the patient's menstrual cycle and variability in AMH has been shown to be associated to the day of the menstrual cycle. Hadlow *et al.*[17] reported that although the absolute changes in low AMH levels were less pronounced, the percent change could be high and therefore, especially in case AMH, can push the result closer to a clinical cutoff. Given this, clinicians should consider variability in a patient's serum AMH level during their menstrual cycle when assessing a patient for the possibility of becoming pregnant.

Second, the patient described in this report was diagnosed with POI due to amenorrhea with raised gonadotrophins and low estradiol at 33 years of age. However, the ESHRE Guideline Group on POI recommends the following diagnostic criteria for POI: oligo/amenorrhea for at least 4 months and an elevated FSH level of more than 25 IU/L on two occasions more than 4 weeks apart.[18] FSH levels in this patient was 18.83 IU/L during her first visit and we did not follow up with subsequent tests.

In conclusion, during the course of treatments at our hospital, AMH levels in the patient's serum changed along with her ability to ovulate. It suggests that even if the AMH level immediately after the cancer therapy is low, there is a chance that dormant primordial follicles in the ovary begin to develop after an extended period. Therefore, ovulation and the ability to conceive could be predicted by a change in the serum AMH levels after a successful cancer treatment.

Disclosure

None declared.

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Full length article

Fetal cell-free DNA fraction in maternal plasma for the prediction of hypertensive disorders of pregnancy

Nobuhiro Suzumori[a,*], Akihiko Sekizawa[b], Takeshi Ebara[c], Osamu Samura[d], Aiko Sasaki[e], Rina Akaishi[e], Seiji Wada[e], Haruka Hamanoue[f], Fumiki Hirahara[f], Haruna Izumi[f], Hideaki Sawai[g], Hiroaki Nakamura[h], Takahiro Yamada[i], Kiyonori Miura[j], Hideaki Masuzaki[j], Takahiro Yamashita[k], Takashi Okai[k], Yoshimasa Kamei[l], Akira Namba[l], Jun Murotsuki[m], Tomohiro Tanemoto[d], Akimune Fukushima[n], Kazufumi Haino[o], Shinya Tairaku[p], Keiichi Matsubara[q], Kazutoshi Maeda[r], Takashi Kaji[s], Masanobu Ogawa[t], Hisao Osada[u], Haruki Nishizawa[v], Yoko Okamoto[w], Takeshi Kanagawa[w], Aiko Kakigano[x], Michihiro Kitagawa[y], Masaki Ogawa[z], Shunichiro Izumi[A], Yukiko Katagiri[B], Naoki Takeshita[B], Yasuyo Kasai[C], Katsuhiko Naruse[D], Reiko Neki[E], Hisashi Masuyama[F], Maki Hyodo[G], Yukie Kawano[H], Takashi Ohba[I], Kiyotake Ichizuka[J], Takeshi Nagamatsu[K], Atsushi Watanabe[L], Nahoko Shirato[b], Junko Yotsumoto[M], Miyuki Nishiyama[e], Tatsuko Hirose[b], Haruhiko Sago[b]

[a] Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[b] Department of Obstetrics and Gynecology, Showa University School of Medicine, Tokyo, Japan
[c] Department of Occupational and Environmental Health, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
[d] Department of Obstetrics and Gynecology, Jikei University School of Medicine, Tokyo, Japan
[e] Center of Maternal-Fetal, Neonatal and Reproductive Medicine, National Center for Child Health and Development, Tokyo, Japan
[f] Department of Clinical Genetics, Yokohama City University Hospital, Yokohama, Japan
[g] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan
[h] Department of Obstetrics, Osaka City General Hospital, Osaka, Japan
[i] Department of Obstetrics and Gynecology, Hokkaido University Graduate School of Medicine, Sapporo, Japan
[j] Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
[k] Maternal and Child Health Center, Aiiku Hospital, Tokyo, Japan
[l] Departments of Obstetrics and Gynecology, Saitama Medical University School of Medicine, Saitama, Japan
[m] Department of Maternal and Fetal Medicine, Tohoku University Graduate School of Medicine, Miyagi Children's Hospital, Sendai, Japan
[n] Department of Clinical Genetics, School of Medicine, Iwate Medical University, Morioka, Morioka, Japan
[o] Department of Obstetrics and Gynecology, Niigata University Medical and Dental Hospital, Niigata, Japan
[p] Department of Obstetrics and Gynecology, Kobe University Graduate School of Medicine, Kobe, Japan
[q] Department of Obstetrics and Gynecology, Ehime University School of Medicine, Matsuyama, Japan
[r] Department of Obstetrics and Gynecology, Shikoku Medical Center for Children and Adults, Kagawa, Japan
[s] Department of Obstetrics and Gynecology, The University of Tokushima, Faculty of Medicine, Tokushima, Japan
[t] Department of Obstetrics and Gynecology, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan
[u] Department of Maternal-fetal Medicine, Chiba University Hospital, Chiba, Japan
[v] Department of Obstetrics and Gynecology, Fujita Health University, Aichi, Japan
[w] Department of Obstetrics, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan
[x] Department of Obstetrics and Gynecology, Osaka University Faculty of Medicine, Osaka, Japan
[y] Sanno Hospital, Tokyo, Japan
[z] Perinatal Medical Center, Tokyo Women's Medical University Hospital, Tokyo, Japan
[A] Department of Obstetrics and Gynecology, Tokai University School of Medicine, Kanagawa, Japan
[B] Department of Obstetrics and Gynecology, School of Medicine, Faculty of Medicine, Toho University, Tokyo, Japan
[C] Department of Obstetrics and Gynecology, Japanese Red Cross Medical Center, Tokyo, Japan
[D] Department of Obstetrics and Gynecology, Nara Medical University, Nara, Japan
[E] Department of Perinatology and Gynecology, National Cerebral and Cardiovascular Center, Osaka, Japan

* Corresponding author.
E-mail address: og.n.suz@med.nagoya-cu.ac.jp (N. Suzumori).

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[F] Department of Obstetrics and Gynecology, Okayama University Graduate School of Medicine, Okayama, Japan
[G] Department of Obstetrics and Gynecology, Hiroshima University Graduate School of Medicine, Hiroshima, Japan
[H] Department of Molecular Pathology, Faculty of Medicine, Oita University, Oita, Japan
[I] Department of Obstetrics and Gynecology, Kumamoto University, Kumamoto, Japan
[J] Department of Obstetrics and Gynecology, Showa University Northern Yokohama Hospital, Yokohama, Japan
[K] Departments of Obstetrics and Gynecology, Tokyo University Hospital, Tokyo, Japan
[L] Division of Clinical Genetics, Nippon Medical School Hospital, Tokyo, Japan
[M] Departments of Genetic Counseling, Ochanomizu University, Tokyo, Japan

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ABSTRACT

Objective: The purpose of this study is to compare the fetal fractions during non-invasive prenatal testing (NIPT) in singleton pregnancies according to gestational age and maternal characteristics to evaluate the utility of this parameter for the prediction of pregnancy complications including gestational diabetes mellitus (GDM) and hypertensive disorders of pregnancy (HDP).
Study design: This study was a multicenter prospective cohort study. The present data were collected from women whose NIPT results were negative. The relationships between the fetal fractions and the gestational age, maternal weight and height, and incidences of miscarriage, preterm delivery, and pregnancy complications including GDM, HDP and placental abruption were assessed.
Results: A total of 5582 pregnant women with verified NIPT negative results were registered in the study. The demographic characteristics of the study populations were statistically analyzed, and the women with HDP tended to have a low fetal fraction in samples taken during early gestation. The area under the curve (AUC) in a receiver operating characteristic curve (ROC) analysis was 0.608 for women with HDP.
Conclusion: A low fetal fraction on NIPT might be correlated with future HDP. However, predicting HDP during early pregnancy in women with a low fetal fraction might be difficult.
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Introduction

Non-invasive prenatal testing (NIPT) with use of massively parallel sequencing has become a clinical certainty and is being progressively used for the detection of common chromosomal aneuploidies [1–3]. In Japan, NIPT for trisomies 21, 18, and 13 was started in 2013, and a primary nationwide trial was accompanied by the Japan NIPT consortium [4]. Between April 2013 and March 2016, the Japan NIPT consortium piloted 30,613 tests at 55 medical centers contributing to a multicenter clinical investigation [5]. We assumed that, in our prospective study, the evaluation of the fetal DNA fraction at an early gestational age might offer significant information not only for screening for fetal aneuploidy but also for the prediction of pregnancy complications [6,7].

During pregnancy, the placenta shed large amount of fetal/placental DNA into the maternal circulation. The cell-free fetal DNA (cffDNA) originates from apoptotic trophoblastic cells [1–3]. The fetal fraction, which is a key factor that confirms adequate fetal chromosomal representation, is affected by maternal weight, the maternal body mass index (BMI), gestational age, fetal aneuploidy, mode of conception and samples transportation [2,3,7]. For pregnancy complications, Quezada et al. described that quantity of the fetal fraction in maternal plasma during the 11th to 13th week of gestation is not predictive of a preterm delivery [8]. Previous publication suggested that preeclampsia is associated with both high and low levels of cffDNA (<5th percentile) [9].

For the prediction of pregnancy complications, a latest report has suggested the application of the ratio of sFlt-1 (soluble fms-like tyrosine kinase 1) to placenta growth factor (PLGF) in maternal serum as a screening for preeclampsia at 20 weeks of gestation [10]. Antwi et al. reported the development and validation of a prediction example for gestational hypertension in a cohort study [11]. In gestational diabetes (GDM), circulating concentrations of Sex hormone binding globulin (SHBG) characterize a potentially helpful new biomarker for predicting the risk of GDM [12]. However, for the prediction of pregnancy complications, prospective studies using cfDNA during early gestation have so far been limited.

The objective of this study was to search further whether maternal cell-free DNA and the fetal fraction in NIPT are increased or decreased in pregnancies which ended in miscarriage, preterm delivery, or pregnancy complications including placental abruption, GDM and hypertensive disorders of pregnancy (HDP), the latter includes gestational hypertension, preeclampsia and eclampsia, and to determine whether these measurements are beneficial for the prediction of pregnancy complications.

Methods

Study population

This was a case-control study represented from a prospective observational study of adverse gestation outcome in pregnant women undergoing NIPT. The data originated from a clinical study of cfDNA testing for fetal aneuploidy and singleton between 10 and 20 weeks' gestation. It was the data recorded which included maternal characteristics and medical history, including maternal age, ultrasounds findings, abnormal serum screening results etc. The study design was supported by all the hospitals' Institutional Review Boards, and all the women provided written informed consent to participate. The aspects of the Japan NIPT consortium practice, including the enrollment of high-risk pregnant women who had requested testing, are provided on the website (http://www.nipt.jp/).

Sample collection and test methods

Between April 2013 and March 2014, blood samples were collected from pregnant women at each medical center and were referred to Sequenom Laboratories (San Diego, CA) for MaterniT21 Plus tests within 7 days of collection. The cases with missing information regarding maternal information, multiple gestation and fetal demise before NIPT were excluded.

Cell-free maternal plasma DNA extracted from each sample was subjected to library preparation and massively parallel sequencing with an Illumina HiSeq 2000 (San Diego, USA) as

described previously [1,5,7]. The SeqFF method is a multivariate regression model that verifies the fetal DNA fraction [13]. As the magnitude of copy number variation can also be used to estimate the fetal DNA fraction, bins located on chromosomes 13, 18, 21, X, and Y were eliminated from the SeqFF method to prevent issues of model over-fitting and circular evidence [13]. The sequencing data were concerned to calculate the *z*-score, which is a robust assessment of normalized chromosomal representation compared with euploid genome.

Follow-up and confirmation afterbirth

Cases with negative NIPT results were followed with use of a personal questionnaire to find pregnancy and postpartum data. The clinical data, test results and pregnancy outcomes were collected and aggregated every month at the data center of the secretariat. The pregnancy outcomes included miscarriage (<22 weeks), preterm delivery (<37 weeks), hypertensive disorders of pregnancy (HDP), gestational diabetes mellitus (GDM), and placental abruption. HDP including gestational hypertension, preeclampsia and eclampsia, was defined as hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg, twice) with or without proteinuria. A diagnosis of GDM was determined by the criteria of the Japan Diabetes Society (JDS) [14].

Normal pregnancy was defined as term delivery (≥37 weeks of gestation) without medical or obstetric problems. The multiple imputation data were studied to estimate the pooled beta coefficients of maternal age, maternal weight, fetal fraction affecting outcome. This study was part of a clinical trial registered with the University Medical Information Network clinical trials registry (UMIN000009338).

Table 1
Characteristics of missing values of the study population.

Variables	n	Number of missing values	(%)
Maternal age	5582	1	0
Maternal weight	5238	345	6.2
Fetal fraction (square root transformed)	5051	532	9.5
Outcome(Normal · Miscarriage)	5307	276	4.9
Outcome(Normal · Preterm)	5314	269	4.8
Outcome(Normal · HDP)	5401	182	3.3
Outcome(Normal · GDM)	5370	213	3.8
Outcome(Normal · Abruption)	5280	303	5.4

Statistical analysis

The measured fetal fraction was signified as the square root (√) transformed distribution to ensure normality as described previously [7].

We used a multiple imputation method to impute missing data [15]. The percentage of missing values among all the variables varied from 0% to 9.5% (Table 1). Five imputed data sets were made and combined to assess the pooled means and standard errors for demographic statistics. We also applied multiple imputation data for the logistic regression analysis to estimate the pooled beta coefficients for maternal age, maternal weight, and fetal fraction affecting each outcome. To evaluate how well the model fit the data, the Cox-Snell R-squared and Nagelkerke R-squared values were calculated. We also calculated the area under the curve (AUC) by means of a receiver operating characteristic curve (ROC) analysis to validate the accuracy of the predictive models. All the statistical analyses were performed by statistical software package SPSS 22.0 (SPSS, Chicago, IL, USA) [16–18].

Results

Of the 7740 women who participated in this study, 747 were excluded because of a lack of details, such as maternal and gestational age. Of the 6993 high-risk pregnant women tested in the study, two cases had a fetal fraction over 60% and were excluded from the study of fetal fraction metrics. More than 99.8% of the samples (n = 6981) had a fetal fraction above the lower acceptable limit for the accurate interpretation of fetal aneuploidy. A total of 5582 pregnant women with NIPT negative results were enrolled, and none of the women had a false-negative NIPT result. Most of the women (>94%) were 35 years old or older; the median gestational age at the time of examination was 13.0 (10.0–20.2) weeks; and the median maternal weight was 52.8 (34.0–115.0) kg. The characteristics of the missing values of the study population are presented in Table 1, and the frequency distribution of the maternal plasma √fetal DNA fraction is showed in Fig. 1a (crude) and b (adjusted; one of the five distributions that were replaced by imputed values). Both of the √fetal fractions had similar bell-shaped distributions with peaks between 20% and 40% at 10–20 weeks of gestation.

The median fetal DNA fraction within 10–20 weeks of gestation was 15.2%, with an interquartile range of 11.4%–18.8%. The demographic characteristics of the study populations were statistically analyzed in relation to miscarriage, preterm delivery,

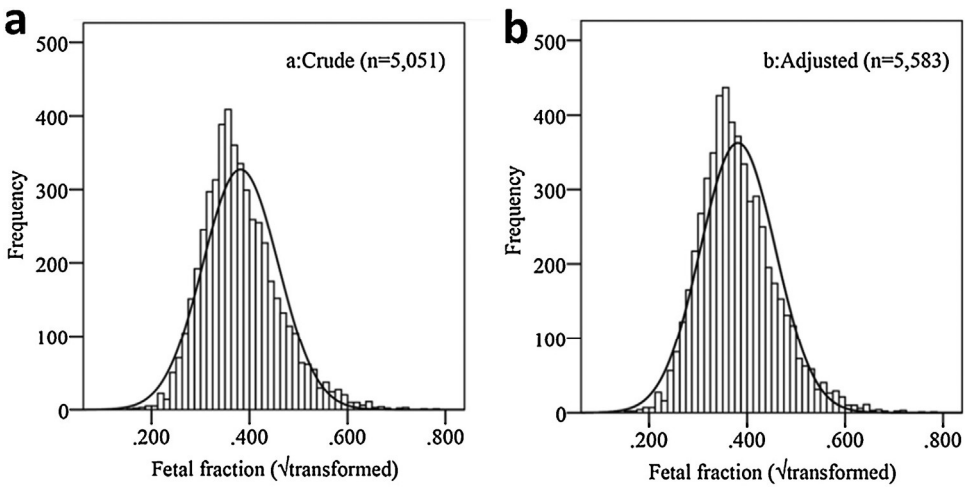


Fig. 1. Frequency distribution of square root of fetal fraction in maternal plasma DNA.

Table 2
Demographic characteristics of the study populations according to miscarriage, preterm delivery HDP, GDM and abruption.

	Outcomes										
	Normal (n = 5270)	Miscarriage (n = 37)	*P*	Preterm (n = 44)	*P*	HDP (n = 131)	*P*	GDM (n = 100)	*P*	Abruption (n = 10)	*P*
Maternal age (years)	38.37 (0.03)	38.81 (0.42)	0.308	38.25 (0.41)	0.776	38.82 (0.23)	0.060	38.58 (0.29)	0.471	37.80 (0.77)	0.436
Maternal weight (kg)	52.80 (0.10)	52.89 (1.36)	0.951	53.25 (1.46)	0.761	55.83 (0.84)	**<0.001**	58.22 (1.07)	**<0.001**	53.56 (2.91)	0.795
Fetal fraction (%)	0.152 (0.0008)	0.136 (0.011)	0.177	0.139 (0.009)	0.151	0.134 (0.006)	**0.003**	0.140 (0.005)	0.045	0.134 (0.016)	0.273
Fetal fraction ($\sqrt{}$transformed)	0.382 (0.001)	0.360 (0.014)	0.152	0.364 (0.011)	0.139	0.359 (0.007)	**0.005**	0.367 (0.007)	0.061	0.358 (0.023)	0.304

Data are shown as the pooled mean (pooled SE).
Statistically significant difference ($P < 0.01$) for comparison between each outcome with the control (Welch's *t*-test).
Multiple imputation for missing data was applied to the following variables; Maternal age, Maternal weight, fetal fraction (%), fetal fraction (square root transformed).
The bold data show statistically significant.

hypertensive disorders of pregnancy (HDP), gestational diabetes mellitus (GDM) and placental abruption (Table 2). The maternal weights of the women with HPD and/or GDM were significantly higher than those of controls ($P < 0.001$). Additionally the fetal fraction percentages in the HDP women were significantly lower than those of the controls ($P < 0.003$).

Table 3 presents the multiple imputation data for the logistic regression analysis to estimate the pooled beta coefficients for maternal age, maternal weight, and fetal fraction affecting each outcome. To assess how well the model fits the data, the Cox-Snell R-squared and Nagelkerke R-squared values were calculated and showed. Using maternal age, maternal weight, and the fetal fraction of an NIPT performed during early pregnancy, we found that it was difficult to predict miscarriage, preterm delivery, HDP, GDM, or placental abruption because the Cox-Snell R-squared and Nagelkerke R-squared values were relatively low. However, based on the Cox-Snell R-squared and Nagelkerke R-squared data for HDP, we found a correlation between the low fetal fractions and maternal HDP.

As for HDP, the area under the curve (AUC) of the receiver operating characteristic curve (ROC) analysis was calculated to validate the accuracy of the predictive models and is showed in Fig. 2. The AUC in women with HDP was 0.608 (95%CI: 0.554–0.662).

Discussion

In this multicenter prospective cohort study, a total of 5582 women with confirmed NIPT negative results among 7740 high-risk women who experienced NIPT were included. The fetal fraction is an essential quality metric for the reliable detection of aneuploidy using NIPT, but the relationship between the fetal fraction during early pregnancy and the prediction of pregnancy complications remains unclear. Our present data suggest that it may be difficult to predict HDP in women with a low fetal fraction during early pregnancy, although we found a correlation between the low fetal fractions and maternal HDP.

Spontaneous preterm birth is a complex syndrome with multiple pathway interactions that regulate its occurrence, as well as genetic, immunological, physiological and environmental factors [19]. Recent studies recommend the use of several markers such as C-reactive protein and alkaline phosphatase to predict preterm delivery [20]. As demonstrated in this study, the use of cfDNA testing is unlikely to be beneficial for the prediction of preterm delivery, and this supports the conclusion of a previous report by Quezada et al. [8]. In addition, recent data did not demonstrate that a low fetal fraction on the NIPT could suggest a risk of miscarriage (less than 20 weeks of gestation) [9].

In this study, the demographic characteristics of the study populations were statistically analyzed, and women with subsequent HDP tended to have a low fetal fraction in samples gotten during early gestation, although Farina et al. reported higher levels of maternal plasma cfDNA during late gestation in preeclampsia,

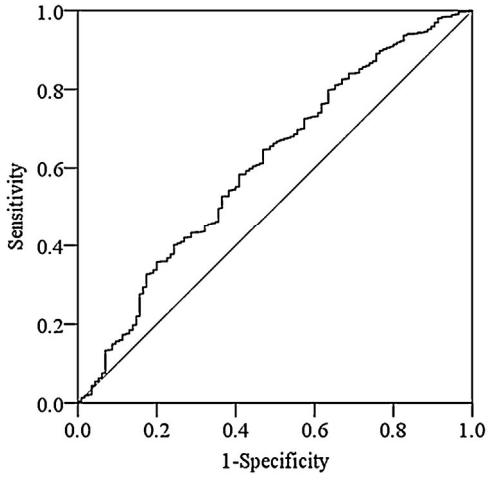


Fig. 2. Calculated the area under curve (AUC) using receiver operating characteristic curve (ROC) analysis for validating the accuracy of predictive models in HDP.

Table 3
The multiple imputation data for logistic regression analysis to estimate the pooled beta coefficients of maternal age, maternal weight, fetal fraction affecting outcome.

Outcome	n	Cox-Snell R-squared	Nagelkerke R-squared	Pooled beta coefficients							
				Maternal age	*P*	Maternal weight	*P*	Fetal fraction ($\sqrt{}$transformed)	*P*	Constant	*P*
Miscarriage	5307	0.001	0.019	0.064	####	−0.011	0.641	−4.350	0.154	−5.216	0.084
Preterm	5314	0.001	0.007	−0.02	####	0.00	0.962	−3.28	0.157	−2.81	0.292
HDP	5401	0.005	0.026	0.05	####	0.04	**0.001**	−2.83	0.054	−6.67	**0.000**
GDM	5370	0.010	0.061	0.008	####	0.071	**0.000**	0.093	0.953	−8.244	**0.000**
Abruption	5280	0.000	0.009	−0.082	####	0.004	0.926	−4.421	0.378	−1.739	0.746

The bold data show statistically significant.

compared with the levels in matched controls [21], a recent report suggested. A recent report suggested that a small placenta volume was noticed more often in cases with early HDP than in unaffected pregnancies [22]; therefore we assumed that the fetal fraction taken before the occurrence of HDP would be low. In contrast, the area under the curve (AUC) using a receiver operating characteristic curve (ROC) analysis was 0.608 for the women with HDP in our study. Although Rolnik et al. reported that the amount of cfDNA in maternal plasma and the fetal fraction at 11–13 and 20–24 weeks of gestation are not predictive of preeclampsia [23], a recent report demonstrates the potential benefit of combined first-trimester cffDNA, total cell-free DNA, and PAPP-A for the early detection of preeclampsia [24]. With angiogenic and antiangiogenic factors as biomarkers, the AUC value for preeclampsia was 0.91 [25].

The maternal weights of women with GDM and HDP were significantly higher than those of controls, and the fetal fraction percentages of GDM women were not significantly different, compared with those of controls. Higher body weights in women with forthcoming HDP may be lower the fetal fraction on NIPT. In obese pregnant women, the circulating DNA is partly come from apoptotic and necrotic cells of adipose and stromal vascular tissues, although circulating DNA in healthy women is mostly derived from hematopoietic cells undergoing apoptosis [26]. We assumed that GDM would be difficult to predict using fetal fraction data gained during early pregnancy. Lu et al. reported that specific early second trimester lipid biomarkers can forecast the maternal GDM status independent of the maternal age and BMI, possibly enhancing risk factor-based assessment [27].

The risk factors for placental abruption contain advanced maternal age, abdominal trauma, hypertension, preterm delivery, preeclampsia, oxidative stress, and intrauterine infections [28–30]. Our present data for placental abruption cases were limited, and we could not find a predictive relationship between cffDNA during early gestation and placental abruption. Recent data suggests that the metabolomic profiles of early pregnancy maternal serum samples in cases with vaginal bleeding may serve as important markers for the prediction of abruption [28].

As of the end of March 2017, NIPT was being performed at 80 institutions, of which 73 were participants in the Japan NIPT consortium. Our prospective nationwide files on NIPT performance in Japan will be important in studying the associations between NIPT result and the postnatal outcome. Further studies using fetal DNA combined with other markers obtained at early gestational age may help in estimating the risk for developing HDP.

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● 原　著 ●

1型糖尿病合併妊娠に関する Sensor Augmented Pump 療法の有用性に関する検討

Efficacy of sensor augmented pump therapy in pregnant women with type 1 diabetes mellitus

小熊　朋子[1)] Tomoko Oguma
楠　　宜樹[2)] Yoshiki Kusunoki
原田佳世子[1)] Kayoko Harada
松岡　理恵[1)] Michie Matsuoka
藤井　雄太[1)] Yuuta Fujii
大杉　敬子[2)] Keiko Osugi
大東　真菜[2)] Mana Oohigashi
鷲尾佳穂里[2)] Kahori Washio
小西　康輔[2)] Kousuke Konishi
田中　宏幸[1)] Hiroyuki Tanaka
小山　英則[2)] Hidenori Koyama
柴原　浩章[1)] Hiroaki Shibahara
難波　光義[2)] Mitsuyoshi Namba

1）兵庫医科大学産科婦人科学講座
Department of obstetrics and Gynecology, Hyogo College of Medicine
2）兵庫医科大学内科学糖尿病・内分泌・代謝科
Division of Diabetes, Endocrinology and Metabolism, Department of Internal Medicine, Hyogo College of Medicine

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【要約】 糖尿病合併妊娠では、母児合併症を予防するために妊娠前から厳格な血糖管理を維持する必要がある。Sensor Augmented Pump（SAP）療法とは、持続グルコースモニター（Continuous glucose monitoring: CGM）機能が備わったインスリンポンプを用いて血糖管理を行う治療法である。今回、日本人 1 型糖尿病合併妊娠における SAP 療法の有用性について検討した。方法は、2012 年 6 月から 2017 年 12 月までに当院で分娩に至った 1 型糖尿病合併妊娠の分娩記録および母児の診療録を後方視的に検討した。SAP 療法群 10 例、MDI 療法群（Multiple Daily Injection：MDI）11 例を検討した。結果は中央値（四分位範囲）で示す。年齢は S 群 33（29 ～ 37）歳、M 群 31（29 ～ 35）歳と両群で有意な差を認めなかったが（P = 0.832）、罹病期間は S 群 19（16 ～ 21）年、M 群 4（2 ～ 14）年と S 群で長い傾向を認めた（P = 0.061）。妊娠前の HbA1c は S 群 7.7（6.6 ～ 8.3）%、M 群 6.6（6.2 ～ 6.8）% と S 群で高い傾向を認めた（P = 0.056）。HbA1c は S 群では妊娠前と比較して妊娠後期 6.6（5.9 ～ 6.8）% と有意な改善を認めたものの（P = 0.021）、M 群では妊娠後期 HbA1c は 6.1（6.0 ～ 6.5）% と有意な変化を認めなかった（P = 0.103）。妊娠中母体の体重変化、母児の周産期帰結には両群で有意な差を認めなかった。本検討の結果から、1 型糖尿病合併妊娠において SAP 療法により母体の重症低血糖や過度な体重増加のリスクを高めることなく安定した血糖管理ができることが明らかとなった。

●キーワード：1 型糖尿病合併妊娠、Sensor augmented pump、Continuous subcutaneous insulin infusion、Continuous glucose monitoring

緒　　言

糖尿病合併妊娠は胎児奇形、妊娠高血圧症候群、胎児胎盤機能不全、巨大児による肩甲難産、新生児低血糖、新生児黄疸など様々な母児合併症をきたすため、妊娠前から厳格な血糖管理を維持することが求められる[1-3]。とりわけ内因性インスリン分泌が枯渇した 1 型糖尿病合併妊娠においては、重症低血糖など不安定な血糖変動を呈することが問題となりやすい[4]。

厳格な血糖管理を行うためには日々の血糖変動を正確に把握することが求められる。そのためには、血糖自己測定（Self-monitoring of blood glucose: SMBG）および持続血糖モニター（Continuous glucose monitoring:

CGM）が用いられてきた。CGM では皮下組織間液中のグルコース濃度を持続的に測定できるため、SMBG よりも日々の血糖変動を詳細に評価することができる[5,6]。CGM にはおもに医療従事者が過去のデータを用いて血糖管理に使用するレトロスペクティブ（プロフェッショナル）CGM と、患者がおもに在宅で使用し、リアルタイムのグルコース値を用いて自己管理に使用するリアルタイム（パーソナル）CGM とがある。

リアルタイム CGM 機能が備わったインスリンポンプを用いて血糖管理を行う治療法を Sensor Augmented Pump（SAP）療法と呼び、1 型糖尿病患者では SAP 療法によって重症低血糖を回避しつつ血糖管理が改善することが示されている[7]。1 型糖尿病合併妊娠の血糖管理においてもリアルタイム CGM や SAP 療法が有用とする海外の報告もあるが[8,9]、わが国において SAP 療法が 1 型糖尿病合併妊娠の血糖管理や周産期合併症の発症予防において有用かどうかについて検討した報告はない。

今回、当院で SAP 療法もしくは頻回注射療法 (multiple daily injection: MDI) で管理され分娩に至った 1 型糖尿病合併妊娠において、母体血糖管理および母児合併症を含む周産期帰結について調査し、SAP 療法の有用性について検討を行った。

対象と方法

1．対象

2012 年 6 月から 2017 年 12 月までに当院で分娩に至った 1 型糖尿病合併妊娠の 21 例（SAP 療法群 10 例、MDI 療法群 11 例）の分娩記録および母児の診療録を後方視的に調査した。MDI 療法群は全例妊娠中 MDI および SMBG で血糖管理を行っていた。SAP 療法群のうち、妊娠前から SAP 療法を導入したのは 4 例、妊娠初期に CSII から SAP 療法を導入した症例が 6 例であった。本検討の内容については、兵庫医科大学倫理委員会の審査を受け承認を得た（承認番号 2586）。

2．調査項目

SAP 療法の有用性を検討するために、おもな調査項目として患者背景、母体の血糖管理指標の推移（HbA1c およびグリコアルブミン（GA））、年齢、罹病期間、母体の体重変化、母児の周産期帰結について調査した。あわせて、インスリン量の変化、重症低血糖や糖尿病ケトアシドーシス発症の有無などについても調査した。

本検討では妊娠初期を妊娠 4 ～ 15 週、妊娠中期を 16 ～ 27 週、妊娠後期を 28 週以降とした。妊娠前の HbA1c は妊娠判明直前の測定値を使用した。HbA1c および GA については、妊娠初期については妊娠 12 週 ± 3 週、妊娠中期については妊娠 24 週 ± 3 週、妊娠後期については分娩前最終の測定値をそれぞれ使用した。

3．解析方法

結果は中央値（四分位範囲）で示した。SAP 療法群と MDI 療法群の比較において、年齢、罹病期間、HbA1c、GA、体重、インスリン量、出生体重、および Apgar Score などについては Mann-Whitney U test を用いて検討を行った。また、妊娠前から分娩直前の HbA1c の変化および妊娠初期から分娩直前の GA の変化については Wilcoxon signed-rank test を用いて検討を行った。SAP 療法群および MDI 療法群の経産婦の割合、随時血清 C- ペプチドが 0.30 ng/mL 未満の割合、糖尿病腎症 2 期以降の割合、巨大児分娩の割合、早産率、帝王切開率などの検定には Fisher's exact test を用いた。本検討では $P < 0.05$ を統計学的有意とした。解析ソフトには BellCurve software（Social Survey Research Information Co. Ltd.; 東京、日本）を使用した。

結　果

1．臨床背景

患者の臨床背景を**表 1** に示す。年齢は SAP 療法群 33（29 ～ 37）歳、MDI 療法群 31（29 ～ 35）歳と両群で有意な差を認めなかった（$P = 0.832$）。罹病期間は SAP 療法群 19（16 ～ 21）年に対し、MDI 療法群は 4（2 ～ 14）年と SAP 療法群で罹病期間が長い傾向を認めた（$P = 0.061$）。妊娠前の HbA1c は SAP 療法群 7.7（6.6 ～ 8.3）%、MDI 療法群 6.6（6.2 ～ 6.8）% と SAP 療法群で高い傾向を認めた（$P = 0.061$）。妊娠前の BMI は SAP 療法群 23.0（19.9 ～ 24.5）kg/m^2、MDI 療法群 20.7（20.2 ～ 21.3）kg/m^2 と両群で有意な差を認めなかった（$P = 0.149$）。妊娠中に測定された随時血清 C- ペプチドは SAP 療法群では全例 0.30 ng/mL 未満、MDI 療法群は 9 例（69%）が 0.30 ng/mL 未満であった（$P = 0.262$）。腎症に関しては、SAP 療法群では 2 例が腎症 2 期、1 例が腎症 3 期で残り 7 例は腎症 1 期、MDI 療法群では全例腎症 1 期であった（$P = 0.090$）。

表1　患者の臨床背景

	SAP療法群	MDI療法群	P値
症例数	10	11	
年齢（歳）	33（29 ～ 37）	31（29 ～ 35）	0.832
罹病期間（年）	19（16 ～ 21）	4（2 ～ 14）	0.061
経妊婦：n（%）	2（20%）	6（55%）	0.119
妊娠前HbA1c（%）	7.7（6.6 ～ 8.3）	6.6（6.2 ～ 6.8）	0.056
妊娠前BMI（kg/m²）	23.0（19.9 ～ 24.5）	20.7（20.2 ～ 21.3）	0.149
血清CPR ＜ 0.30 ng/mL：n（%）	10（100%）	9（82%）	0.262
糖尿病腎症 1期：2期以降	7 ： 3	11 ： 0	0.090
妊娠前TDD（単位/日）	32（26 ～ 42）	27（20 ～ 30）	0.091
TBD/TDD（%）	35（30 ～ 41）	34（25 ～ 37）	0.398

CPR：C-peptide immunoreactivity
TDD: Total daily dose of insulin（総インスリン量）
TBD: Total basal insulin dose（総基礎インスリン量）
中央値(四分位範囲)、または症例数(比率)で示した。
SAP療法群、MDI療法群の比較にはMann-Whitney U testまたはFisher's exact testを用いて検討を行った。

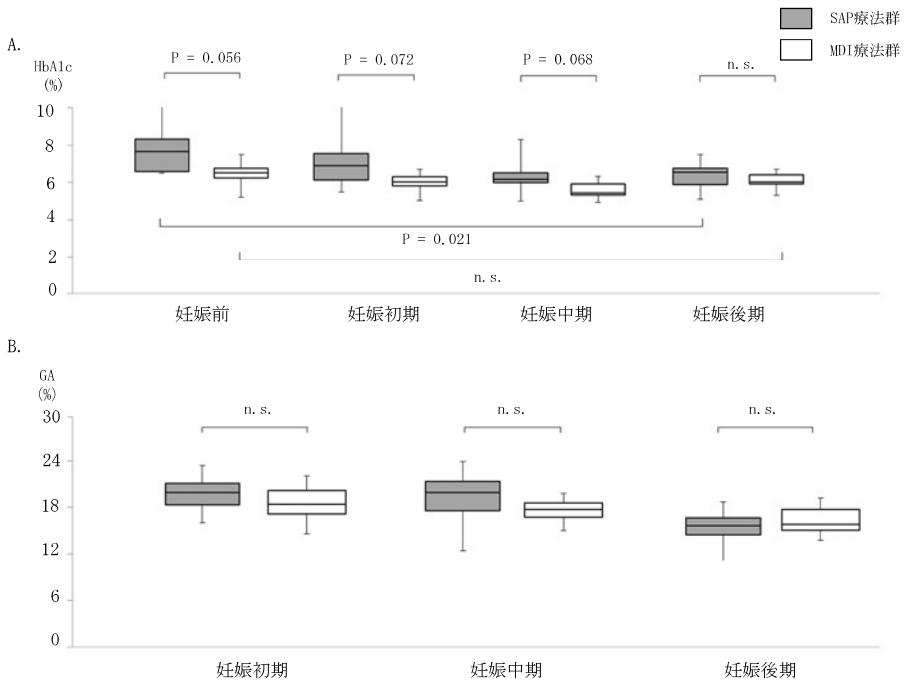


図1　母体血糖変動指標の推移

A：HbA1c、B：GA
妊娠時期ごとのSAP療法群、MDI療法群の比較にはMann-Whitney U testを用いて検討を行った。妊娠前もしくは妊娠初期と妊娠後期の比較にはWilcoxon signed-rank testを用いて検討を行った。n.s; not significant。

2. 血糖管理指標の推移

妊娠経過中の母体のHbA1cおよびGAの推移を図1に示す。本検討の対象者は、日本糖尿病学会のガイドラインにしたがって空腹時血糖値70 ～ 100 mg/dL、食後2時間血糖値120 mg/dL未満を目標として血糖管理されていた[9,10]。

HbA1c は SAP 療法群では妊娠前と比較して妊娠後期に有意に改善を認めた（SAP 療法群 ; 妊娠前 7.7（6.6 ～ 8.3）%、妊娠後期 6.6（5.9 ～ 6.8）%, P = 0.021）。一方で、MDI 療法群では、妊娠前 6.6（6.2 ～ 6.8）% から妊娠後期 6.1（6.0 ～ 6.5）% と改善を認めたものの有意な差を認めなかった（P = 0.103）。妊娠後期の HbA1c については SAP 療法群および MDI 療法群ともに同程度であり、有意な差を認めなかった（P = 0.417）。GA については、SAP 療法群および MDI 療法群とも妊娠初期と比較して分娩前に有意に改善を認めた（SAP 療法群 ; 妊娠初期 19.9（18.3 ～ 21.1）%、妊娠後期 15.7（14.4 ～ 16.7）%、P ＜ 0.01: MDI 療法群 ; 妊娠初期 18.5（17.6 ～ 20.3）%、妊娠後期 16.0（15.2 ～ 18.4）%, P ＜ 0.01）。妊娠後期の GA において SAP 療法群と MDI 療法群との間に有意な差を認めなかった（P = 0.775）。

3. 母体の体重推移

妊娠経過中の母体の体重変化の推移について**図２**に示す。本検討対象者の食事療法は、非肥満妊婦の摂取エネルギーに関しては標準体重× 30 kcal を基本とし、妊娠中に増大するエネルギー需要量の増大に対しては付加量（初期＋ 50 kcal、中期＋ 250 kcal、末期＋ 450 kcal）を加え、肥満妊婦に対しては標準体重 ×30 kcal を基本とし、エネルギー付加は行わないように指導されていた[10-12]。さらに、母体の血糖および体重管理や胎児成長などを参考に妊娠経過ごとに個別に摂取エネルギーや栄養素の配分を調節するように医師や栄養士などが中心となって個別に指導されていた。また、全例当院でカーボカウント法について指導されていた。

SAP 療法群では妊娠経過中の母体の体重変化の中央値は +10.6（+9.6 ～ +12.9）kg、MDI 療法群では母体の体重変化の中央値は +12.5（+10.7 ～ +13.1）kg と両群で有意な差を認めなかった（P = 0.916）。

4. 母体の周産期帰結

母体の周産期帰結について**表２**に示す。妊娠経過中 SAP 療法群では 2 例（20%）、MDI 療法群では 1 例（9%）に妊娠高血圧症候群の発症を認めた（P = 0.462）。分娩週数は SAP 療法群 37 週 2 日、MDI 療法群 38 週 2 日と両群の間に有意な差を認めなかった（P = 0.383）。早産についても SAP 療法群 4 例（40%）、MDI 療法群 2 例（18%）と両群の間に有意な差を認めなかった（P = 0.268）。そのほか、帝王切開率などについても両群で有意な差を認めなかった。重症低血糖による当院への救急搬送例は MDI 療法群で 1 例認めた。糖尿病ケトアシドーシスの発症については両群ともに認めなかった。

5. 児の周産期帰結

児の周産期帰結について**表３**に示す。児の出生体重の中央値は SAP 療法群 3518（3027 ～ 3814）g、MDI 療法群 3160（2978 ～ 3449）g と両群で有意な差を認めなかった（P = 0.504）。4000 g 以上の巨大児については SAP 群と 1 例（10%）、MDI 療法群 1 例（9%）に認め

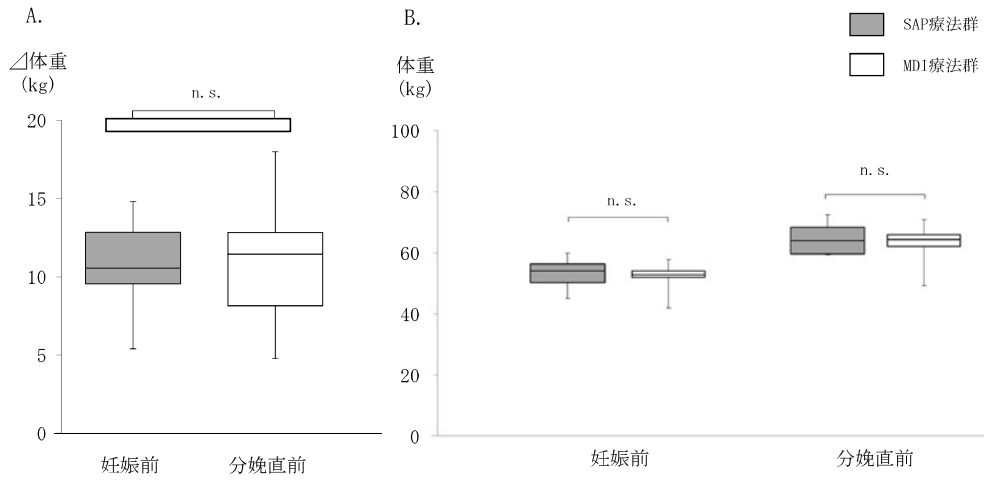


図２　母体体重の推移
A：妊娠前から分娩直前にかけての母体の体重増加量
B：母体体重の推移
SAP 療法群、MDI 療法群の比較には Mann–Whitney U を用いて検討を行った。n.s; not significant

表２　母体の周産期帰結

	SAP療法群（n = 10）	MDI療法群（n = 11）	P値
妊娠高血圧症候群：n（%）	2（20%）	1（9%）	0.462
分娩週数	37週2日 （36週1日～38週4日）	38週2日 （37週5日～39週1日）	0.383
早産；n（%）	4（40%）	2（18%）	0.268
予定日超過：n（%）	0（0%）	0（0%）	0.621
前期破水：n（%）	2（20%）	1（9%）	0.462
帝王切開：n（%）	7（70%） （うち緊急帝王切開2例）	8（73%） （うち緊急帝王切開4例）	0.633
糖尿病ケトアシドーシス（%）	0（0%）	0（0%）	0.621
重症低血糖：n（%）	0（0%）	1（9%）	0.524
分娩直前TDD（単位/日）	46（39 ～ 52）	43（40 ～ 50）	0.503
分娩直前TBD/TDD（%）	31（28 ～ 38）	28（24 ～ 38）	0.230

TDD：総インスリン量、TBD：総基礎インスリン量
中央値(四分位範囲)または症例数(比率)で記した。
SAP療法群、MDI療法群の比較にはMann-Whitney U testまたはFisher's exact testを用いて検討を行った。

表３　児の周産期帰結

	SAP療法群（n = 10）	MDI療法群（n = 11）	P値
出生体重（g）	3518 （3027 ～ 3814）	3160 （2978 ～ 3449）	0.504
Apgar score（1分値）	8（8 ～ 8）	8（8 ～ 9）	0.123
Apgar Score（5分値）	9（9 ～ 9）	9（9 ～ 9）	0.741
巨大児：n（%）	1（10%）	1（9%）	0.738
LGA：n（%）	6（60%）	6（55%）	0.575
胎児死亡：n（%）	0（0%）	0（0%）	0.621
先天奇形：n（%）	0（0%）	0（0%）	0.621
新生児黄疸：n（%）	4（40%）	3（27%）	0.438
呼吸障害：n（%）	1（10%）	1（9%）	0.738
新生児低血糖：n（%）	3（30%）	5（38%）	0.392
心筋症：n（%）	0（0%）	0（0%）	0.621
NICU入室：n（%）	3（30%）	3（27%）	0.633

中央値(四分位範囲)または症例数(比率)で記した。
SAP療法群、MDI療法群の比較にはMann-Whitney U testまたはFisher's exact testを用いて検討を行った。

た（P＝ 0.738）。また、胎児死亡、胎児先天奇形、新生児黄疸、呼吸障害、ブドウ糖補液を必要とした新生児低血糖の有無を比較検討した結果、それぞれに有意な差を認めなかった（表３）。

考　察

SAP 療法ではリアルタイム CGM 機能を使用することができるが、リアルタイム CGM ではリアルタイムのグルコース値やグルコースの流れを患者自身が評価し、自己管理に使用することができる。さらに、低グ

ルコースや高グルコースに到達したことを知らせるアラート機能や、低グルコースや高グルコースに到達することを予測するアラート機能などを使用することができ、重症低血糖などの予防にもつながる[7、13]。実際に 1 型糖尿病合併妊娠においてリアルタイム CGM を使用することで母体の血糖管理は改善し、LGA（Large for Gestational Age）のリスクが低下することが示されている[8]。さらに、CSII では、MDI よりも生理的なインスリン分泌の再現が可能となるため、1 型糖尿病および糖尿病合併妊娠においては MDI よりも血糖管理が改善することが示されている[14、15]。本検討の対象においては、罹病期間が長く、内因性インスリン分泌が枯渇し、不安定な血糖変動を呈している 1 型糖尿病症例に対して SAP をよく使用されていたこともあったため、罹患期間については SAP 療法群で長い傾向を認め、妊娠前の HbA1c についても SAP 療法群が高い傾向を認めたことなど患者背景に差を認めた。しかし、本検討において、SAP 療法群では妊娠前と比較して妊娠後期には母体の HbA1c が有意に改善することが示され、さらに妊娠後期の HbA1c や GA については両群で有意な差を認めなかった。以上より、妊娠前もしくは妊娠初期までに SAP 療法を導入することは内因性インスリン分泌が枯渇した 1 型糖尿病合併妊娠の血糖管理に有用である可能性が示された。

インスリンを用いて厳格な血糖管理を行ううえで、重症低血糖のリスクが増加することが問題となるが[16]、本検討では両群あわせて 1 例に認めたのみであった。とくに SAP 療法では、低グルコースに関するアラート機能などを使用することができるため重症低血糖の予防にもつながることが報告されている[7、13]。本検討の対象者においても SAP 療法群では低グルコースアラートなどを使用することができたため重症低血糖の予防につながったと考えられた。

妊娠経過中の母体の過度な体重増加は巨大児分娩のリスクとなることが報告されている[17、18]。インスリンを用いて厳格な血糖管理を行ううえで母体の体重増加が問題となりやすく[16]、とくに CSII では MDI よりも体重増加が問題となることがあるが[19]、本検討の結果からは妊娠経過中母体の体重変化量は両群で有意な差を認めなかった。本検討の対象者は、妊娠 30 週までは 2 ～ 4 週に 1 度、妊娠 31 週以降は毎週外来または療養指導を受けており、過度な体重増加抑制につながった可能性があるが、単施設の検討であり今後 SAP 療法群の体重変化についてより大規模な検討が必要と考えられる。

本検討において、LGA を SAP 療法群、MDI 療法群ともに 6 例認めた。糖代謝異常合併妊娠では、母体の平均血糖値のみならず食後血糖値や血糖変動指標と児出生体重が関連することが報告されている[6、20、21]。HbA1c は同程度でも 2 型糖尿病と比較して 1 型糖尿病では血糖変動幅の指標となる標準偏差が高値となることが報告されている[22]。実際に、1 型糖尿病妊娠においては HbA1c が良好な血糖管理が得られても巨大児のリスクが上昇することが報告されており[23]、今後 1 型糖尿病合併妊娠において LGA や巨大児分娩と母体の血糖管理を含めたさまざまな要因についてさらなる検討を進めていくことが必要である。また、児の周産期帰結の検討では、先天奇形や胎児死亡、新生児黄疸、呼吸障害、新生児低血糖などにおいて両群で有意な差を認めなかったが、今後症例数をさらに増やし検討していく必要がある。

本検討の限界としては、単施設で後方視的に調査している点が挙げられる。今回の解析では SAP 療法群がより病期が進行していた背景より、母体の血糖管理および児帰結に与える影響についてさらに検討するためには、妊娠前から SAP による血糖管理を行っている糖尿病患者を対象とした、さらに大規模な前向き研究が必要であると考えられた。

結論としては、本検討の結果から 1 型糖尿病合併妊娠において SAP 療法により母体の重症低血糖や過度な体重増加のリスクを高めることなく安定した血糖管理ができる可能性があることが明らかとなった。しかし、1 型糖尿病合併妊娠における SAP 療法の有用性を検討するためには妊娠前から SAP 療法を導入し、さらに症例数を増やして検討する必要があると考えられた。

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Paternal occupational exposure to chemicals and secondary sex ratio: results from the Japan Environment and Children's Study

*Sho Adachi, Junko Sawaki, Narumi Tokuda, Hiroyuki Tanaka, Hideaki Sawai, Yasuhiro Takeshima, Hiroaki Shibahara, Masayuki Shima, the Japan Environment and Children's Study Group**

Summary

Background Declining proportions of male births have been reported in several industrialised countries. Previous reports have shown that exposure to certain chemical substances might influence the secondary sex ratio (SSR). We assessed the associations between paternal occupational exposure to chemicals and the SSR of their children using the Japan Environment and Children's Study (JECS), a large-scale birth cohort study.

Methods Data on paternal occupational exposure to various agents and other covariates were collected using a self-administered questionnaire to partners of pregnant female participants enrolled in the JECS. After adjusting for potential confounders, multivariable modified Poisson regression models were used to evaluate associations between paternal occupational exposures and the SSR of their children. This study was registered in the UMIN Clinical Trials Registry, number UMIN000030786.

Findings The JECS study gathered data on 103 062 pregnancies, 104 065 fetuses, and 51 898 partners of pregnant women. Among 50 283 children with data on paternal occupational exposures, 25 657 were male and 24 626 were female. The proportion of boys whose fathers were regularly occupationally exposed to insecticides was 0·445 (males, n=293; females, n=366; 95% CI 0·406–0·483), which was lower than the proportion of boys whose fathers were not exposed to insecticides. After adjusting for confounding factors, regular paternal occupational exposure to insecticides (adjusted relative risk 0·86, 95% CI 0·78–0·96) and medical disinfectants (0·95, 0·90–1·00) were significantly associated with lower SSRs among their offspring compared with the offspring of fathers not exposed to these substances.

Interpretation A declining proportion of boys could potentially be due to fathers working in environments in which they are exposed to chemicals. The associations between poorer semen quality and levels of reproductive and thyroid hormones require investigation.

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*Study group members are listed at the end of the Article

Hyogo Regional Center for the Japan Environment and Children's Study (S Adachi PhD, J Sawaki PhD, N Tokuda MS, Prof Y Takeshima PhD, Prof H Shibahara PhD, Prof M Shima PhD), **Department of Clinical Genetics** (Prof H Sawai PhD), **Department of Pediatrics** (Prof Y Takeshima), **Department of Obstetrics and Gynecology** (Prof H Shibahara), **and Department of Public Health** (S Adachi, Prof M Shima), **Hyogo College of Medicine, Nishinomiya, Japan; and Department of General Medicine and Community Health Science, Sasayama Medical Center, Sasayama, Japan** (Prof H Tanaka PhD)

Correspondence to:
Prof Masayuki Shima, Department of Public Health, Hyogo College of Medicine, Nishinomiya 663-8501, Japan
shima-m@hyo-med.ac.jp

Introduction

According to the Japanese Government's Specified Report of Vital Statistics, published in 2017,[1] the sex ratio at birth in Japan has ranged from 104·9 to 106·0 males for every 100 females since 1981. Although this ratio is similar to that observed worldwide,[2] the ratio between 1968 and 1980 in Japan has been reported to be slightly higher (106·0–107·2).[1] In the past 50 years, a trend towards a declining proportion of male births has been reported in several other industrialised countries;[3] exposures to oestrogenic or other hormonally active environmental chemicals during fetal and childhood development have been speculated to adversely affect male reproductive health.[4] In some countries, the male-to-female ratio at birth is biased owing to prenatal sex determination and selective abortion of fetuses of particular sexes.[5] The sex of a child is determined through the sex-determining region on the Y chromosome,[6] but factors that affect the sex ratio of infants are largely unknown.

People worldwide are exposed to harmful substances in many workplaces, including chemical substances[7] and radiation.[8] Chemical substances are classified according to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS).[9] The GHS evaluates reproductive toxicities, including adverse effects on the sexual function and fertility of adults and the development of offspring. However, the GHS does not consider the influence on the secondary sex ratio (SSR); ie, the ratio of male to female offspring.[9]

Previous studies[10–17] have shown that some chemical substances influence the SSR. Girls were reportedly more likely to be born to fathers with increased serum dioxin concentrations.[10,11] Exposures to polychlorinated biphenyls[12–14] and mercury[13] have also been shown to affect the SSR. Gupta and colleagues[15] reported that parents

Research in context

Evidence before this study

We searched PubMed using the keywords "sex ratio (including "gender ratio") " and "exposure (including "exposed" or "chemical")", for articles published in English from inception until Sept 19, 2019. Of 1874 articles, 118 relevant articles were identified. In studies including small numbers of exposed parents, exposures to various substances, including polychlorinated biphenyl, mercury, dioxin, inhalation anaesthetics, and ionising radiation have been reported to affect the secondary sex ratio (SSR). However, findings from large-scale birth cohort studies have been limited, and few studies have comprehensively investigated the relationship between occupational exposures and SSR.

Added value of this study

In this study, high-quality data were obtained from the Japan Environment and Children's Study (JECS), a large-scale prospective national birth cohort study that collected data on 103 062 pregnancies, 104 065 fetuses, and 51 898 partners of pregnant women enrolled between Jan 24, 2011, and March 31, 2014. The association between paternal occupational exposures to 23 chemical substances and SSR in their offspring was comprehensively investigated. Our findings indicate that regular paternal occupational exposures to insecticides lowered the SSRs in their offspring compared with fathers without exposure. To our knowledge, this is the first study to report an association between paternal exposure to insecticides and the SSR in a large-scale birth cohort study.

Implications of all the available evidence

A declining proportion of boys among fathers occupationally exposed to insecticides might be related to poorer semen quality and alterations in reproductive hormone levels. Our findings provide valuable evidence for the considerable effect of paternal occupational exposure to insecticides on SSR. The decline in the proportion of boys could potentially be affected by fathers working in environments that commonly involve insecticide use. Care should be taken to prevent occupational exposure to insecticides, especially in male workers of reproductive age.

who worked in anaesthesiology and used inhalational anaesthetic agents in their workplace were more likely to have female offspring. Moreover, Alexopoulos and colleagues[17] reported that belonging to groups such as sandblasters or painters and ship carpenters was significantly associated with lower offspring SSRs.

We used data from a large-scale nationwide birth cohort study to examine associations between paternal occupational exposures to various substances and the SSR of their children.

Methods

Study design and participants

We obtained data from the Japan Environment and Children's Study (JECS), a large-scale prospective birth cohort study.[18] Pregnant women were recruited between Jan 24, 2011, and March 31, 2014. This study was registered in the UMIN Clinical Trials Registry (number UMIN000030786). The study design,[18] baseline profile of participants[19] in the JECS, and study protocol are described elsewhere. The JECS protocol was approved by the institutional review board on epidemiological studies of the Ministry of the Environment of Japan and by the ethics review boards of all participating institutions. The JECS was done in accordance with the Declaration of Helsinki and the Japanese Ethical Guidelines for Epidemiological Research published by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor, and Welfare, Japan. Written informed consent was obtained from all participants.

For the **study protocol** see https://www.env.go.jp/chemi/ceh/en/about/index.html

After registration, the mothers' data were collected using self-administered questionnaires during the first trimester. Fathers were recruited in the period between recruitment of the mother and the postnatal check-up of the child, 1 month after birth. The fathers' data were also collected using a self-administrated questionnaire. The children's medical records at birth, including sex, were transcribed by physicians, midwives, nurses, or research coordinators.

We investigated the relationship between the occupational exposures of fathers in the period around the onset of pregnancy and the proportions of boys using data from the JECS study. Data for current analyses were extracted from the dataset released in March, 2018 (dataset jecs-an-20180131).

Variables

Data regarding the occupational exposures of the parents around the onset of pregnancy were obtained from responses to the questionnaires. Fathers responded to questions concerning the frequency of exposure to various chemicals at work during the 3 months before their child's conception. Alternatively, mothers responded to questions concerning their own exposure to various chemicals at work during the first trimester. The questionnaire assessed occupational exposures to kerosene, petroleum, benzene, or gasoline; chlorine bleach or germicides; medical disinfectants; permanent markers; water-based paints or inkjet printers; organic solvents (including paint thinner; solvents for examination, analysis, or extraction; dry-cleaning detergents; stain-removing agents; paint coatings; and nail polish removers); photocopiers or laser printers; engine oil; formalin or formaldehyde; anticancer drugs (excluding those prescribed to the participants as part of their treatment protocol); general anaesthetics for surgery; insecticides; herbicides; unidentified or other agricultural chemicals; radiation, radioactive substances,

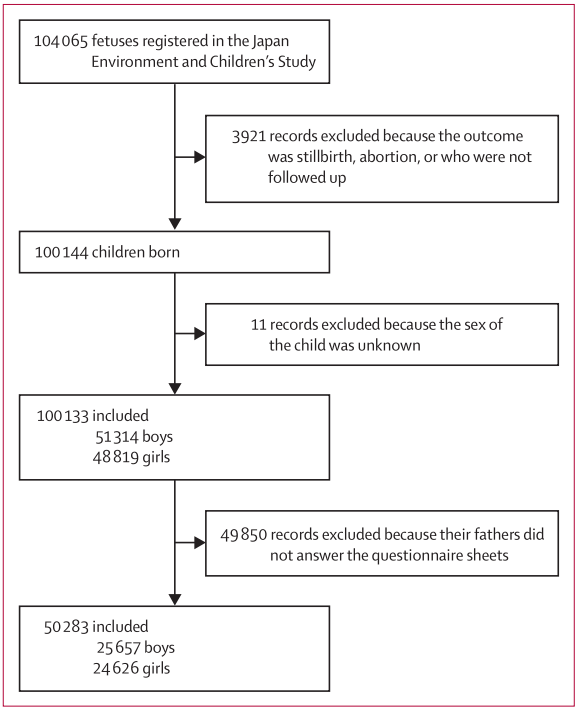


Figure: **Flowchart of study participant selection**

or isotopes; microbes; any products containing lead (including solder); lead-free solder; chromium, arsenic, or cadmium; mercury; dyestuffs (for hair colouring); gases generated during welding; and other chemical substances. Exposures were defined as those occurring for more than half a day at work. The frequencies of exposure were categorised into three groups: no exposure, occasional exposure (one to three times a month), and regular exposure (once a week or more). Data on other parental covariates including age, alcohol consumption status, smoking status, and occupational classification were collected on the basis of the parents' responses to the questionnaires. The economic status was evaluated based on data pertaining to household income, and data were obtained from the responses to questionnaires during the second or third trimester. Data on the sex of the children were collected at birth by obstetricians.

Statistical analysis

The proportions of male offspring were compared among the frequencies of each occupational exposure, and the corresponding trends were estimated using the Cochran-Armitage test for linear trends. A modified Poisson regression approach (ie, a Poisson regression model with a robust error variance)[20] was used to evaluate the factors that affected the male proportions after adjusting for paternal age (<25 years, 25–34 years, 35–44 years, and ≥45 years), maternal age (<25 years, 25–34 years, and ≥35 years), parental alcohol consumption status (non-drinkers, ex-drinkers, and current drinkers), smoking status (non-smokers, ex-smokers [quit before

	Children (n=50 283)
Age, years	32 (29–36)
Smoking status	
Non-smokers	14 375 (28·6%)
Ex-smokers (quit before pregnancy)	11 582 (23·0%)
Ex-smokers (quit during early pregnancy)	2391 (4·8%)
Current smokers	21 092 (41·9%)
Alcohol consumption status	
Non-drinkers	10 646 (21·2%)
Ex-drinkers	1881 (3·7%)
Current drinkers	37 578 (74·7%)
Paternal occupational exposure	
Kerosene, petroleum, benzene, or gasoline	11 073 (23·2%)
Chlorine bleach or germicide	5906 (12·5%)
Medical disinfectant	4119 (8·7%)
Permanent marker	17 992 (37·6%)
Water-based paint or inkjet printer	14 046 (29·6%)
Organic solvents	7556 (15·9%)
Photocopier or laser printer	19 846 (41·4%)
Engine oil	5409 (11·4%)
Formalin or formaldehyde	550 (1·2%)
Anticancer drug	289 (0·6%)
General anaesthetic for surgery	344 (0·7%)
Insecticide	5210 (11·0%)
Herbicide	1902 (4·0%)
Unidentified or other agricultural chemicals	565 (1·2%)
Radiation, radioactive substances, or isotopes	1536 (3·3%)
Microbes	555 (1·2%)
Any products containing lead	2267 (4·8%)
Lead-free solder	1552 (3·3%)
Chromium, arsenic, or cadmium	259 (0·6%)
Mercury	156 (0·3%)
Dyestuffs	2790 (5·9%)
Gases generated during welding	3290 (7·0%)
Other chemical substances	1408 (3·0%)
Paternal occupational category	
Managers	2004 (4·1%)
Professionals and technicians	14 769 (30·0%)
Clerical support workers	4584 (9·3%)
Sales workers	5267 (10·7%)
Service workers	5504 (11·2%)
Protective service workers	2020 (4·1%)
Skilled agricultural, forestry, and fisheries workers	861 (1·8%)
Craft and manufacturing workers	6489 (13·2%)
Drivers and machine operators	1968 (4·0%)
Construction or mining workers	3229 (6·6%)
Package deliverers, cleaners, hand packers, and other elementary workers	669 (1·4%)
Full-time homemakers	65 (0·1%)
Students	282 (0·6%)
Unemployed	385 (0·8%)
Occupations not classified above	1095 (2·2%)

Data are median (IQR) or n (%).

Table 1: **Paternal characteristics and occupational exposures**

	Male	Female	Proportion of male births (95% CI)	χ^2 statistic*	p value*
Kerosene, petroleum, benzene, or gasoline				0·08	0·78
No	18 692	17 958	0·510 (0·505–0·515)	..	..
Occasional	3506	3354	0·511 (0·499–0·523)	..	..
Regular	2157	2056	0·512 (0·497–0·527)	..	..
Chlorine bleach or germicides				1·28	0·26
No	21 118	20 389	0·509 (0·504–0·514)	..	..
Occasional	2170	1999	0·521 (0·505–0·536)	..	..
Regular	892	845	0·514 (0·490–0·537)	..	..
Medical disinfectants				2·78	0·095
No	22 070	21 144	0·511 (0·506–0·515)		
Occasional	879	812	0·520 (0·496–0·544)	..	..
Regular	1187	1241	0·489 (0·469–0·509)	..	..
Permanent marker				3·42	0·065
No	15 112	14 713	0·507 (0·501–0·512)	..	..
Occasional	4801	4514	0·515 (0·505–0·526)	..	..
Regular	4481	4196	0·516 (0·506–0·527)	..	..
Water-based paint or inkjet printers				0·34	0·56
No	16 987	16 436	0·508 (0·503–0·514)	..	..
Occasional	3776	3535	0·516 (0·505–0·528)	..	..
Regular	3431	3304	0·509 (0·497–0·521)	..	..
Organic solvents				1·05	0·30
No	20 348	19 633	0·509 (0·504–0·514)	..	..
Occasional	1951	1836	0·515 (0·499–0·531)	..	..
Regular	1945	1824	0·516 (0·500–0·532)	..	..
Photocopier or laser printers				2·32	0·13
No	14 263	13 868	0·507 (0·501–0·513)	..	..
Occasional	2652	2505	0·514 (0·501–0·528)	..	..
Regular	7557	7132	0·514 (0·506–0·523)	..	..
Engine oil				0·30	0·59
No	21 452	20 659	0·509 (0·505–0·514)	..	..
Occasional	1744	1634	0·516 (0·499–0·533)	..	..
Regular	1039	992	0·512 (0·490–0·534)	..	..
Formalin or formaldehyde				0·24	0·62
No	23 844	22 907	0·510 (0·505–0·515)	..	..
Occasional	164	182	0·474 (0·420–0·528)	..	..
Regular	106	98	0·520 (0·449–0·590)	..	..
Anticancer drugs				0·27	0·60
No	23 967	23 034	0·510 (0·505–0·514)	..	..
Occasional	98	81	0·547 (0·472–0·622)	..	..
Regular	56	54	0·509 (0·412–0·606)	..	..
General anaesthetic for surgery				6·00	0·014
No	23 968	22 994	0·510 (0·506–0·515)	..	..
Occasional	66	91	0·420 (0·342–0·502)	..	..
Regular	84	103	0·449 (0·377–0·523)	..	..
Insecticides				6·37	0·012
No	21 574	20 611	0·511 (0·507–0·516)	..	..
Occasional	2306	2245	0·507 (0·492–0·521)	..	..
Regular	293	366	0·445 (0·406–0·483)	..	..
Herbicides				0·52	0·47
No	23 144	22 209	0·510 (0·506–0·515)	..	..
Occasional	894	865	0·508 (0·485–0·532)	..	.
Regular	66	77	0·462 (0·378–0·547)	..	.

(Table 2 continues on next page)

	Male	Female	Proportion of male births (95% CI)	χ^2 statistic*	p value*
(Continued from previous page)					
Unidentified or other agricultural chemicals				0·76	0·38
No	23 474	22 547	0·510 (0·505–0·515)	..	..
Occasional	227	208	0·522 (0·474–0·570)	..	..
Regular	57	73	0·438 (0·352–0·528)	..	..
Radiation, radioactive substances, or isotopes				2·28	0·13
No	23 211	22 258	0·510 (0·506–0·515)	..	..
Occasional	314	337	0·482 (0·443–0·521)	..	..
Regular	436	449	0·493 (0·459–0·526)	..	..
Microbes				0·48	0·49
No	23 654	22 756	0·510 (0·505–0·514)	..	..
Occasional	140	117	0·545 (0·482–0·607)	..	..
Regular	154	144	0·517 (0·458–0·575)	..	..
Any products containing lead				0·04	0·84
No	22 798	21 956	0·509 (0·505–0·514)	..	..
Occasional	815	729	0·528 (0·503–0·553)	..	..
Regular	358	365	0·495 (0·458–0·532)	..	..
Lead-free solder				0·03	0·86
No	23 145	22 273	0·510 (0·505–0·514)	..	..
Occasional	542	500	0·520 (0·489–0·551)	..	..
Regular	257	253	0·504 (0·460–0·548)	..	..
Chromium, arsenic, or cadmium				0·73	0·39
No	23 818	22 905	0·510 (0·505–0·514)	..	..
Occasional	66	69	0·489 (0·402–0·576)	..	..
Regular	70	54	0·565 (0·473–0·653)	..	..
Mercury				0·005	0·95
No	23 847	22 934	0·510 (0·505–0·514)	..	..
Occasional	63	52	0·548 (0·452–0·641)	..	..
Regular	19	22	0·463 (0·307–0·626)	..	..
Dyestuffs				0·54	0·46
No	22 533	21 685	0·510 (0·505–0·514)	..	..
Occasional	1242	1170	0·515 (0·495–0·535)	..	..
Regular	198	180	0·524 (0·472–0·575)	..	..
Gases generated during welding				0·23	0·63
No	22 318	21 517	0·509 (0·504–0·514)	..	..
Occasional	1021	912	0·528 (0·506–0·551)	..	..
Regular	684	673	0·504 (0·477–0·531)	..	..
Other chemical substances				2·05	0·15
No	23 044	22 188	0·509 (0·505–0·514)	..	..
Occasional	242	236	0·506 (0·460–0·552)	..	..
Regular	498	432	0·535 (0·503–0·568)	..	..

*Cochran–Armitage test for linear trend among three categories (no, occasional, and regular exposure; df is 1).

Table 2: **Associations between paternal occupational exposure and proportion of boys**

pregnancy], ex-smokers [quit during early pregnancy], and current smokers), economic status (<¥4 million and ≥¥4 million), occupational classification, and occupational exposure to other chemical substances. A modified Poisson regression model with a robust error variance is considered to be appropriate and frequently applied to estimate relative risk for common binary outcomes.[20,21] In multivariable analyses, cases with missing values (n=10 744) were excluded and 39 539 cases were included. The trends in male proportions between ordinal categories of exposure frequencies were investigated using the categories of exposure frequency as continuous variables in the modified Poisson regression model. For sensitivity analyses, using a multiple imputation model based on the expectation maximisation with bootstrapping algorithm, the model was analysed to avoid possible selection bias (n=50 283). In addition, the modified Poisson regression models were rerun after additionally

adjusting for maternal occupational exposures (n=34 010). Models excluding multiple births (n=943) and children born after blastocyst transfer (n=1231) were also analysed

See Online for appendix

	Adjusted relative risk (95% CI)	Wald χ^2 statistic	p value
Kerosene, petroleum, benzene, or gasoline		1·27	0·26
No	1 (ref)	..	..
Occasional	0·99 (0·96–1·02)	..	..
Regular	0·97 (0·93–1·01)	..	..
Chlorine bleach or germicides		3·58	0·058
No	1 (ref)	..	..
Occasional	1·02 (0·98–1·06)	..	..
Regular	1·05 (0·99–1·11)	..	..
Medical disinfectants		2·69	0·10
No	1 (ref)	..	..
Occasional	1·02 (0·97–1·08)	..	..
Regular	0·95 (0·90–1·00)	..	..
Permanent marker		1·88	0·17
No	1 (ref)	..	..
Occasional	1·02 (0·99–1·06)	..	..
Regular	1·02 (0·99–1·06)	..	..
Water-based paint or inkjet printers		0·46	0·50
No	1 (ref)	..	..
Occasional	1·00 (0·97–1·04)	..	..
Regular	0·98 (0·95–1·02)	..	..
Organic solvents		0·06	0·81
No	1 (ref)	..	..
Occasional	0·99 (0·95–1·03)	..	..
Regular	1·00 (0·95–1·04)	..	..
Photocopier or laser printers		1·24	0·27
No	1 (ref)	..	..
Occasional	1·02 (0·98–1·06)	..	..
Regular	1·01 (0·98–1·04)	..	..
Engine oil		0·54	0·46
No	1 (ref)	..	..
Occasional	0·99 (0·95–1·04)	..	..
Regular	1·03 (0·97–1·09)	..	..
Formalin or formaldehyde		0·09	0·76
No	1 (ref)	..	..
Occasional	0·94 (0·82–1·07)	..	..
Regular	1·08 (0·93–1·26)	..	..
Anticancer drugs		1·88	0·17
No	1 (ref)	..	..
Occasional	1·17 (1·00–1·37)	..	..
Regular	1·08 (0·86–1·35)	..	..
General anaesthetic for surgery		3·32	0·068
No	1 (ref)	..	..
Occasional	0·85 (0·69–1·05)	..	..
Regular	0·87 (0·71–1·06)	..	..
Insecticides		7·56	0·0060
No	1 (ref)	..	..
Occasional	0·96 (0·93–1·00)	..	..
Regular	0·86 (0·78–0·96)	..	..

(Table 3 continues on next page)

in view of a reported association between these factors and high SSR.[22] Furthermore, the proportion of male offspring in each occupational exposure group was compared with fathers exposed to none of the 23 substances as a reference group. The modified Poisson regression models were also done for each substance to compare with fathers exposed to none of the substances as a reference group. The significance level for all statistical analyses was set at $p<0·05$. All statistical analyses were done using the R software (version 3.6.1) package.

Role of the funding source

The funder of the Japan Environment and Children's Study (JECS) participated in the creation of the study design and data collection of the JECS. However, it had no role in the study design, data analysis, data interpretation, or Article preparation for the present study. The corresponding author had full access to all data in the study, and had final responsibility for the decision to submit for publication.

Results

The JECS study gathered data concerning 103 062 pregnancies, 104 065 fetuses, and 51 898 partners of pregnant women. Of these, 50 283 children (25 657 and 24 626 boys and girls, respectively), whose sex was determined at birth by an obstetrician, whose fathers consented to participate in the JECS and completed the questionnaire, were analysed (figure). The fathers' characteristics and occupational exposures are shown in table 1. The fathers' median age was 32 years (IQR 29–36). Among 23 types of exposure, the prevalence of paternal occupational exposure to photocopiers or laser printers was the highest (41·4%). Professionals and technicians comprised the highest proportion (30·0%) among the occupational categories. Characteristics of the children included in this analysis (n=50 283) were similar to those of children whose fathers were not participants in this study (n=49 850; appendix p 1).

The associations between paternal occupational exposures and the proportions of boys are shown in table 2. For paternal occupational exposure to insecticides, the proportions of boys were 0·511 (95% CI 0·507–0·516), 0·507 (0·492–0·521), and 0·445 (0·406–0·483) for none, occasional, and regular exposure to insecticides, respectively; and the proportion in the regularly exposed group was smaller than that of the group not exposed to insecticides. The proportion of boys declined significantly with increasing frequencies of paternal exposure to insecticides (p=0·012). The proportion of boys was also more likely to further decrease significantly in fathers with paternal occupational exposure to general surgical anaesthetics for surgery (p=0·014). Among fathers with regular occupational exposure to medical disinfectants, the proportion of boys was lower than the proportion in fathers with no exposure to medical disinfectants;

however, the trend in the exposure frequency was not significant (p=0·095). The association between paternal occupational exposures to other chemical substances and the proportion of boys was not significant.

The results of the multivariable analysis using a modified Poisson regression model are shown in table 3. Compared with fathers with no exposure to insecticides, fathers with regular occupational exposure to insecticides had lower proportions of boys (adjusted relative risk [RR] 0·86, 95% CI 0·78–0·96), and we found a declining trend in the proportion of boys at birth with higher frequencies of paternal exposure to insecticides (p=0·0060). An association between regular paternal occupational exposure to medical disinfectants and low proportion of boys was also observed (adjusted RR 0·95, 0·90–1·00); however, the trend in exposure frequency was not significant (p=0·10). Occasional exposures to insecticides or medical disinfectants did not significantly affect the SSR.

In a sensitivity analysis based on multiple imputation to avoid possible selection bias, the association between regular paternal occupational exposure to insecticides and a decrease in the proportion of boys remained significant (appendix pp 2–3). With further adjustment for maternal occupational exposures, the association between regular paternal occupational exposure to insecticides and reductions in the proportion of boys remained significant, and a significant decline in the proportion of boys in fathers with higher exposure to general anaesthetic for surgery was also observed (appendix pp 4–5). In the model excluding multiple births, both occasional and regular paternal occupational exposures to insecticides were still associated with low proportions of boys (appendix pp 6–7). Additionally, in the model excluding children born after blastocyst transfer, associations between regular paternal occupational exposures to insecticides and medical disinfectants and a decrease in the proportion of boys remained significant (appendix pp 8–9). Furthermore, even when the reference group was fathers exposed to none of the 23 substances, the proportion of boys was smaller in fathers regularly exposed to insecticides (appendix pp 10–12). In a multivariable analysis, the association with exposure to insecticides was similar (appendix pp 13–15).

	Adjusted relative risk (95% CI)	Wald χ^2 statistic	p value
(Continued from previous page)			
Herbicides		0·77	0·38
No	1 (ref)	..	..
Occasional	0·97 (0·92–1·04)	..	..
Regular	0·94 (0·73–1·20)	..	..
Unidentified or other agricultural chemicals		0·16	0·69
No	1 (ref)	..	..
Occasional	1·01 (0·90–1·14)	..	..
Regular	0·94 (0·74–1·20)	..	..
Radiation, radioactive substances, or isotopes		1·14	0·29
No	1 (ref)	..	..
Occasional	0·95 (0·87–1·04)	..	..
Regular	0·97 (0·89–1·05)	..	..
Microbes		0·24	0·62
No	1 (ref)	..	..
Occasional	1·07 (0·93–1·21)	..	..
Regular	1·02 (0·90–1·16)	..	..
Any products containing lead		0·005	0·95
No	1 (ref)	..	..
Occasional	1·03 (0·97–1·11)	..	..
Regular	0·96 (0·86–1·07)	..	..
Lead-free solder		0·01	0·91
No	1 (ref)	..	..
Occasional	1·00 (0·93–1·08)	..	..
Regular	1·01 (0·90–1·14)	..	..
Chromium, arsenic, or cadmium		0·20	0·66
No	1 (ref)	..	..
Occasional	0·96 (0·79–1·17)	..	..
Regular	1·08 (0·89–1·30)	..	..
Mercury		0·003	0·96
No	1 (ref)	..	..
Occasional	1·16 (0·96–1·39)	..	..
Regular	0·80 (0·51–1·25)	..	..
Dyestuffs		0·42	0·52
No	1 (ref)	..	..
Occasional	1·01 (0·97–1·06)	..	..
Regular	1·00 (0·89–1·13)	..	..
Gases generated during welding		0·95	0·33
No	1 (ref)	..	..
Occasional	1·05 (0·99–1·10)	..	..
Regular	1·01 (0·95–1·08)	..	..
Other chemical substances		0·12	0·73
No	1 (ref)	..	..
Occasional	0·97 (0·87–1·08)	..	..
Regular	1·02 (0·95–1·10)	..	..

Adjusted for economic status, paternal age, smoking status, alcohol consumption status, occupational classification, and occupational exposures to 23 chemical substances; and maternal age, smoking status, and alcohol consumption status. Test for linear trend was calculated in a modified Poisson regression model using the categories of exposure frequencies as continuous variables.

Table 3: **Adjusted relative risks for having a boy (n=39 539)**

Discussion

In this large-scale birth cohort study, we examined the relationship between paternal occupational exposures to various substances and the SSR in their offspring. Our findings showed that paternal occupational exposures to insecticides and medical disinfectants were associated with low SSRs. To our knowledge, this is the first study to report an association between paternal exposure to insecticides and SSR.

Bae and colleagues[16] previously reported that the maternal serum concentrations of mirex, which has

never been registered as an insecticide in Japan, were associated with an altered SSR; however, association with paternal exposure has not been reported. Exposure to pesticides has reportedly been associated with poorer semen quality, including a high rate of sperm sex chromosome disomy[23] and reduced fertility in male workers.[24] In the present study, the proportions of boys were low not only among those exposed to insecticides, but also among regular users of herbicides or unidentified or other agricultural chemicals. These chemicals are frequently used by farmers, but the proportion of boys among fathers engaged in agriculture was similar to that of all the study participants (data not shown), Additionally, after adjusting for covariates and other occupational exposures, relative risks for boys among fathers regularly exposed to herbicides or unidentified or other agricultural chemicals became close to one. However, occupational exposure to insecticides was robustly associated with a decreased proportion of boys. Therefore, we consider that the lower proportion of boys was due to regular use of insecticides, and not due to working as a farmer.

The most frequently used medical disinfectants in Japan include iodine preparations and ethanol. The association between iodine and the proportion of boys and semen quality remains unclear. However, an excessive intake of iodine is known to be responsible for thyroid dysfunction,[25] and free thyroxine concentrations have been reported to be associated with semen quality.[26] Therefore, our results might have been related to thyroid dysfunction. A subcutaneous injection of ethanol has been shown to lower the SSR in mice,[27] and semen quality has been reported to be poorer in alcoholic men.[28] However, the absorption of ethanol from occupational exposure is expected to be minimal.

Gupta and colleagues[14] reported that although anaesthesia care providers are significantly more likely to have firstborn female offspring, the mechanism is unknown. In this study, univariable analyses showed that paternal occupational occasional exposure to general anaesthetics for surgery was associated with a low proportion of boys at birth. Although not significant, the relative risks for having a boy were less than one in both the occasional and regular exposure groups after adjusting for potential confounders. In the sensitivity analysis after additionally adjusting for maternal occupational exposures, increasing frequencies of paternal exposure to general anaesthetics significantly decreased the trend for having a boy (appendix pp 4–5). This suggests the presence of confounding factors, such as other exposures and maternal exposures. Given medical disinfectants and anaesthetics for surgery are both usually used in the same medical field, exposures to either agent might affect the proportion of boys among exposed fathers, which could have been a confounding factor.

Exposures to mercury[13] and radiation[29] have been reported to affect the proportion of boys and semen quality. At present, these substances are regulated in Japan and the exposure levels among workers are minimal.[30] We did not observe a relationship between these substances and the proportion of boys.

We identified that paternal occupational exposure to some substances was associated with low SSRs. The effect of exposure to insecticides on low SSR was substantial. Further studies are needed to clarify the mechanisms of the lower SSR observed following exposure to these substances. In particular, the association between insecticides and medical disinfectants and poorer semen quality and levels of reproductive and thyroid hormones should be evaluated. In addition to other reproductive toxicities that are routinely examined, it might be necessary to evaluate the effect of new substances used in the workplace on SSRs.

In a previous study on the JECS cohort, Hattori and colleagues[19] found that blastocyst transfer was associated with a higher SSR. After excluding children born after blastocyst transfer, the association between regular paternal occupational exposure to insecticides and medical disinfectants and a decrease in the proportion of male offspring was still significant (appendix pp 8–9).

This study had several limitations. First, information concerning occupational exposures was obtained from responses to self-administrated questionnaires; therefore, the exposures might not have been evaluated objectively. Additionally, exposure-response trends were estimated with only three ordinal categories using a test for linear trend. Therefore, careful attention must be paid to the results of the tests. However, for exposure to insecticides, we consider that an exposure-response relationship was shown. Second, the analyses included only 50·2% of the children who were born in the JECS, and whose sex had been determined. This might have led to a selection bias; however, the proportions of males to females were similar between children included and not included in this study (appendix p 1). Moreover, on multivariable analysis, 10744 cases with missing values were excluded, and only 39539 cases were analysed. Furthermore, between fathers included and excluded during multivariable analyses, the proportion of boys was similar, although there were some differences in the other characteristics, including household income, and educational and smoking status (appendix p 16). A model with multiple imputation was used to avoid selection bias and showed almost the same results as the complete case analyses (appendix pp 2–3). Third, no biomarkers, such as 3-phenoxybenzoic acid, a metabolite of pyrethroid insecticides, were examined. Finally, this study did not evaluate the semen quality and levels of reproductive and thyroid hormones in the fathers. Therefore, the design of our study might not be suitable for evaluating the dose-response relationships between paternal occupational exposures to various substances and SSRs, and the mechanisms for the observed associations. Despite these limitations, the results of this nationwide birth cohort study, obtained from a large

sample recruited from the general population across Japan, provide evidence that paternal occupational exposure to insecticides affects the SSR in terms of male births. To our knowledge, our study is the first large-scale cohort study to have examined the relationship between paternal occupational exposures and the SSR of their offspring, and is the first study to report an association between paternal exposure to insecticides and the SSR.

In conclusion, our results suggest that paternal occupational exposures to insecticides, and possibly to medical disinfectants, lower the proportions of male offspring. This association was particularly strong for insecticides. Further studies are needed to evaluate the mechanisms for the lowered SSR. In particular, the associations between poorer semen quality and levels of reproductive and thyroid hormones require investigation.

Contributors

SA conceptualised the study. SA and MS drafted the initial Article. SA analysed the data. SA and MS prepared the final version of the Article. SA, JS, NT, HT, HSa, YT, HSh, and MS collected and verified the data. SA, YT, HSh, and MS interpreted the data. All authors reviewed and approved the final version.

Members of the Japan Environment and Children's Study (JECS) Group as of 2019

Michihiro Kamijima (principal investigator, Nagoya City University, Nagoya, Japan), Shin Yamazaki (National Institute for Environmental Studies, Tsukuba, Japan), Yukihiro Ohya (National Centre for Child Health and Development, Tokyo, Japan), Reiko Kishi (Hokkaido University, Sapporo, Japan), Nobuo Yaegashi (Tohoku University, Sendai, Japan), Koichi Hashimoto (Fukushima Medical University, Fukushima, Japan), Chisato Mori (Chiba University, Chiba, Japan), Shuichi Ito (Yokohama City University, Yokohama, Japan), Zentaro Yamagata (University of Yamanashi, Chuo, Japan), Hidekuni Inadera (University of Toyama, Toyama, Japan), Takeo Nakayama (Kyoto University, Kyoto, Japan), Hiroyasu Iso (Osaka University, Suita, Japan), Masayuki Shima (Hyogo College of Medicine, Nishinomiya, Japan), Youichi Kurozawa (Tottori University, Yonago, Japan), Narufumi Suganuma (Kochi University, Nankoku, Japan), Koichi Kusuhara (University of Occupational and Environmental Health, Kitakyushu, Japan), and Takahiko Katoh (Kumamoto University, Kumamoto, Japan).

Declaration of interests

YT reports personal fees from Daiichi Sankyo, outside the submitted work. All other authors declare no competing interests.

Data sharing

Data sharing is not permitted by the JECS owing to a government policy restricting the deposition of data containing personal information. More details are available at https://www.env.go.jp/chemi/ceh/en.

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Prevention and practice during the COVID-19 emergency declaration period in Japanese obstetrical/gynecological facilities

Hiroaki Komatsu[1], Kouji Banno[2], Nozomu Yanaihara[3], and Tadashi Kimura[4], Board Members of Japan Society of Obstetrics and Gynecology*

[1]Department of Obstetrics and Gynecology, Tottori University School of Medicine, Yonago, Tottori, Japan
[2]Department of Obstetrics and Gynecology, Keio University School of Medicine, Shinjyuku-ku, Tokyo, Japan
[3]Department of Obstetrics and Gynecology, The Jikei University School of Medicine, Minato-ku, Tokyo, Japan
[4]Department of Obstetrics and Gynecology, Graduate School of Medicine, Osaka University, Suita, Osaka, Japan

Abstract

Aim: A nationwide questionnaire survey was performed to evaluate how Japanese Society of Obstetrics and Gynecology (JSOG) members dealt with the coronavirus disease (COVID-19) pandemic during the declared nationwide emergency.

Methods: We sent questionnaires to members of JSOG via official email. Participants answered anonymously using Google forms.

Results: Two (0.08%) JSOG members had contracted COVID-19. There was a clear decrease in the number of patients scheduled for operation, not only for malignant but also for benign diseases. A decrease in the number of outpatients for infertility treatment was also observed. Polymerase chain reaction testing for severe acute respiratory syndrome coronavirus 2 was available in 20.4% of the facilities. Inpatients and outpatients were requested to wear masks, limit the number of contacts and check body temperature when visiting hospitals/clinics. During parturition care, caregivers and physicians wore gloves, masks (other than N-95), face shields and gowns. About 66% and 80% of the facilities decided to transfer pregnant women if they had asymptomatic and symptomatic infection, respectively. Cesarean section was typically chosen as delivery mode in infected women.

Conclusion: The COVID-19 pandemic provoked significant changes in obstetrics and gynecology practices in Japan. Apparently, nosocomial infections were largely prevented due to these changes, although some of them might not have been necessary.

Key words: coronavirus disease, COVID-19, gynecology, obstetrics.

Introduction

The Japanese government declared a nationwide emergency due to the pandemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection causing coronavirus disease (COVID-19) from 4 April 2020 to 25 May 2020. The government did not choose a lock down of cities by law; instead,

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Correspondence: Professor Tadashi Kimura, Department of Obstetrics and Gynecology, Graduate School of Medicine, Osaka University 2-2, Yamadaoka, Suita, Osaka. Email: tadashi@gyne.med.osaka-u.ac.jp
*Board members of Japan Society of Obstetrics and Gynecology: Daisuke Aoki, Kiyoko Kato, Tomoaki Ikeda, Yutaka Osuga, Aikou Okamoto, Masaki Mandai, Nobuo Yaegashi, Mikio Mikami, Yoshiki Kudo, Akihiko Sekizawa, Takayuki Enomoto, Yoshimasa Kamei, Yoichi Kobayashi, Masayasu Koyama, Hiroshi Sameshima, Hiroaki Shibahara, Makio Shozu, Norihiro Sugino, Toshiyuki Takeshita, Satoru Nagase, Takuma Fujii, Nagamasa Maeda, Shingo Miyamoto and Hidemichi Watari.

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it requested self-restraint to ensure reduced social interaction (about 80% reduction), hand hygiene and mask wearing. Polymerase chain reaction (PCR)-based testing was restricted and used only for patients with symptoms. On a national scale, there were 15 463 PCR-confirmed COVID-19 cases and nearly 550 COVID-19-related deaths during this period.

There are 2210 birth facilities in Japan and around 860 000 births/year.[1] Half of these facilities are private units operated by few physicians. Even in the departments of obstetrics and gynecology in general hospitals, the mean number of physicians is typically less than five.[2] Most facilities do not have negative-pressure isolation rooms, neither do they store N-95 masks. To avoid nosocomial infection not only between patients but also between patients and medical staff members, our society members had to enforce a strict infection control policy. Fortunately, the pandemic was relatively mild in Japan, and the infection appeared to be quite well controlled, even in pregnant women and patients. Therefore, it is important to describe how our society members responded to the COVID-19 threat during the period of nationwide emergency.

Methods

The cross-sectional study was carried out between 18 May 2020 and 31 May 2020. We sent questionnaires to almost all members of Japanese Society of Obstetrics and Gynecology (JSOG) (more than 16 000 members were reachable via the internet), and the responses were submitted anonymously using Google Forms. The questionnaire was designed by JSOG board members. The diagnosis of COVID-19 was defined by a positive PCR test for SARS-CoV-2 infection. In the questionnaire, physicians were requested to report the places (prefectures) where they were working.

Results

Respondent characteristics

Responses were received from 2446 JSOG members. Only facility managers (n = 1160; 47%) were requested to answer questions about facility policies. Among the institutions where these managers worked, 766 (66.0%) were birth facilities.

Table 1 Number of institutions with confirmed SARS-CoV-2 infection among staff members and patients

SARS-CoV-2 infection	Number of facilities
Pregnant women	40
Patients with gynecological cancer	9
Patients newly diagnosed as SARS-CoV-2 positive during their stay in obstetrics and gynecology wards	3
Midwives	5
Obstetrics-related staff other than midwives (e.g., nurses, nurse assistants, cleaners)	7
Gynecology-related staff (e.g., nurses, nursing assistants, cleaners)	8

SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

The age-stratified proportions of the respondents were 19.7%, 27.4%, 30.1%, 16.4% and 3.3% in the age groups of 31–40, 41–50, 51–60, 61–70 and ≥71 years, respectively.

Survey of infection among member physicians

Only two of all respondents (0.08%) had been confirmed PCR positive for SARS-CoV-2 infection, although 25 respondents were suspected to have COVID-19, of whom 20 worked for general hospitals and 5 worked for clinics.

Survey of medical staff and patients

COVID-19 in midwives or other medical staff members was reported from five and seven birth facilities, respectively (Table 1). COVID-19 cases in gynecological nursing staff members were reported from eight institutions. Infected pregnant women and patients with gynecological cancer were identified in 40 and 9 institutions, respectively. Three patients were newly diagnosed as SARS-CoV-2 positive during their stay in obstetrics and gynecology wards.

Survey of changes in the number of regular patients

The number of (i) pregnant women visiting outpatient clinics, (ii) operations for gynecological malignancies, (iii) operations for benign gynecological conditions, (iv) treatments for gynecological malignancy (chemotherapy, radiation therapy) and (v) treatments using advanced reproductive technologies decreased in

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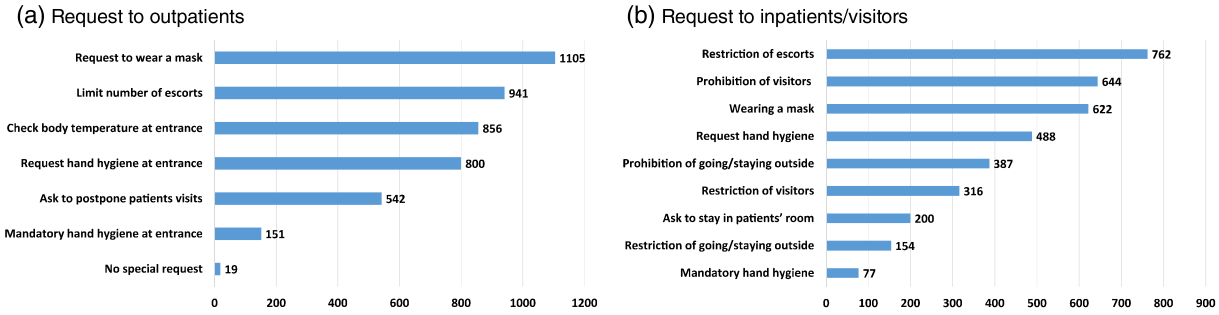


Figure 1 Questions about the facility's response (N = 1160). (a) What we request outpatients to follow. (b) What we request inpatients and visitors to follow.

38.1, 57.6, 23.9, 71.8 and 74.3% of the facilities, respectively (Supplementary Figure 1).

Survey of countermeasures to COVID-19

For institutional infection control, our members enforced various policies for both inpatients and outpatients. The requests for both inpatients and outpatients were, in descending order, to wear masks, limit the number of contacts, monitor body temperature and ensure proper hand hygiene (Figure 1).

PCR and SARS-CoV-2 screening policy

In-house PCR was available in 20.4% of the facilities. Overall, 76/1160, 47/1160 and 38/1160 facilities chose to implement a universal SARS-CoV-2 screening policy for pregnant women (mainly at 37–38 weeks of gestation), patients who would undergo scheduled general anesthesia and patients undergoing gynecologic surgery, respectively.

Screening practices for SARS-CoV-2 in pregnant women

As a means of infection control, birth facilities withdrew several services other than birth care. In descending order, the following services were withdrawn: "parents' classes" for group education and communication; postpartum visits by relatives, friends, and family members; birth attendance by partner; and acceptance of pregnant women moving from other prefectures (in Japan, a pregnant woman may give birth in her hometown nearby her parents). (Supplementary Figure 2). The personal protection equipment (PPE) used by midwives and physicians to protect against infection during delivery included gloves, masks (other than N-95), face shields, and gowns (Figure 2). This was followed by the use of gloves and masks (other than N-95). In 64.6% of the facilities, pregnant women were requested to wear a mask during labor and delivery.

Survey of delivery modes in SARS-CoV-2-positive pregnant women

We investigated how our member facilities dealt with SARS-CoV-2-infected pregnant women according to the severity of their condition (Figure 3). Approximately 80% of the facilities declared that they would transfer symptomatic women to other facilities. Even for asymptomatic women, 66% of the facilities chose to transfer the women. Most institutions accepting SARS-CoV-2-infected pregnant women had implemented a cesarean section policy for delivery. A couple of institutions answered that they accepted vaginal delivery in a private room (3.5%) or in a negative pressure room (1.8%) depending on the women's condition.

Discussion

The results of this questionnaire survey with data collected from 1160 facility managers representing 2446 members of the JSOG provides valuable information on the prevailing situation in the fields of obstetrics and gynecology at the time of the declared state of emergency by the Japanese government during the COVID-19 pandemic. They provide insight into patient and clinical trends in gynecology and how medical staff members responded to the emergency.

The survey suggests that only few infections may have been passed on from patients to medical staff members during this period.

The supply of high-performance masks (N-95) was limited in this period. During delivery, pregnant

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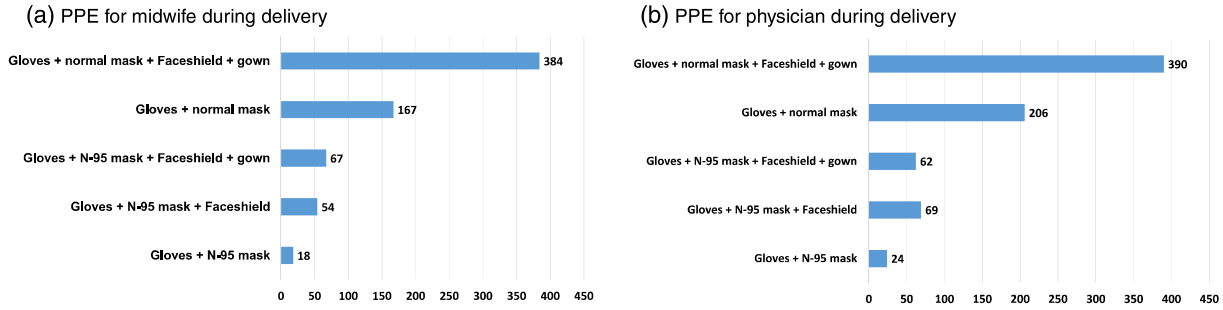


Figure 2 Questions about infection protection during delivery (N = 766). (a) Infection protection during delivery for the midwife. (b) Infection protection during delivery for OB/GYNs. OB/GYNs, obstetricians and gynecologists.

women were asked to wear surgical masks, and healthcare providers used standard universal precautions such as gloves in addition to the mask normally used. According to a Cochrane Review, the odds ratio of acquiring infection decreased by 0.32 by wearing a mask against respiratory disease (primarily influenza virus infection).[3] Many Japanese physicians expected that both they and the pregnant woman wear masks to further reduce the risk of SARS-CoV-2 infection. In fact, the effectiveness of wearing masks to prevent SARS-CoV-2 transmission was estimated to be even higher, and it is presumed that wearing a mask was effective in terms of preventing infection. Thus, they required pregnant women to wear a mask during delivery in the hope that the risk of infection would be reduced.

The majority of the facilities chose to restrict visitors from outside given the restricted PCR test policy. The majority of the facilities could not complete the entire infection control process, and so they could not manage deliveries of infected pregnant woman; therefore, it was necessary to transfer pregnant women to another facility.

The delivery mode of COVID-19-positive pregnant women has been discussed worldwide. Chen *et al.* proposed a protocol for the treatment of pregnant women with COVID-19 and issued a statement about their babies.[3] In the initial period of the pandemic, several articles described cesarean section for COVID-19-positive pregnant women.[4–6] Although practice guidelines from the United States and the United Kingdom did not recommend elective cesarean section,[7,8]

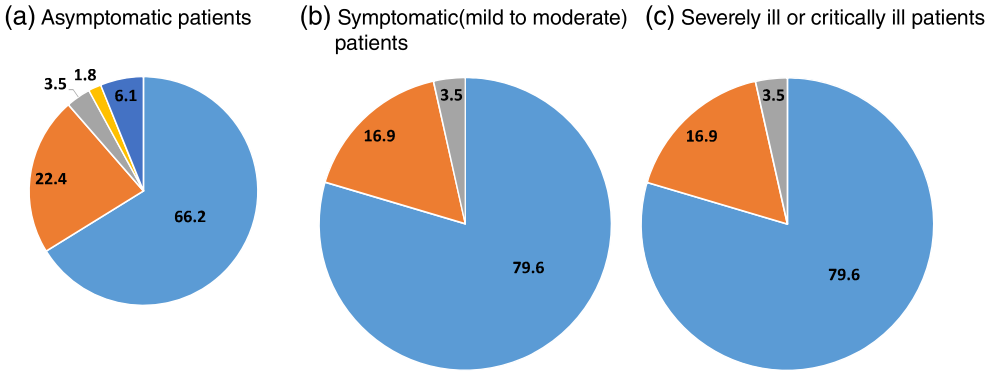


Figure 3 Delivery policy for pregnant women who tested positive for SARS-CoV-2 infection by polymerase chain reaction. (a) Asymptomatic patients. (b) Symptomatic (mild to moderately) ill patients. (c) Severely ill or critically ill patients. Colors indicated as below; (■) Transported to another hospital, (■) cesarean section, (■) vaginal delivery in personal room and PPE protection, (■) vaginal delivery with PPE protection in negative pressure room and (■) others.

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little evidence has appeared as to how to best manage aerosols emerging from rough breath during labor.[9]

Although we might not have the right answer for the correct management of obstetrical/gynecological patients, what was done in Japan during this period was very fundamental, that is, to practice social distancing by limiting visitors, including birth attendants, to wear a mask and to practice proper hand hygiene.[10,11] Moreover, the management of infected pregnant women and patients was limited to specialized and appropriately equipped facilities.

Although the general infection rate, which was low compared with other countries, helped to reduce nosocomial infection, our series of strategic measures against COVID-19 evidenced by this nationwide questionnaire survey should be helpful for future risk reduction.

Importantly, the potential value of the universal use of masks by both patients and caregivers and providing healthcare workers the confidence to absorb and implement fundamental infection–prevention practices may be the greatest contributions.[12]

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Disclosure

None declared.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Figure 1 Survey of the changes in the number of admitted patients. (a) Pregnant women presenting to outpatient clinics (N = 1160). (b) Gynecological malignancy surgery (N = 425). (c) Benign tumor surgery (N = 385). (d) Patients with gynecological malignancy (chemotherapy, radiation therapy; N = 599). (e) Advanced reproductive healthcare patients (N = 405).

Supplementary Figure 2 Questions about the facility's response for pregnant woman (n = 766).

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症例報告

母体Basedow病治療中に胎児甲状腺腫を認め胎内治療を行った一例

（平成28年11月7日受付）
（平成29年5月22日受理）

兵庫医科大学産科婦人科学講座
小熊　朋子　　浮田　祐司　　上田　真子　　原田佳世子
田中　宏幸　　澤井　英明　　柴原　浩章

Key words
Basedow disease
Fetal goiter
In utero treatment
Hypothyroidism
Umbilical blood sampling

概要　Basedow病合併妊娠治療中胎児に甲状腺機能低下症による甲状腺腫を認め，胎内治療を行った1症例を報告する

症例は既往にBasedow病と2型糖尿病があり，自然妊娠成立後PTUおよびヨウ化カリウム丸を内服していた．妊娠32週羊水過多を契機に超音波とMRIで胎児の甲状腺腫を確認した．内服薬減量を開始し，妊娠34週5日に臍帯血を採取し胎児甲状腺機能低下症と診断した．そこで妊娠35週よりレボサイロキシン200 μg/週を計2回羊水内に投与したところ，気管の通過性および羊水量の正常化を認め妊娠37週4日経腟分娩となった．

胎児甲状腺腫は嚥下困難による羊水過多，回旋異常による分娩障害，出生後には気道閉塞で呼吸障害を呈する可能性がある．胎内治療を行い甲状腺腫の縮小を図ることにより経腟分娩，非挿管での新生児管理が可能になることから，確定診断を行い出生前から治療を開始する意義があると考える．

緒言

甲状腺腫は出生後に発症することが多く胎児の甲状腺腫は出生1/3,000～5,000に発症する稀な疾患である[1]．しかし母体に甲状腺疾患がある場合胎児の2.6%に，また母体にBasedow病がある場合は胎児の19%に甲状腺腫が認められると報告されており[2]，母体に甲状腺疾患を認める場合母体の甲状腺ホルモン値を安定させるだけでなく胎児の状態を注意深く観察することが重要である．

今回我々は母体Basedow病合併妊娠で抗甲状腺薬治療中に胎児甲状腺腫を認め胎内治療を行った症例を経験したので報告する．

症例

41歳　女性　7経妊1経産（3自然流産3人工流産）
既往歴：Basedow病（PTU 600mg/日，ヨウ化カリウム丸100mg/日）
2型糖尿病（インスリン療法）
家族歴：高血圧・糖尿病
現病歴：自然妊娠成立後，PTUを300mg/日に減量し当科で妊婦健診を行っていた．妊娠29週の経腹超音波で羊水過多（AFI 32cm）を認め，妊娠32週のMRIおよび経腹超音波で胎児の頚部に6×5cm大の腫瘤性病変を認め甲状腺腫を疑った（図1）．精査・加療目的に妊娠33週当科入院となった．
妊娠32週胎児MRI所見（図2）：
頸部腹側に約64×53×31mmの軟部陰影を認めた．腫瘍体積は36.12mLと推定された．内部は左右二葉に分かれており，信号は均一でT1WIで高信号，T2WIで低信号であった．気道及び食道は腫瘤により圧排され狭窄していた．胃泡は確認できるが下腹部は平坦でvolumeが少なく，胎便は少ない印象であった．その他異常を認めなかった．
妊娠32週時超音波所見（図1）：
胎児：骨盤位．推定体重2,514g（+2.3SD），BPD

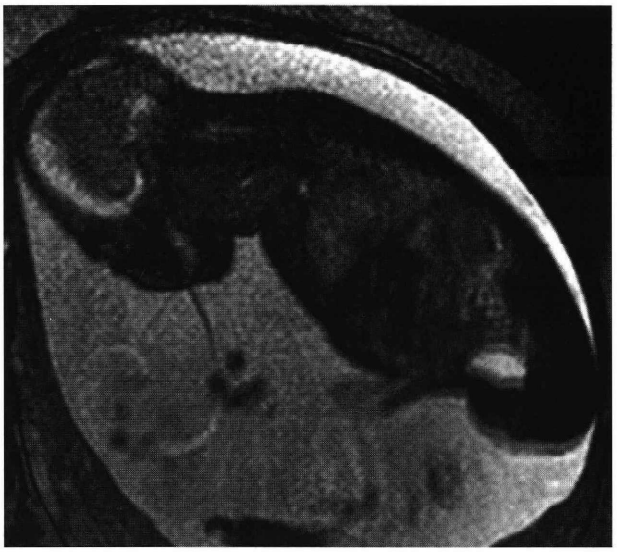
兵庫医科大学産科婦人科学講座
〒663-8501　兵庫県西宮市武庫川町1-1
Department of Obstetrics and Gynecology, Hyogo Colledge of Medicine
1-1 Mukogawa-cyo, Nishinomiya-city, Hyogo 663-8501, Japan

図1 妊娠32週 経腹超音波
胎児の頚部に6×5×3cm大の気管を囲むような，左右対称に均一でややhypoechoicな充実性腫瘤を認めた．カラードップラーでは腫瘤の末梢部に血流を認めた





図2 妊娠32週 胎児MRI
頸部腹側に約64×53×31mmのT1WIで高信号，T2WIで低信号を呈する腫瘤を認めた．内部は均一で左右二葉に分かれており，腫瘤により気道及び食道は腫瘤により圧排され狭窄していた．胃泡は確認できるが下腹部は平坦でvolumeが少なく，胎便は少ない印象であった．



86.4mm（+0.9SD），FTA 78.6cm²（+2.8SD），FL 61.6mm（+0.9SD），胎児の頚部に気管を囲むような，左右対称に均一でややhypoechoicな6×5×3cm大の充実性腫瘤を認めた．カラードップラーでは腫瘤の末梢部に血流を認めた．AFI 23cmと羊水量の改善を認め嚥下機能の改善があったと考えたが，気道の開通は確認できなかった．胎児水腫や心拡大，不整脈は認めずBiophysical profile scoring（BPS）は8点であった．

【CTG所見】基線140bpm，基線細変動は正常，一過性頻脈を認め，一過性徐脈を認めなかった．子宮収縮を認めなかった．

入院時所見：妊娠33週0日，身長164cm，体重74kg（非妊娠時体重67kg），体温37.0℃．血圧127/79mmHg．脈拍95回/分，腹囲106cm．外診所見；子宮は軟，収縮認めず．内診所見；子宮口閉鎖，展退0%，帯下白色中等量

入院時血液検査：

（生化）FBS：166mg/dL，HbA1c：5.4%と糖尿病の所見を認めた．その他異常認めず（血算）異常認めず（凝固）異常認めず（甲状腺機能）freeT3：5.12ng/dL，freeT4：1.75ng/dL，TSH：0.013μIU/mL，TSAb：141%，TRAb：1.4IU/L

入院後経過：入院前Basedow病に対しPTU 300mg/日＋ヨウ化カリウム丸50mg 2錠/日内服していたが，妊娠31週よりヨウ化カリウム丸を漸減し妊娠32週3日にヨウ化カリウム丸を中止した．入院後は内服薬減量による効果をみるため超音波で経過観察していた．AFI 23cmと羊水量の改善を認めた嚥下機能の改善があったと考えたが，超音波で気道の開通は確認できなかった．児は頭位で胎動はやや乏しかったが，BPSは8点であり浮腫や心拡大，心肥大，頻脈等は認めなかった．しかし妊娠34週にはAFI 23cmと再び羊水過多の所見を認め，必ずしも嚥下機能が改善したと断言できなかった．

一方母体の採血ではTSH：0.014μIU/mL，freeT3：3.81ng/dL，freeT4：1.65ng/dLと著変はなかったが，freeT4は正常値上限でありこれ以上の内服薬減量は困難と判断した．糖尿病に対する血糖管理は引き続きインスリンで行っており，妊娠中HbA1cは5.5%前後に安定しており増悪はなかった．そこで妊娠34週5日に臍帯血採取を施行したところ，TSH：60.04μIU/mL，freeT3：2.54ng/dL，freeT4：0.75ng/dLと胎児の甲状腺機能低下を示す所見であった．患者および御家族に十分な説明をした上で，妊娠35週より1週間毎にレボサイロキシンを200μg/回，計2回経腹的超音波ガイド下で羊水内に投与した．

図3　妊娠36週　胎児MRI
羊水は減少傾向であり，頸部に43×40×40mmの軟部陰影を認め，体積は妊娠32週と比較し79%とサイズの縮小を確認した．気道はMRI上も走行確認可能となった．

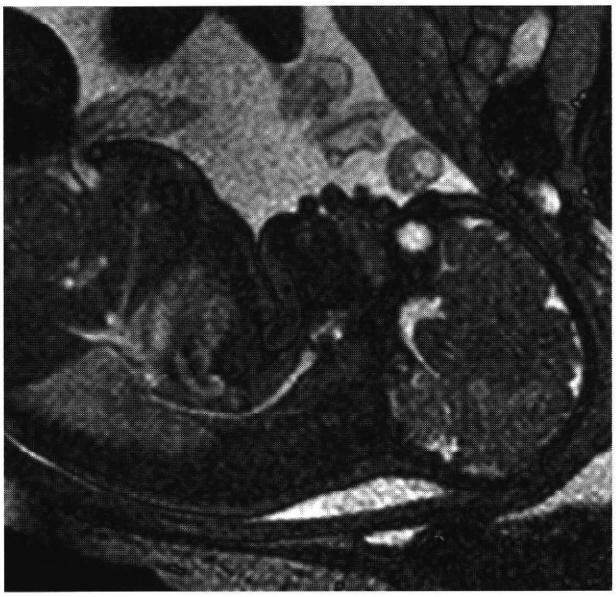


図4　出生後新生児
児は出生直後より啼泣・自発呼吸を認めた．頸部には軟性の腫瘤および伸展した皮膚を認めた．

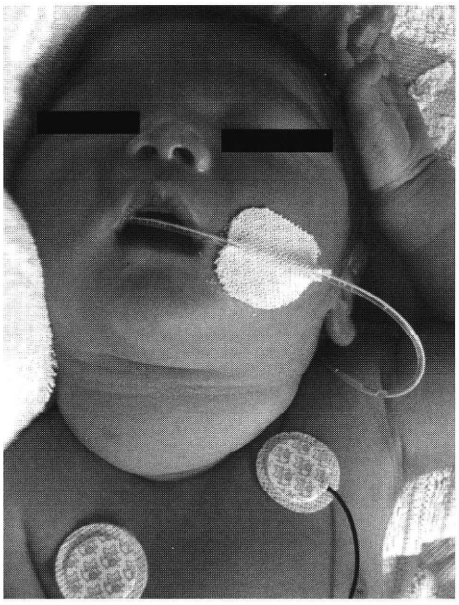
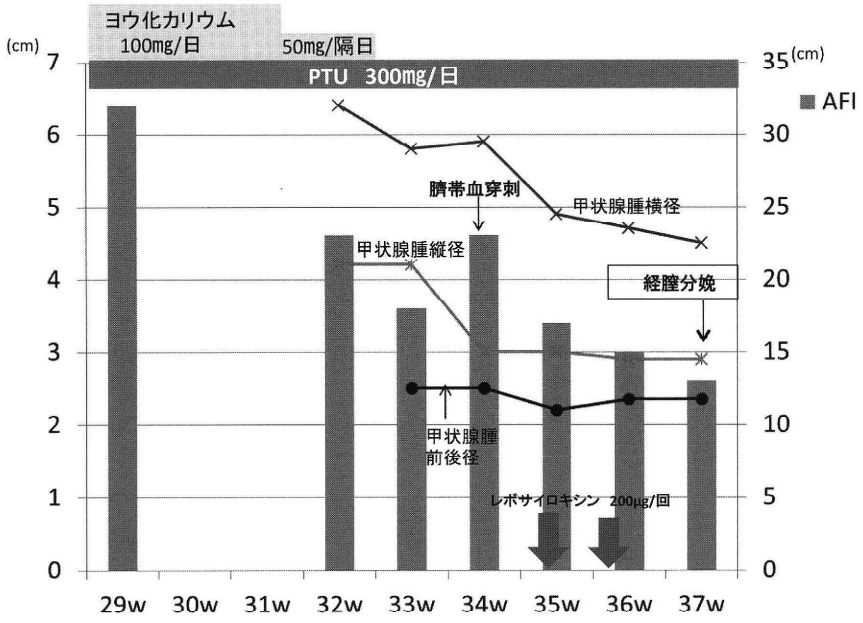


図5　胎児甲状腺腫と羊水量（AFI）の推移
ヨウ化カリウムの漸減後，羊水量は改善傾向であった．妊娠34週に再び羊水量の増加があったが血糖管理に大きな変化はみられず，嚥下機能の改善があったと断言できなかったため，臍帯血採取のうえレボサイロキシンを羊水内に投与した．レボサイロキシン投与後羊水量は改善し甲状腺腫も縮小した．



レボサイロキシン投与後胎児の頻脈・不整脈や浮腫は認めなかった．超音波で妊娠36週頃より径5mmの気管が確認できるようになった．妊娠36週の胎児MRIでも羊水は減少傾向であり，頸部に43×40×40mmの軟部陰影を認め，体積は28.78mLとサイズの縮小を確認した（図3）．気道はMRI上も走行確認可能となった．

37週4日　自然陣痛発来し，3,518gの女児をアプガースコア9/9点で娩出となった．児は出生直後より啼泣・自発呼吸を認めた．頸部には軟性の腫瘤および伸展した皮膚を認めた（図4）．臍帯血動脈pHは7.315と異常を認めなかった（図5）．

分娩後母体の経過は良好で，甲状腺ホルモンの値に著明な変動なく分娩後5日目に退院となった．

児はNICU管理となり，採血にてTSH；30.06 μIU/mL, freeT4：1.19ng/dLと甲状腺機能低下症であった．TSAb，TRAbは上昇を認めておらず，今回の胎児甲状腺機能低下症・甲状腺腫は母体のPTU内服によるものと考えられた．日齢0日の超音波にて，甲状腺腫のサイズは右葉25×16×41mm，左葉24×11×40mm，峡部の厚さは3.6mmと縮小を認め，実質の血流シグナルはやや亢進していた．児の大腿骨レントゲンでは大腿骨遠位端の骨化は正常であった．日齢3日目からfreeT4の上昇がみられたが，症状認めず無投薬で経過観察となり日齢34日目に退院となった．

考察

胎児の甲状腺腫は，出生1/3,000～5,000に発症する稀な疾患である[1]．原因としては，Basedow病などの甲状腺機能亢進症の母体に投与された抗甲状腺薬の経胎盤移行，ヨード欠乏症，ヨード中毒，抗甲状腺抗体の経胎盤移行，甲状腺ホルモン合成の先天性代謝異常，視床下部－下垂体機能低下症性の甲状腺機能低下症などがある[3)～8)]．甲状腺腫そのものによって気管圧迫を伴い羊水過多を引き起こすだけでなく，胎児甲状腺機能亢進症の場合，子宮内胎児発育遅延や骨成熟の加速，心不全や甲状腺中毒症，頭蓋骨癒合症，知力低下[9)]，新生児甲状腺中毒症[10)]の原因となる．胎児甲状腺機能低下症である場合は，骨成熟の遅延や精神運動発達の遅滞がみられる[11) 14)～16)]．

甲状腺腫大により気道の開通が確認できない場合は分娩方式を帝王切開とし，Ex utero intrapartum treatment（EXIT法）にて児を娩出することで術中に気道を確保する方法が必要である．胎児甲状腺腫の治療を行うには，まずその甲状腺の機能を評価する必要がある．現在では確定診断方法は臍帯血採取を行う．また，治療効果の判定には，超音波での腫瘤のサイズを評価する他に，臍帯血を定期的に採取することによってより治療判定を正確に行うことができる．しかし臍帯穿刺はIUFDなどの合併症を認める手技であり頻回に行うことは懸念されてきており，より非侵襲的な診断方法が模索されている．超音波でBPSや骨成熟・頸部腫瘤への血流分布等をスコアリングする方法がある[2)]．MRIによる評価ではT2強調画像でhigh densityを示すと甲状腺機能低下症であるといった報告[12)]がある一方胎児MRIのT2強調画像の高信号は必ずしも胎児甲状腺機能低下を示唆するのもではないという報告[13)]もある．実際本症例では甲状腺腫はT2強調画像は低信号を呈しており，MRIによる甲状腺機能評価は困難であった．よって現在のところ確定診断方法としては臍帯血による評価以外に存在しないのが現状である．

確定診断後は胎児甲状腺腫が甲状腺機能亢進に伴う場合は母体に抗甲状腺薬を投与することにより改善を図る．一方それが甲状腺機能低下に伴っている場合は，まず母体への抗甲状腺薬の減量を行い，効果が乏しい際には羊水腔内へのレボサイロキシンの投与が考慮されている[11) 14)～17)]．

レボサイロキシンの投与量については現在のところ各施設毎に異なっており，推定体重×投与量（15 μg/kg/週）を投与や[15) 17)]，週数に限らず150～500 μg/body/週としている施設が散見された．しかしながら妊娠33週に250 μg/body1回のみの投与で過剰となり，出生後の児に抗甲状腺薬を投与する必要があったとする報告[14)]もみられたため，本症例においては200 μg/bodyとした．本症例ではレボサイロキシン投与開始後から気管の開通が確認でき，出生後新生児の甲状腺機能の改善を考慮すると有効性があったかと思われるが，母体への投薬減量も行っていたためどちらが有効であったか判断は困難である．

しかしこの母体への投薬減量とレボサイロキシン投与の併用は一連の改善につながったと考える．過剰投与の報告もあることから今後より多くの症例を集め適したレボサイロキシン投与量を検討していく必要がある．

また本邦においてレボサイロキシンは錠剤を溶解し羊水内に投与する必要がある．本症例においては，レボサイロキシン200 μg（チラーヂンS 50 μg×4錠，あすか製薬株式会社）を55℃のヒートブロックで加温した生理食塩水（大塚生食注，大塚製薬株式会社）50mLに適宜撹拌しながら薬剤がほぼ溶けたことを目視にて確認しながら溶解した．作業は全てクリーンな環境下で行い，できる限りの滅菌化を図った．

レボサイロキシンの投与は，半減期を考慮すると週に1回程度の連続した治療を要する症例が多いため，何度も羊水穿刺を行うことにより絨毛膜羊膜炎をきたし早産のリスクが高まると考えられることから胎内治療を行う際は患者の同意とともに家族の理解を求める必要がある．

本症例では胎内治療前後でMRIを用いて立体的に評価することで，気道の確認および甲状腺腫の体積が79%に縮小したと評価することが容易でありMRIが胎児甲状腺腫の経時的な変化を定量的に評価するうえで有用であったと考える．その他羊水TSHを測定し胎児治療の効果を評価する試みがなされている[15)]．

児の出生後の精神運動発達については今後も当院小児科でフォローする必要があると思われるが，現在のところ明らかな異常は認めていない．

各々の施設ごとで麻酔科やNICU科スタッフと綿密に連携し，胎内治療の必要性や分娩時期・方式を検討し決定する必要がある．

利益相反について

今回の論文に関係して，開示すべき利益相反状態はありません．

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A case of fetal goiter and prenatal therapy during pregnancy of a woman with Basedow disease

Tomoko Oguma, Yuji Ukita, Mako Ueda, Kayoko Harada, Hiroyuki Tanaka, Hideaki Sawai, Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo Colledge of Medicine

We are reporting on a case in which a goiter was found due to hypothyroidism in the fetus during treatment for Basedow disease as a complication of pregnancy.

The patient had a history of Basedow disease and type 2 diabetes and had orally taken PTU and potassium iodide capsules after achieving spontaneous pregnancy. Triggered by excessive amniotic fluid during the 32nd week of pregnancy, goiter was confirmed in the fetus upon ultrasound and MRI. First reducing the dose of oral administration medicine, we collected cord blood on the fifth day of the 34th week of pregnancy and made a diagnosis of hypothyroidism of the fetus. Accordingly, she was administered levothyroxine 200 μg/week twice from the 35th week into the amnionic fluid, resulting in normalization of the passage of the trachea along with the amniotic fluid volume and she had a vaginal delivery on the fourth day of the 37th week of pregnancy.

Fetal goiter may cause excessive amniotic fluid due to swallowing difficulty, dystocia due to abnormal rotation, and respiratory disorders due to airway obstruction after birth. Intrauterine treatment to reduce goiter enables vaginal delivery and the management of newborns without intubation, suggesting the significance of making a definitive diagnosis and initiating treatment prior to birth.

【症例報告】

異なる経過をたどった慢性早剥羊水過少症候群の2症例

表　摩耶，脇本　裕，亀井秀剛，浮田祐司
原田佳世子，福井淳史，田中宏幸，柴原浩章
兵庫医科大学産科婦人科学講座

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概要　慢性早剥羊水過少症候群（chronic abruption oligohydramnios sequence；CAOS）は，一般に周産期予後は不良で初回出血の週数が早いほど予後が悪いとされている．今回，われわれは異なる経過をたどり，CAOSの予後規定因子について示唆に富む2症例を経験したので報告する．症例1は妊娠14週4日より性器出血と絨毛膜下血腫（sub-chorionic hematoma；SCH）を認め，妊娠23週4日に腹痛と多量の性器出血により当院に救急搬送され入院管理となった．入院後も性器出血は持続し，妊娠24週1日で羊水過少を認めCAOSと診断した．妊娠31週6日に陣痛が発来し経腟分娩となり健児を得た．胎盤病理は絨毛膜羊膜炎（chorioamnionitis；CAM）を認めなかった．症例2は妊娠15週4日に性器出血を認め当院に受診し，SCHを認めた．妊娠16週1日より持続する性器出血と子宮収縮を認め入院管理とした．同時期より羊水過少を認めCAOSと診断した．妊娠21週3日より子宮内胎児発育停止を認め，妊娠24週1日で子宮内胎児死亡となり，妊娠24週6日に経腟分娩した．胎盤病理はCAM III度であった．2例の経過を比較すると，CAOSにおいてもCAMという炎症の長期持続が児の予後不良因子であった可能性が示唆された．SCHに羊水過少を認めた場合はCAOSを念頭に，児の関連合併症に注目するとNICU併設の高次医療機関での周産期管理が推奨できる．〔産婦の進歩70（2）：134-142，2018（平成30年5月）〕

キーワード：慢性早剥，羊水過少，絨毛膜下血腫，早産，絨毛膜羊膜炎

【CASE REPORT】

Two cases of chronic abruption-oligohydramnios sequence (CAOS) presented different clinical courses

Maya OMOTE, Yu WAKIMOTO, Hidetake KAMEI, Yuji UKITA
Kayoko HARADA, Atsushi FUKUI, Hiroyuki TANAKA and Hiroaki SHIBAHARA
Department of Obstetrics and Gynecology, Hyogo College of Medicine

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Abstract　Here we present two cases of observed chronic abruption-oligohydramnios sequence (CAOS) with different infant outcomes. This report highlights the differences in placental pathology in cases of observed CAOS and the important role played by placental inflammation in the health and survival of the infant. In the first case, the patient presented to hospital complaining of persistent and abnormal vaginal bleeding and upon further investigation, subchorionic hematoma (SCH) was found at 14 weeks and four days of gestation. The patient was treated and sent home with instructions to check for worsening symptoms. At 23 weeks and four days of gestation, the patient returned to hospital with abdominal pain and abundant vaginal bleeding and was admitted. Due to the patient's previous SCH, we observed the amniotic fluid index (AFI) for four days. During this time, there was evidence of oligohydramnios, and a diagnosis of CAOS was made. The patient was treated with antibiotics and tocolytic drugs. At 31 weeks and six days of gestation, the patient went into labor and gave birth vaginally to a normal infant. Placental pathology showed no signs of chorioamnionitis (CAM). In the second case, the patient first visited the hospital reporting persistent vaginal bleeding at 13 weeks and five days of gestation. She was treated and sent home with instructions to monitor

for signs of worsening symptoms. The patient returned to the hospital a second time nine days later with evidence of SCH, and was again sent home after treatment. At 16 weeks and 1 day of gestation, the patient returned for the third time and was admitted for heavy vaginal bleeding and persistent uterine contractions. The AFI was monitored and a diagnosis of CAOS was made. In this case, the fetus showed a growth delay from 21 weeks and three days of gestation and the fetal heartbeat disappeared at 24 weeks and one day of gestation. At 24 weeks and six days of gestation, the infant was vaginally delivered. Placental histopathology found evidence of third degree CAM. It has been previously reported that long-term persistence of inflammation along with CAM is correlated with poor prognosis in infants. If cases of SCH present with evidence of oligohydramnios, perinatal management at a higher-level medical institution with a neonatal intensive care unit is recommended. [Adv Obstet Gynecol, 70 (2) : 134-142, 2018 (H30.5)]

Key words : chronic abruption, oligohydramnios, sub-chorionic hematoma, preterm labor, chorioamnionitis

緒　言

慢性早剥羊水過少症候群（chronic abruption oligohydramnios sequence；CAOS）は，出血源が不明の性器出血があり，正常範囲であった羊水量（amniotic fluid index；AFI>5 cm）が破水を認めないのにもかかわらず過少になると定義される病態で，Elliotらによって1998年に報告された[1]．一般に周産期予後は不良で初回出血の週数が早いほど予後が悪い[1]．CAOSを発症した場合は羊膜，絨毛膜への血流や栄養供給が減少し，胎盤機能の低下や胎児の腎機能の悪化などにより羊水過少を生じる．また，羊膜の脆弱化による前期破水を引き起こすとされている[1-5]．

今回われわれは，異なる経過をたどったCAOSの2症例を経験したので報告する．

症　例

【症例1】症例は35歳，2妊1産である．早産既往はなく，前回妊娠時は妊娠39週0日で3066gの女児を経腟分娩している．今回，自然妊娠が成立しA病院で妊婦健診を受けていた．妊娠14週4日，性器出血を認めたため，A病院を再診した．経腟超音波検査でSCHを認めたが破水所見なく経過観察となった．妊娠16週4日に持続する性器出血とSCHの増大を認め，B病院を紹介受診となり，同日にB病院に入院管理となった．この時の羊水量は正常範囲の5～6 cmで推移し破水所見はなかった．妊娠23週4日に下腹部痛と性器出血の増加を認め，当院に救急搬送で受診となった．受診時に経腟超音波検査で59×24 mm大のSCH（図1）および胎児心拍数陣痛図（cardiotocogram；CTG）で規則的な子宮収縮を認めたため切迫早産の診断で妊娠23週4日に当院に入院管理となった．入院時，腟鏡診で帯下は淡血性で中等量，活動性の出血はなく，内診で子宮口は閉鎖していた．子宮頸管粘液中顆粒球エラスターゼ検出試薬（エラスペック®）は陰性であった．「腟分泌液中ヒトインスリン様成長因子結合蛋白1型（insulin-like growth factor binding protein-1；IGFBP-1）検出試薬（チェックPROM®）」は陰性で破水所見を認めなかった．経腟超音波検査で子宮頸管長は50 mm以上でAFIは6 cmであり，羊水腔内には羊水中のechogenicな沈殿物であるamniotic fluid sludgeを認めた．バイタルサインは異常なく，血液検査では炎症反

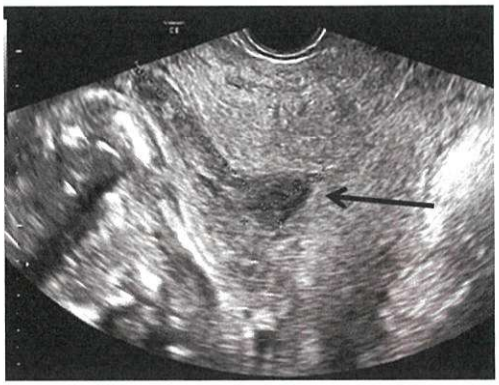


図1　症例1における受診時の経腟超音波検査
矢印の位置に59×24 mm大の絨毛膜下血腫（SCH）を認める．

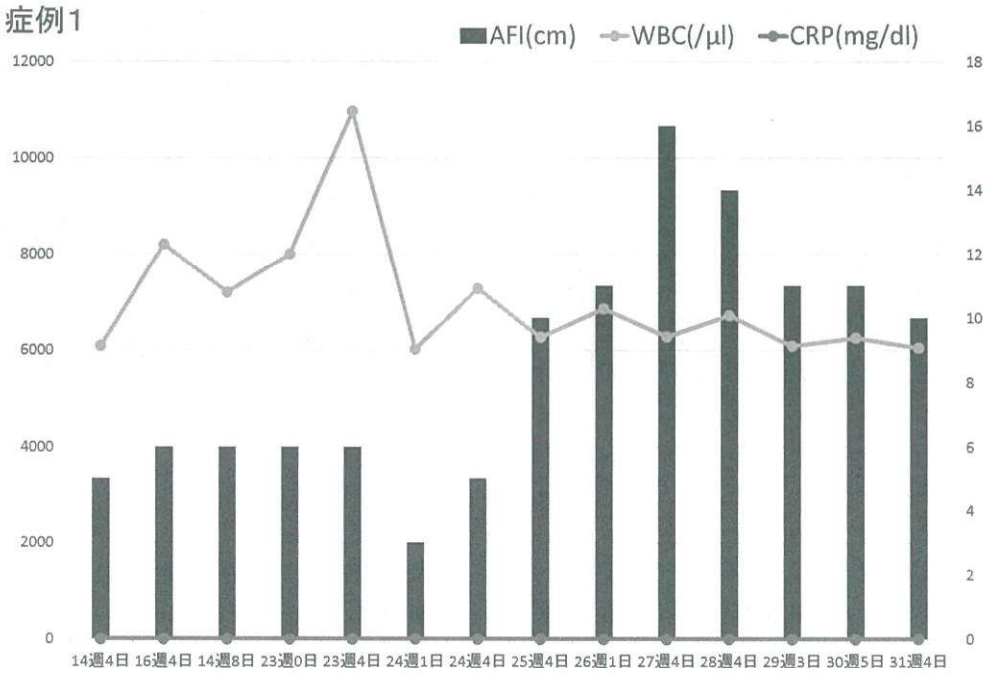


図2　症例1における血液検査（WBC，CRP），AFIの経時的推移

応上昇を認めず（WBC；10970/1 μl，CRP；0.06 mg/dl），感染徴候は否定的であった（図2）．腟分泌物培養検査ではescherichia coliとC. pseudodiphtheriticumを検出した．入院した妊娠23週4日からの連日の腟鏡診での水様性帯下の流出はなく，BTB（brom-thymol-blue）法において青色変化はなかった．入院時に6 cmあったAFIは徐々に減少し，妊娠24週1日では3 cmとなった．妊娠24週1日でのチェックPROM®は陰性で明らかな破水所見を認めず，軽度の出血のみで高位破水は否定的であった．持続する性器出血および羊水過少があることからCAOSと診断し，入院時からリトドリン塩酸塩持続点滴（50 μg/分）に加えて感染予防目的にフロモキセフナトリウム（1g×3回/日）を投与した．経過中の性器出血は増減しながら持続した．出血はあったが妊娠24週4日から徐々に羊水は増加し，AFIは5 cmと正常範囲に改善し，妊娠25週4日にはAFIは10 cmまで回復した．妊娠25週4日に約2 cm大の凝血塊を排出したが，性器出血は少量となり持続した．経腹超音波検査でSCHの増大は認めなかった．妊娠30週5日，子宮収縮回数が増加したため，リトドリン塩酸塩の投与量を増量し（166 μg/分），硫酸マグネシウムの点滴を1g/時で開始し，2g/時まで増量した．また，今後分娩に至る可能性を考慮してベタメタゾン投与を行った．妊娠31週6日，腹部緊満感の増強および子宮口は3 cmと開大を認めたため，リトドリン塩酸塩と硫酸マグネシウム持続点滴の投与を中止し，経腟的に児を娩出した．児は1914g，Apgar score 8点/9点（1分値/5分値）の女児であった．なお，当院で入院中の妊娠24週から31週にかけての性器出血は褐色帯下程度であることが多かったが，分娩時まで持続していた．

肉眼的所見では羊水は血性で，卵膜の黄染は認めずやや赤色で，胎盤の一部に約6 cm大の暗赤色の血腫を認めた（図3）．病理組織学的検査では，絨毛組織，脱落膜組織，卵膜組織および臍帯に炎症細胞の浸潤を認めず，CAMは否定的であった．卵膜の胎児面にびまん性のヘモジデリン沈着（diffuse chorioamniotic

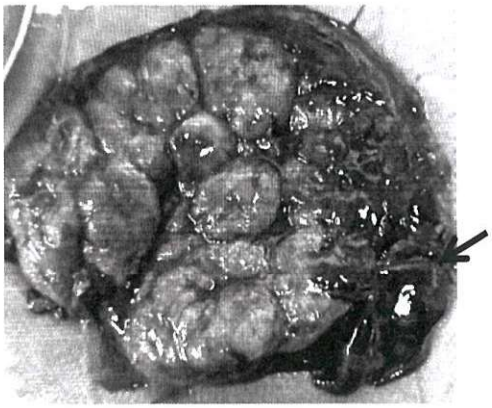


図3　症例1における娩出後胎盤の肉眼像
矢印の位置で胎盤辺縁に約6 cm大の暗赤色の血腫を認める．



図5　症例2における受診時の経腟超音波検査
矢印の位置に24×22 mm大の絨毛膜下血腫（SCH）を認める．



図4　症例1における胎盤病理組織像（×400倍，HE染色）
矢印の位置で絨毛膜板にヘモジデリンを貪食した黄褐色成分を細胞質に含むマクロファージを認める．

hemosiderosis；DCH）を認めた（図4）．

児は出生後，多呼吸を認め，新生児一過性多呼吸の診断にて，NICUで管理した．生後よりマスク型のcontinuous positive airway pressure；CPAP（fraction of inspiratory oxygen：FiO_2 30％，Flow：8 l/分，positive end expiratory pressure：PEEP 5 cmH_2O）を施行したが，人工サーファクタントの投与や気管内挿管を必要とはしなかった．日齢1でCPAPを終了し，日齢8までFiO_2＝0.3にて保育器内酸素放流を行ったが，呼吸状態が安定したため日齢9でコット移床した．経過良好であったため日齢26より自律哺乳開始した．日齢50で体重増加良好で退院となった．

【症例2】症例は34歳，1妊0産である．性交のタイミング指導を受け妊娠が成立し，C病院でとくに問題なく妊婦健診を受けていた．妊娠15週4日に性器出血を認めたため，C病院を再診した．経腟超音波検査でSCHを認め，当院を紹介受診となった．当院受診時，経腟超音波検査で24×22 mmのSCH（図5）を認めた．妊娠16週1日に持続する性器出血および規則的な腹部緊満感を認め，切迫流産の診断にて入院管理となった．入院時，腟鏡診で帯下は褐色少量で活動性の出血はなく，内診で子宮口は閉鎖していた．エラスペック®は陰性であった．チェックPROM®は陰性で破水所見は認めなかった．超音波検査で子宮頸管長は37 mmで，AFIは6 cmであった．血液検査は軽度の炎症反応を認めた（WBC；8810/1 μl，CRP；0.97 mg/dl）（図6）．腟分泌物培養検査ではgardnerella vaginalisとstaphylococcus（CNS）を検出した．入院時からリトドリン塩酸塩持続点滴（50 μg/分）とフロモキセフナトリウム（1g×3回/日）の投与を行った．妊娠17週1日で多量の活動性の性器出血および経腟超音波検査で30×27×22 cm大のSCHの増大があり，妊娠18週5日には77×32×20 cm大のSCHを認めた．経腹超音波検査での臍帯動脈血流異常は認めず，児の発育は保

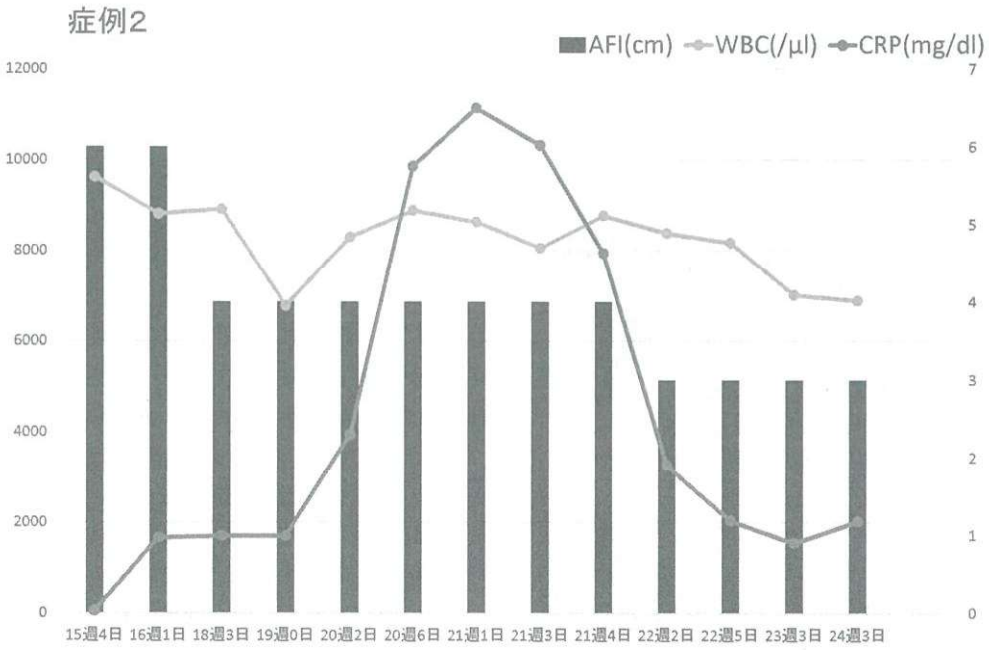


図6　症例2における血液検査（WBC，CRP），AFIの経時的推移



図7　症例2における経腹超音波検査
臍帯動脈血流の途絶を認める．

たれていた．性器出血は増減を繰り返しながら持続した．入院時に6 cmあったAFIは，妊娠18週3日でAFI 4 cmとなり妊娠22週3日で3 cmと減少した．児の推定体重は妊娠21週3日で376g（－0.7SD），妊娠23週3日に355g（－2.7SD）であり，子宮内胎児発育停止を認めた．妊娠23週3日の超音波検査で臍帯動脈血流の途絶（図7）を認めた．今後妊娠の継続をした場合に子宮内胎児死亡（intrauterine fetal death；IUFD）をきたす可能性およびこの週数の新生児の予後に

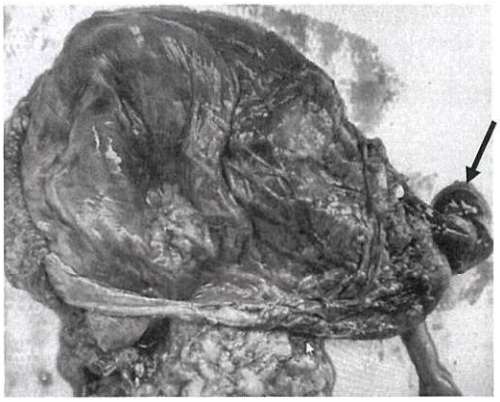


図8　症例2における娩出後胎盤の肉眼像
卵膜の黄染を認め，矢印に血腫を認める．

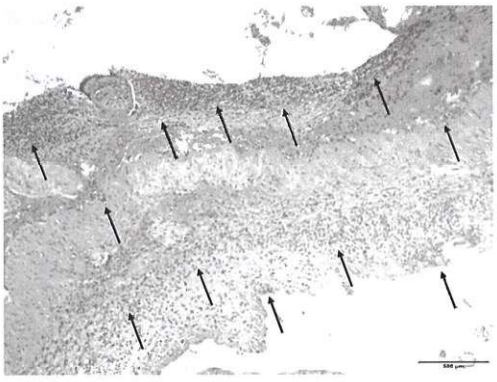


図9　症例2における胎盤病理組織像（×200倍，HE染色）
全層性に矢印の位置の好中球などの炎症細胞の浸潤を認めCAMIII度である．

ついて本人および家族に入念なインフォームドコンセントを行い経過観察する方針となり，リトドリン塩酸塩の投与を行いながら在胎週数の延長を試みたが，妊娠24週1日にIUFDを確認した．妊娠24週6日に279gの性別不詳の児を自然経腟分娩した．児には明らかな体表奇形を認めなかった．

肉眼的所見では羊水は血性で卵膜に黄染を認め，胎盤は暗赤色で一部に約3 cm大の血腫があった（図8）．胎盤病理ではヘモジデリン沈着は認めず，全層性に炎症細胞の浸潤を認め，Blanc分類でCAMIII度であった（図9）．なお，経過中には臨床的CAMの所見を認めなかった．

考　察

CAOSは慢性の常位胎盤早期剥離（慢性早剥）に羊水過少を合併するものとされ，1998年にElliotらにより提唱された[1]．いまだ診断基準や周産期管理について統一した見解はない[3]．慢性早剥[6-9]の概念としては，母体の凝固系異常がなく軽症の部分的な常位胎盤の剥離で，急速遂娩を要さず数日以上の経過をたどる状態，さらに，持続的あるいは断続的な性器出血を認めるが，前置胎盤をはじめとした他の出血源を認めず常位胎盤からの出血と考えられる状態と報告されている[8]．なお，胎盤辺縁や胎盤以外からの出血など真の胎盤剥離がない場合も含むとされている[8]．Harris[10]は慢性早剥の病態生理として，静脈性出血により羊膜，絨毛膜と脱落膜の間が剥離し血腫が形成され，剥離面が拡大せずに胎盤の辺縁に血腫が限局すると定義し[10]，急速遂娩を要するような動脈性出血による急性の胎盤早期剥離と異なるとしている．CAOSを発症した場合は羊膜，絨毛膜への血流や栄養供給が減少し，胎盤機能の低下や胎児の腎機能の悪化などにより羊水過少を生じ，羊膜の脆弱化による前期破水を引き起こすとされている[1-5]．

今回の2症例の主要所見の比較を示す（表1）．2例とも初回出血が妊娠20週未満で，破水所見はなく羊水過少となった．CAOSの周産期予後は初回出血を20週未満に認めた場合，周産期死亡率は43％に至り[1]，20週以降の発症の場合でも周産期死亡率は10％と報告され，一般的に予後不良とされている[1]．ただし，小野寺ら[11]の報告によると，CAOSと診断後に約8～10週の経過で羊水量が回復した場合，妊娠継続により健児を得られる可能性[11,12]が指摘されており，初回出血が妊娠初期であっても臨床的CAMを疑う子宮内感染や経腹超音波検査で胎児発育停止などの胎児胎盤機能不全の所見がない場合には，中期中絶[13]や人工早産は控えるべきであると述べられている[11]．この2症例においては，症例1は入院管理中に羊水量が回復し生児を得たが，症例2は羊水量が回復せず死産となった．

妊娠中の性器出血が観察された場合には，

表1　症例1と症例2の主要所見の比較

	症例1	症例2
初回性器出血	14週	15週
SCH	5-6cm ↓ 消失	2-3cm ↓ 7-8cmに増大
DCH	あり	なし
CAM	なし	あり III度
エラスペック	0.73	0.90
膣培養	Escherichia coli,C.pseudodiphtheriticum →Lactobacillus,Staphylococcus aureus	Gardnerella vaginalis,Staphylococcus(CNS) →Enterococcus faecalis, Candida albicans
チェックPROM	陽転化なし	陽転化あり （妊娠23週3日）
転帰	経膣分娩 生児	経膣分娩 死産

SCHを認めることがある．SCHは胎盤辺縁部において，絨毛膜が脱落膜から剥離した部分に母体血流が貯留した状態である．多くは妊娠第1三半期に発生し，頻度は全妊娠の4%から48%と，報告により大きく異なっている．多くの例では自然に血腫は消失するが，場合により流産の原因となる[14]．また，妊娠中期を超えて存続したものは妊娠32週未満の早産率が有意に高く[15]，新たにSCHが発生した症例ではCAMや破水の原因となり早産と関連すると述べられている[14]．そして，CAOSの症例の75%に合併するとされるSCHはCAMや破水の原因となるが[16]，SCHの予後には一定の見解が得られていない[14]．またSCHが長期化し，慢性早剥となる症例が存在することも報告されている[5]．したがって，性器出血が遷延し妊娠中期までSCHを認めた場合は，羊水過少やCAOSの発症に注意して妊娠を管理する必要がある．

症例2はSCH増大とCAMに伴う炎症反応が持続し，胎児胎盤機能不全の徴候を認めた．持続出血[16]に伴い羊水量の回復なく感染徴候をみた症例であり，従来の報告[3-5,16-19]と同様に予後不良であった．CAMは病理組織学的に絨毛膜・羊膜周辺への好中球の浸潤像にて診断される[20]．CAOSの発症はCAMに伴う絨毛膜羊膜の脆弱化による胎盤機能不全に関与している可能性があると述べられている[21]．Kobayashiら[16]はCAOS 15例中全例にCAMを認め，そのうち7例が重症のCAMであったと報告している．また，Elliotら[1]は24例中10例に，興石ら[5]は3例中2例に，鈴木ら[3]は4例中全例に，奥野ら[17]は8例中5例にCAMを認めていると報告している．このようにCAOSを認めた場合は，CAMの発症を念頭に妊娠管理する必要がある．CAOSの治療としては，子宮収縮抑制剤による可及的な子宮収縮の抑制に加えて，抗生剤の使用による臨床的CAMの予防を行うとされている[3,4]．ただし，CAMに対する適切な抗生剤の投与時期，投与期間，種類については，依然エビデンスに欠けるとされている[22]．今回の2症例においてはフロモキセフナトリウムによる抗生剤の予防的投与を実施した．

症例1はCAMを発症せず，妊娠31週6日まで妊娠管理し得たことが児のintact survivalに寄与したと考える．ただし，症例1は早産であり，胎盤の病理組織学的検査でDCHを認めている．Redlineら[2]は絨毛膜板および絨毛膜・羊膜において鉄染色陽性があるものをDCHと称し，DCHの存在は少なくとも分娩より24～48時間前の子宮内出血を表し[23]，早産との因

果関係が指摘されている[2]．DCHは辺縁出血やSCHが羊水腔内に移行拡散し，赤血球中のヘモグロビンの成分である鉄が色素顆粒のまま，びまん性に絨毛膜や卵膜に沈着し貪食細胞に取り込まれるものと考えられている[2,6-10,23]．したがって，CAOSは胎盤外壁につながる母体側の静脈叢の破綻が原因で慢性早剥となると考えられており[18]，静脈叢の破綻に伴い母体血が羊水中に流出し，DCHの原因になると推測される[18]．DCH46例の検討では26例（57％）にCAOSを認め[24]，CAOS15例の検討では8例（53％）にDCHを認めている[16]．症例1はCAMを発症しなかったが，DCHを認めたことで早産の一因となった可能性があると考える[2,23]．このDCHは早産ばかりでなく，児の慢性肺疾患のリスク因子であるとOhyamaらは報告している[24]．胎児が血性羊水中に長期間存在すると，羊水中のヘモジデリンなどの血液分解産物[3,25]が児の気管支上皮や肺胞を損傷する[3]ことで，新生児肺高血圧，dry-lung症候群，新生児慢性肺疾患（chronic lung disease；CLD）[26,27]などを出生後に生じ死亡している報告もある[5,16,24]．したがって，COASのようなDCHの存在が疑われる症例では，早産に至る可能性と出生後の新生児の呼吸状態を注意深く観察する必要があり，高次医療機関での総合的な妊娠管理が望ましいと考える[19]．

症例2において，妊娠23週3日に胎児機能不全を認めた際に，十分なインフォームドコンセントを施行のうえ妊娠継続を試みた．板橋らの検討によると児の在胎期間別死亡率は妊娠23週では早期新生児死亡が11％，新生児死亡が20.2％，死亡退院が25.5％にみられ，さらに推定体重が400g未満の際は早期新生児死亡が22.6％，新生児死亡が37.7％，死亡退院が52.8％と報告されている[28]．Inoueらの検討によると出生体重別の児の生存率では，300g以下で18％，301～400gで41％，401～500gで60％と報告されている[29]．CAOSの症例では，平均在胎週数は27～28週といわれている[18]．しかし，在胎週数26週で死産に至った症例報告や在胎週数23週で脳性麻痺となった症例報告もある[3,5,16,17,19]．したがって，児の娩出のタイミングは児の予後を含めた十分なインフォームドコンセントを施行のうえ，慎重に決定する必要があると考える[10,30]．

結　語

CAOSは周産期予後不良な病態として広く知られているが，症例1はCAMを認めず，周産期予後は良好で生児を得た．一方で，症例2は重度のCAMを認め死産に至った．従来の報告と同様にCAOSにおいてもCAMの存在という炎症の長期持続は児の予後不良因子に関連した．

今後さらなるCAOSの症例集積および検討が望まれ，周産期管理について統一した見解が得られることを期待する．

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Case Report

A Case of a Pregnant Woman with Thrombosis in an Artificial Aortic Valve Resulting in Severe Cerebral Hemorrhage in the Newborn

Hidetake Kamei, Yu Wakimoto, Yumi Murakami, Maya Omote, Kayoko Harada, Atsushi Fukui, Hiroyuki Tanaka, Hideaki Sawai, and Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan

Correspondence should be addressed to Yu Wakimoto; yuuwakimoto@gmail.com

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Many patients, after artificial valve replacement surgery, receive warfarin anticoagulant therapy. However, it has been reported that warfarin administration during pregnancy can cause fetal teratogenicity. With reference to this case, we will discuss how warfarin administration in mid-pregnancy caused severe cerebral hemorrhage in the newborn child. The 36-year-old patient in this case underwent aortic valve replacement surgery when she was 11 years old; this requires the continued use of warfarin after surgery. Although she was advised otherwise, the patient became pregnant. The warfarin treatment was discontinued at 5 weeks of gestation and she began self-injection of heparin; however, her health quickly deteriorated requiring an emergency, warfarin treatment. On gestation week 21, she was admitted to our hospital with a high likelihood of a spontaneous abortion. A week later, transesophageal ultrasonography revealed a thrombus in the patient's aortic valve. Because of this finding, we re-started warfarin administration. At 32 weeks of gestation, cardiotocography showed decreased fetal heart rate; thus, an emergency Cesarean section was performed. A baby was delivered, weighing 1,702 g with an Apgar Score of 1 at 1 minute and 4 at 5 minutes. Cranial computed tomography of the infant showed bilateral intraventricular hemorrhage and ventricular dilation. In order to protect the mother and prevent hemorrhage in the newborn, it is recommended that a continuous heparin infusion should be administered to the pregnant woman after the 36th week of gestation. Regarding the impact on the infant, it is considered that continuous intravenous administration of heparin is safer during the third trimester of pregnancy. However, administration of heparin alone makes the preventive effect of thrombosis uncertain. When warfarin is administered in pregnancy, pregnancy management should be performed bearing the risk of fetal cerebral hemorrhage in mind.

1. Introduction

Recent advances in cardiac surgical techniques have enabled women to carry pregnancy to full term [1]. However, there are risks involved; thus, continuous perinatal care is required. We report a case of the perinatal management of a patient with aortic valve thrombosis occurring after mechanical valve replacement.

2. Case

The patient was a 36-year-old gravida 0 woman. At the age of 7, she underwent ventricular septal defect closure for the right ventricular outflow tract. At the age of 11, she received a mechanical aortic valve replacement. Since after the replacement, she has been receiving warfarin orally at a dosage of 4.5 mg/day. She conceived naturally and she was referred to our hospital for perinatal management. Oral administration of warfarin was discontinued at 5 weeks of gestation and she began self-injection of heparin. At 21 weeks and 5 days of gestation, she was admitted to our hospital with a high risk of spontaneous abortion and was put on intravenous ritodrine. This successfully prevented a miscarriage. At 21 weeks and 6 days of gestation, we started a continuous infusion of 25,000 units of heparin daily. On the 22nd week, transesophageal echocardiography showed a movable thrombus in the aortic valve. The size of the biggest thrombus

Table 1: Blood sample from neonate immediately after birth and at 1 day of age.

Postpartum	Day 0	Day 1
AT-3(%)	70	72
D-dimmer (μg/ml)	1.2	1.5
Fibrinogen (mg/dl)	553	621
APTT (seconds)	≧180	37.8
PT-INR	Unmeasurable	1.02
Platelet ($\times 10^4/\mu$l)	50.3	53.2
Hb (g/dl)	6.8	7.1

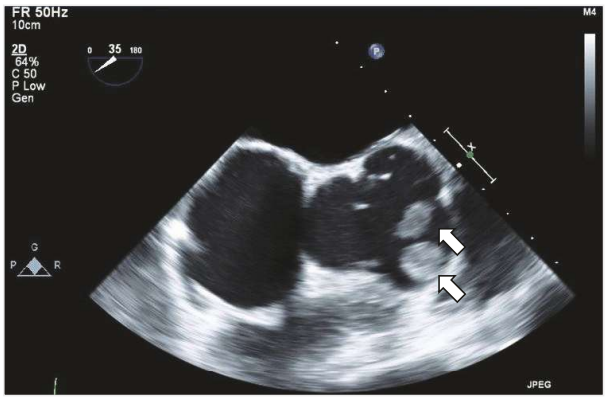


Figure 1: Transesophageal echocardiography. A transesophageal echocardiography of the mother's heart showed a movable thrombus on the aortic valve at 22 weeks of gestation. The size of the biggest thrombus was 26 × 8 mm (arrows).

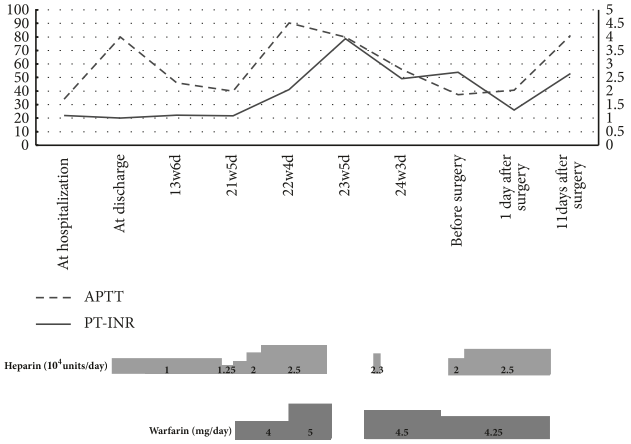


Figure 2: The change of APTT and PT-INR. This figure shows the change of APTT and PT-INR in the mother's blood in relation to dosage of heparin and warfarin.

was 26 × 8 mm (Figure 1). We increased the dosage of heparin to 28,000 units daily and restarted the administration of warfarin. Following this, the thrombus reduced in size, and at 23 weeks and 5 days transesophageal echocardiography showed no signs of thrombosis in the patient. At 32 weeks and 2 days of gestation, a routine cardiotocography showed a decreased fetal heart rate; thus, an emergency Cesarean section was performed under general anesthesia because of the presence of warfarin in the blood. The baby was delivered, weighing 1,702 g, with an Apgar Score of l at l minute, and 4 at 5 minutes. The total amount of blood loss during the surgery was 1,410 ml. During the surgery, 16 units of fresh frozen plasma (FFP) was transfused; and after surgery, we continued to infuse 20,000 units of heparin daily. On the 11th day after surgery, owing to continuous genital bleeding, heparin administration was discontinued and uterine artery embolization was performed. This treatment stopped the bleeding and on the 21st postsurgical day; we started warfarin administration at 5 mg/day. She was discharged on the 34th postoperative day due to the stable PT-INR levels (Figure 2).

The newly born infant was intubated and admitted to the newborn intensive care unit. At the time of admission, activated partial thromboplastin time was 180 seconds or more and bilateral intracerebral ventricular hemorrhage was detected using ultrasonography. On the first day of life, anemia was observed in the infant and red cell concentrate and FFP were transfused (Table 1). We attempted to reduce the infant's dependence on the ventilator and at 8 days of age the infant was extubated. On the postnatal 10th day, a cranial CT scan showed bilateral intraventricular hemorrhage with ventricular dilation and midline shift (Figure 3). Although convulsions accompanying the intracranial hemorrhage were observed, the infant's general condition was stable and oral feeding was started on postnatal day 10. The newborn was discharged on postnatal day 54. However, the infant later developed cerebral palsy and is currently receiving treatment at our hospital.

3. Discussion

In pregnant women who have undergone artificial valve replacement surgery, mortality rate is high for both mother and fetus during pregnancy. Treatment requires perinatal care with special attention to the possible onset of thromboembolism [2, 3]. During late pregnancy, fibrinogen, von Willebrand factor, factors VIII, IX, X, and XII, are increased and activated; thus, the risk of thrombosis and embolism increases [4]. Therefore, continuous assessment of this risk is needed for women in the later stage of pregnancy with mechanical valves [5]. For a woman who wishes to have a baby after aortic valve replacement surgery, a biological valve is often selected, but in some women, a mechanical

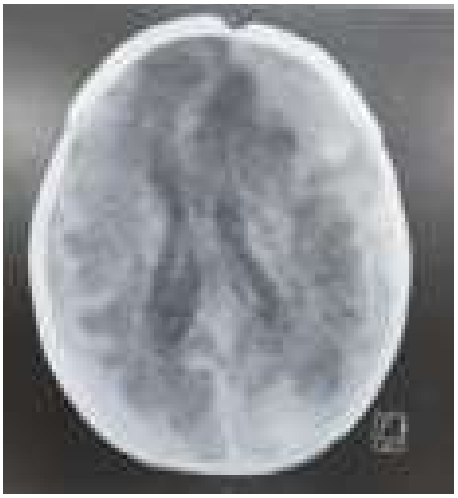


Figure 3: Cranial CT of the newborn. Cranial CT scan of the newborn showed bilateral intraventricular hemorrhage with ventricular dilatation and midline shift.

valve is used, to reduce the possibility of reoperation due to ageing and, thus, the deterioration of a biological valve [6, 7]. In this case, the aortic valve replacement surgery was performed at the age of 11 years and mechanical valves were used. For pregnant women with mechanical valves, it is reported that the dosage of the anticoagulant should be adjusted so that APTT is 1.5 to 2.5 times the normal value. Moreover, it is reported that the effect of heparin will result in a vast change in APTT; therefore, strict monitoring is necessary. In this case, autologous injection of heparin began from 5 weeks of gestation, and continuous infusion of heparin started from 21 weeks of gestation. APTT was measured twice a week and the values of around 40-80 seconds were maintained, but several thrombi appeared on the mechanical valve at 22 weeks of gestation. It has been reported that the incidence of thrombosis in pregnant women with mechanical valves was 3.9% in the warfarin continuation group, 9.2% in the warfarin use after unfractionated heparin use for 12 weeks of pregnancy group, and 25% in the unfractionated heparin use throughout pregnancy group [8]. Comparing between heparin and warfarin anticoagulant therapy during pregnancy, thrombosis occurred significantly in the heparin group, ranging from 12 to 24 percent, indicating that serious complications may occur [9]. Therefore, in this case, because of the high risk of developing maternal cerebral infarction, warfarin administration was started after informing the patient about the side effects of abnormal blood coagulation in the fetus. Thus, after restarting warfarin administration, the multiple thrombi on the mechanical valve disappeared, but intraventricular hemorrhage appeared in the newborn. Blood collection data at the birth showed an abnormally high PT-INR value; the enzyme system of the fetus was underdeveloped and vitamin K-dependent coagulation factor was lower than the normal range. The influence of warfarin was apparent. In Japan, pregnant women with artificial valves are permitted to use warfarin for thrombus treatment during pregnancy in life-threatening circumstances [10]. However, as anticoagulation therapy and antiplatelet therapy during pregnancy affect not only the mother but also the infant, it is necessary to perform pregnancy management, bearing in mind the risk of fetal cerebral hemorrhage during the perinatal management.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Resuscitative hysterotomy in a patient with peripartum cardiomyopathy

Hidetake Kamei, Yu Wakimoto, Kayoko Harada, Atsushi Fukui, Hiroyuki Tanaka and Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan

Abstract

Resuscitative hysterotomy (RH) is a resuscitation technique, allowing the restoration of a pregnant patient's heartbeat. Here, we reported a case of RH performed in a patient with cardiac arrest as a complication of a peripartum cardiomyopathy. A 29-year-old woman with suspected hemolysis, elevated liver enzymes, low platelet syndrome was admitted to the hospital. Cardiopulmonary resuscitation and RH were initiated at 30 weeks of gestation. The infant was successfully delivered 2 min after the mother's cardiac arrest, weighting 1388 g. At the first minute, the Apgar score was 3 and the 5th minute was 6. After delivery, defibrillation was performed on the mother and restoration of spontaneous circulation was observed. However, she was hemodynamically unstable and approximately 2 months later she died. After cardiac arrest, it is possible that RH could improve the hemodynamic status. The opportunity of performing a RH is rare; however, it is necessary to be familiarized with the technique as a resuscitation method.

Key words: cardiopulmonary resuscitation, maternal cardiac arrest, perimortem cesarean section, resuscitative hysterotomy.

Introduction

Perimortem cesarean section (PCS) is now termed as resuscitative hysterotomy (RH).[1] RH is a viable option if cardiopulmonary resuscitation (CPR) is required in a pregnant woman.[2] The methods of resuscitation for pregnant women are different from those of nonpregnant women because the enlarged gravid uterus of pregnant women decreases venous return from the inferior vena cava.[2,3] Therefore, it is necessary to be familiar with the technique as a possible resuscitation method alongside CPR for pregnant women.

The mechanism of RH, which is performed to save the mother's life, empties the uterus by delivering the baby, relieving large vessels from compression.[4] This improves both the circulation dynamics and the respiratory condition of the mother by increasing the maternal functional residual capacity and decreasing oxygen consumption.[2] Although the opportunities to implement RH are very rare, there are 40 to 60 reported maternal deaths in Japan per year, and some of those cases require RH.[3] In addition, RH is highly urgent, and it is necessary to disseminate knowledge about this procedure so that proper training in hospitals and clinics can occur.[5,6]

Here, we report a case of peripartum cardiomyopathy that resulted in cardiac arrest and required the use of RH. As far as we know, there are no case reports of RH being performed for peripartum cardiomyopathy.

Case Report

The patient was a 29-year-old gravida 1 woman with unspecified medical history and family history. The patient weighed 65 kg and had a height of 162 cm when she entered our hospital. Her reported weight prior to pregnancy was 52 kg. She conceived

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Correspondence: Dr Yu Wakimoto, Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, 1-1 Mukogawacho, Nishinomiya, Hyogo 663-8501, Japan. Email: yuuwakimoto@gmail.com

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naturally and received prenatal checkups in the United States. After returning to Japan, she visited her previous doctor for a prenatal checkup at about 18 weeks of pregnancy without any remarkable changes. At 29 weeks and 4 days of her pregnancy, lower leg edema and weight gain of about 4 kg in 2 weeks were observed by the patient and she consulted her doctor. Her doctor did not think that treatment was necessary at the time. But the lower leg edema did not improve and was followed by the onset of general malaise, so she revisited her doctor at 30 weeks of pregnancy. A blood test showed platelets were at a normal level, but liver function was abnormal and urine protein was found. Hemolysis, elevated liver enzymes, low platelet count (HELLP) syndrome was suspected, and the patient was transported immediately to our hospital at 30 weeks of pregnancy.

During her initial visit to our hospital, the patient was conscious and able to stand and walk on her own. The patient's blood pressure was 95/56 mmHg, pulse was 120 beats per minute, and SpO_2 was 100% on room air. She was admitted with tachycardia. Her abdomen was soft and there was no tenderness as well as no epigastric pain. Lower leg edema in both legs was prominent.

There was no bleeding found during the speculum examination and the uterine cervix was closed. Transabdominal ultrasonography showed the fetal heartbeat to be normal and fetal movement was witnessed. The estimated fetus weight was 1637 g, which was appropriate for the number of weeks of pregnancy. The amniotic fluid index was 16.9 cm, and no abnormal findings were observed in the placenta. A chest X-ray was taken with the possibility of emergency cesarean section. X-ray scans showed that the patient's cardiothoracic ratio was 59%, pulmonary congestion was confirmed and pleural effusions were not observed.

At the time of admission to hospital, as cardiotocography showed decreased fetal heart rate (FHR) baseline variability and variable decelerations, we decided to perform an emergency cesarean section at 30 weeks of pregnancy. In the maternity ward, it was difficult to establish peripheral venous access because of edema of the upper and lower limbs and collapse of the peripheral blood vessels. We tried again to establish peripheral venous access in the operating room and considered the possibility of inserting a central venous catheter.

After entering the operating room, decreased FHR baseline variability and prolonged decelerations were observed on the FHR monitor. The patient lost consciousness immediately after inserting the central venous catheter. Her heart rate became bradycardic and cardiac arrest quickly followed. The anesthesiologists started CPR on the patient in accordance with the Japan Resuscitation Council guideline. Before going into cardiopulmonary arrest, she was restless and spinal anesthesia could not be used, so the patient was placed under general anesthesia. General anesthesia was administered shortly thereafter, along with epinephrine. RH and cardiac massage were then performed simultaneously. Two minutes after cardiac arrest was diagnosed, the baby was born with a birth weight of 1388 g and an Apgar score of 1 at 1 min and 6 at 5 min. We performed defibrillation on the mother and her heartbeat resumed 22 min after the diagnosis of cardiac arrest. Percutaneous cardiopulmonary support (PCPS) was carried out 4 min later. After closing the uterine incision, atonic bleeding was confirmed and we performed a supra hysterectomy. The patient lost 4005 mL of blood, which included amniotic fluid. The patient was transfused with 2240 mL of red cell concentrates (RCC) and 450 mL of fresh frozen plasma (FFP) (Fig. 1).

After surgery, the patient was admitted to the intensive care unit under sedation. At 15 days postsurgery, we started to wean her off sedatives. After she became responsive, we started to wean her off PCPS. PCPS flow was reduced, but echocardiography showed a left ventricular ejection fraction (LVEF) of 20%, showing that cardiac function did not improve. Nineteen days after surgery, PCPS was removed. By day 20, the patient remained on the positive pressure ventilation system. Due to barotrauma, pneumothorax was

Time	Timeline of events in the operating room
0h 0m	enter the operating room
0h 10m	insert the central venous catheter
0h 25m	start general anesthesia
0h 29m	cardiac arrest, start cardiopulmonary resuscitation, start operation
0h 31m	delivery of the baby
0h 42m	ventricular fibrillation(VF), Perform defibrillation
0h 51m	return of spontaneous circulation
0h 54m	Percutaneous cardiopulmonary support(PCPS)start
0h 55m	administration of epinephrine
1h 50m	hysterectomy
2h 59m	completion of surgery
3h 31m	leave the operating room

Figure 1 Timeline of events once the patient entered the operating room. Note that the zero point is set to the time when the patient entered the operating room. PCPS, percutaneous cardiopulmonary support; VF, ventricular fibrillation.

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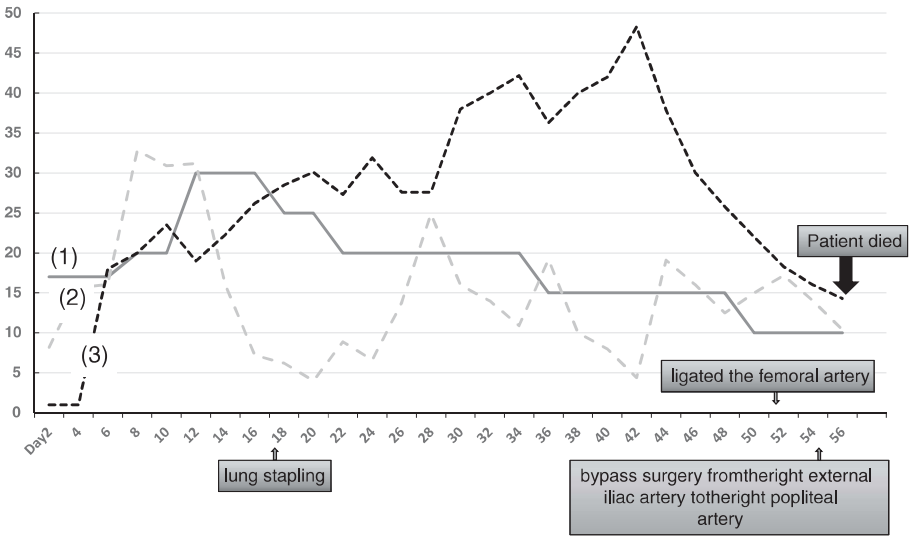


Figure 2 Postoperative course of events leading up to the patient's death. (1) ——A left ventricular ejection fraction (LVEF). (2) - - - - -, C-reactive protein (CRP) as a measure of inflammation. (3) -----, White blood cell (WBC) count.

observed and lung stapling was performed. Afterward, circulation was found to be unstable again and the PCPS was reinserted the next day. Twenty-one days after surgery, fungus was found in the patient's blood culture and antifungal drugs were administered, but they were ineffective. A chest X-ray confirmed exacerbated pulmonary bleeding and withdrawal of PCPS became impossible. On postoperative day 27, we carried out extracorporeal membrane oxygenation (ECMO) and continuous hemodiafiltration. Although multiple antibiotics were administered, inflammatory reactions did not improve. On the 42nd postoperative day, a large amount of melena was observed and we transfused 450 mL of FFP and 560 mL of RCC. At this time, a blood analysis was performed, which revealed the following levels: hemoglobin 8.2 g/dL, platelets 3300/μl, prothrombin time-international normalized ratio 1.65 and D-dimmer 6.9 μg/mL. Fifty-two days after surgery, hemorrhage was observed from the PCPS insertion site and we ligated the femoral artery. Subsequently, a change in the color tone of the lower limb was observed and no pulse could be found in the dorsal artery of the foot. A bypass surgery from the right external iliac artery to the right popliteal artery was performed. On the 55th postoperative day, a bypass surgery from the left common iliac artery to the left popliteal artery was performed due to ischemia of the left lower limb. Over time, the ischemia spread from the patient's lower extremities to the rest of her body, resulting in multiple organ dysfunction. The patient died 56 days after the operation (Fig. 2).

An autopsy showed thinning between the myocardial fiber bundles and atrophy of myocardial fibers. This finding is consistent with peripartum cardiomyopathy. Myocardial tissue was diagnosed as peripartum cardiomyopathy (Fig. 3).

The newborn baby was intubated and admitted to the NICU after birth.

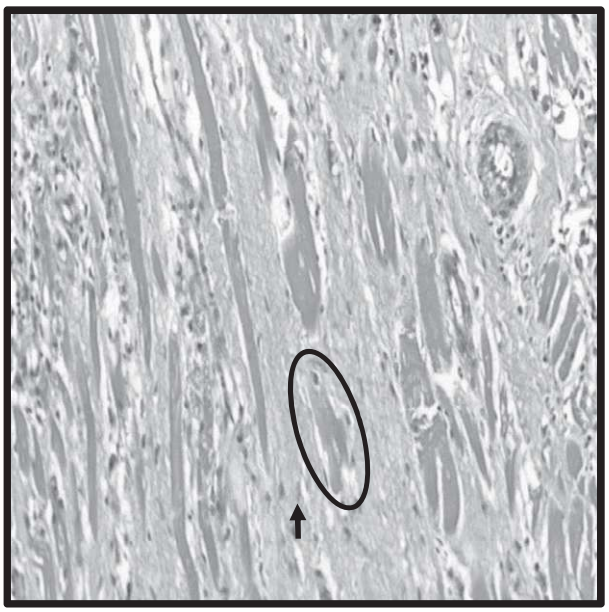


Figure 3 The postmortem micrograph of the myocardial tissue of the patient's left ventricle (×400, hematoxylin and eosin-stain) shows thinning between myocardial fiber bundles (arrow) and atrophy of myocardial fibers (circle).

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At the time of admission, a blood gas analysis was performed which revealed the following levels: pH 7.130, CO_2 22.9, O_2 53.4, BE-20.2, HCO_3 10.1 and Lactate 19. Sodium bicarbonate was administered after the diagnosis of metabolic acidosis. The chest X-P found ground glass opacity in both lung fields, which was diagnosed as respiratory distress syndrome. Surfactant was intratracheally administered. The transcranial doppler did not find evidence of intraventricular hemorrhage, but the baby was sedated. Catecholamine was infused because of circulatory dynamics instability. After the third day of treatment, blood pressure began to stabilize, urine volume increased, and catecholamine administration was terminated. At this point, we attempted to reduce the infant's dependency on the ventilator and on day 17, the infant was extubated. The infant was discharged at 85 days of age. We followed up with the infant 7 years later and sequelae were not found.

Discussion

RH is one form of CPR that can be used on pregnant women suffering from cardiac arrest.[2] Cardiac arrest during pregnancy occurs in approximately 1 out of 12 000 women who have been hospitalized for delivery.[7] Although cardiopulmonary arrest is a rare event during pregnancy and labor, it is necessary to train for CPR for pregnant woman because it is different from CPR for nonpregnant women.

In the third trimester, maternal chest compressions for cardiac arrest are ineffective. Relieving the aortocaval compression from the large gravid uterus of a pregnant women allows for venous return and potentially more effective chest compressions.[8] It is reported that the uterus should be emptied and the baby delivered to achieve an improved maternal hemodynamic status.[8] In the medical literature from 1985 to 2004 using an Ovid Medline search, Katz *et al.*[7] reported that out of 20 mothers who were potentially able to be resuscitated, 12 were resuscitated.[6] Four out of nine cases improved the mother's hemodynamic status even if cardiac arrest occurred more than 15 min prior to delivery by PCS. Dijkman *et al.*[6] reported that in 2 929 289 deliveries from August 1, 1993 to August 1, 2008 in the Netherlands, 55 women had cardiac arrest and 12 of them underwent PCS. Eight of the 12 women regained cardiac output with two maternal and five neonatal survivors. In this report, no maternal survivors were resuscitated after 15 min of CPR and no infant survivors were reported. It is clear that the sooner RH is performed after a mother arrests, the greater the likelihood of survival for both infant and mother.[6,8] If a heartbeat has not resumed after 4 min of CPR, a judgment should be made about whether or not to perform RH.[9] Considering maternal and neonatal outcomes, it is desirable to deliver within about 5 min of maternal cardiac arrest.[8]

In this case report, RH was performed at 30 weeks of pregnancy, and the infant was delivered about 2 min after the cardiac arrest and had no long-term sequelae. Katz *et al.*[10] reported in 2005 that in 38 cases of RH, 34 infants survived (three sets of twins, one set of triplets). Even if RH was performed more than 15 min after cardiac arrest, there were some reported cases of no sequelae (4 out of 7 intact survive, two cases with neurological sequelae, one case of respiratory disorder).[8] However, if RH was performed between 28 and 31 weeks of gestation, the infant survived in 14% of the cases reported.[8] In this case, since the time from maternal cardiac arrest to delivery of the infant was short, the prognosis of the child was good.

In this case report, the mother was already in the operating room when she went into cardiac arrest, so it was possible to start CPR and perform RH immediately, leading to the successful resuscitation of the patient. However, the patient's cardiac function did not improve over time due to peripartum cardiomyopathy. The patient died shortly after due to multiple organ failure from complications with severe infection. It is recommended to introduce ECMO to stabilize the circulation, to perform a RH immediately after the introduction of ECMO and to perform hysterectomy if severe coagulopathy is indicated.[10] In this case report, we performed RH and hysterectomy. Following this, we introduced ECMO after surgery. It may improve the outcome in mother by introduction of ECMO before RH.

It is reported that patient mortality rates from peripartum cardiomyopathy were 4% and the rate of improvement in cardiac function (LVEF ≥ 50%) was 63%.[11] The patient in this case showed rapid weight gain and progressive leg edema. It is difficult to diagnose peripartum cardiomyopathy based on these symptoms because they are not specific to peripartum cardiomyopathy and can also be present in hypertensive disorders of pregnancy. If these symptoms are recognized, it is important to consider the possibility of peripartum cardiomyopathy.[12] In patients with peripartum cardiomyopathy and an LVEF greater than 30% at onset, improvement of left ventricular systolic dysfunction was noted, but in patients with

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peripartum cardiomyopathy with left ventricular thrombus, no improvement of left ventricular systolic dysfunction was observed.[11,12] Therefore, cardiac ultrasound will help to prevent worsening of left ventricular function. A nationwide survey performed in Japan in 2009 showed that the incidence of peripartum cardiomyopathy was 1 in 20 000 pregnancies.[11] Regarding the prognosis of peripartum cardiomyopathy, Amos *et al*.[12] reported that the average of EF, which is 4 years after the onset of peripartum cardiomyopathy, showed that left ventricular function had improved in approximately 60% of patients and worsened in 40%.[6] Less than 10% resulted in a severe case, which required heart transplantation.[6]

Our medical team encountered a situation where RH was required due to cardiopulmonary arrest caused by peripartum cardiomyopathy. Although it was possible to resuscitate the mother after RH was performed, the mother's cardiac function had already decreased to the point that her life could not be saved. Since peripartum cardiomyopathy worsens suddenly, it is important to diagnose and treat it early. We carried out RH immediately after cardiac arrest and the child was delivered shortly afterward, resulting in a good outcome for the infant.

Although the opportunity to perform RH is rare, it is necessary to be familiar with the technique as a resuscitation method for pregnant women because their resuscitation affects the prognosis of both mother and infant.

Disclosure

None declared.

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【症例報告】

体外受精で妊娠成立し硬膜外麻酔を併用し経腟分娩に成功した高位脊髄損傷の1例

藤井　優，加藤　徹，表　摩耶，上東真理子
森本　篤，脇本　裕，原田佳世子，柴原浩章

兵庫医科大学産科婦人科学講座

（受付日　2020/1/8）

概要　近年，救命医学の進歩に伴い，脊髄損傷女性の妊娠は増加傾向にあるが，わが国での分娩症例の報告はまだ少ない．今回われわれは，硬膜外麻酔を併用し経腟分娩に成功した高位脊髄損傷症例を経験した．症例は38歳，未経妊．21歳時に交通事故で第3胸椎を損傷し，第4，5胸椎後方固定術を施行された．受傷後は月経再開し性交渉も可能であった．結婚後，挙児希望のため他院を受診し，タイミング療法では妊娠に至らず，当院の体外受精で妊娠が成立した．妊娠成立後は切迫早産，尿路感染や自律神経過反射（AH）の発症に留意しながら妊婦健診を行い，自覚症状の乏しい患者に子宮収縮や前期破水などを見落とさないための細やかな教育を行った．妊娠31週から切迫早産のため入院管理し，安定後は妊娠37週以降での計画分娩とした．経腟分娩に際して，AH予防のために硬膜外麻酔を施行し，あらかじめ取り決め事項を作成し，他科との連携を密に行った．妊娠37週3日から2日間かけて頸管拡張を行い，陣痛誘発を計画していた妊娠37週5日に自然陣痛が発来し，取り決め事項に沿い分娩管理を開始した．起こりうる合併症をあらかじめ想定し，他科と横断的な連携をはかり，母児共に良好な転帰を迎えることができた．陣痛発来後の早期に硬膜外麻酔を開始したにもかかわらずAHの合併を認め，降圧剤投与を行う必要はあったが，取り決め事項に沿った迅速な対応で脳卒中などの重症AHを発症することなく経腟分娩に成功した．〔産婦の進歩72（3）：280-286，2020（令和2年8月）〕
キーワード：脊髄損傷合併妊娠，高位脊髄損傷，経腟分娩，硬膜外麻酔，自律神経過反射

【CASE REPORT】

A case of transvaginal delivery using epidual anesthesia in a patient with high spinal cord injury

Yu FUJII, Toru KATO, Maya OMOTE, Mariko KAMIHIGASHI
Atsushi MORIMOTO, Yu WAKIMOTO, Kayoko HARADA and Hiroaki SHIBAHARA

Department of Obstetrics and Gynecology, Hyogo College of Medicine

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Abstract　In recent years, the pregnancy of women with spinal cord injuries has increased, with the advancement of critical care medicine. However, there have been few reports of such birth cases in Japan. We experienced a case of a patient with a severe spinal cord injury who achieved a successful transvaginal delivery with epidural anesthesia.The case was 38-year-old woman who was not pregnant. At the age of 21, she injured her third thoracic vertebra in a traffic accident. After the injury, her menstruation resumed and sexual intercourse was possible. After marriage, the patient wished for a baby. She was treated at another hospital with timing therapy, but failed to become pregnant and was referred to our hospital. After sufficient informed consent, in vitro fertilization was performed, and she became pregnant.The patient subsequently experienced premature birth, urinary tract infection, and autonomic hyperreflexia (AH). We educated the patient about uterine contractions and premature rupture, because they had poor subjective symptoms.From the thirty-first week of pregnancy, she was hospitalized for imminent preterm birth, and we planned for delivery after 37 weeks of pregnancy.During vaginal delivery, epidural anesthesia was scheduled to prevent AH. We prepared a protocol

in advance and worked closely with other departments. Her cervix was dilated for two days, from the 37 weeks three days of pregnancy. Spontaneous labor occurred on the 37 weeks five days of pregnancy, and we initiated delivery management according to our protocol.We anticipated possible complications in advance, managed cross-disciplinary cooperation with other departments, and achieved favorable outcomes for both mother and child. We started epidural anesthesia shortly after the onset of labor, but AH developed. We then administered antihypertensive drugs to manage the onset of AH. Our rapid, protocol-based response resulted in a successful vaginal delivery without severe AH complications, such as stroke.
[Adv Obstet Gynecol, 72 (3) : 280-286, 2020 (R2.8)]
Key words : pregnancy, spinal cord injury, vaginal delivery, epidual anesthesia, autonomic hyperreflexia

緒　言

脊髄損傷は交通外傷，スポーツ外傷などを原因とし，救命医学の進歩によりその生存率は改善し，脊髄損傷合併妊娠も増加傾向にあるが，わが国での分娩報告はまだ少ない．脊髄損傷合併妊娠では切迫早産や尿路感染のリスクが高く[1,2]，子宮収縮症状の自覚も乏しい．とくに第6胸椎レベル以上の脊髄損傷の場合，自律神経過反射の予防が重要である[3,4]．今回われわれは，高位脊髄損傷受傷後に不妊治療で妊娠し，硬膜外麻酔併用して経腟分娩に至った症例を経験したので報告する．

症　例

症例は38歳，未経妊であった．家族歴に特記すべきことはなく，21歳時に交通事故で第3胸椎を損傷し，第4，5胸椎後方固定術を施行された．乳頭以下の温痛覚麻痺と運動麻痺があり，深部感覚は保たれていた．日常生活は車椅子で，排泄は自己導尿と下剤で自立していた．受傷後，月経周期は整で28日型，月経量は過多，月経時に冷汗を伴うことがあった．性交渉は可能であった．

近医での6周期のタイミング療法後，36歳時に挙児希望で当院生殖医療センターを受診した．妊娠成立後の切迫早産や尿路感染，排尿障害，自律神経過反射（autonomic hypereflexia；AH）による頭痛，発汗，徐脈，発作性高血圧のための脳卒中のリスク，さらに分娩方式について詳細に説明し，理解されたうえで不妊治療を開始した．

超音波検査上は右卵巣に5 cm大，左卵巣に2 cm大の子宮内膜症性嚢胞をおのおの認めた．それ以外に不妊症検査上の異常は認めず，当院では人工授精から開始した．人工授精を6周期行っても妊娠に至らなかったため，体外受精へ移行した．採卵は静脈麻酔下に行い，初回の採卵で7個の卵子を回収し，体外受精により5個の受精卵を得た．新鮮周期で採卵後3日目に初期胚移植を行ったが妊娠に至らなかった．胚盤胞を3個凍結保存できたため凍結融解胚移植を計画し，2回目の凍結融解胚盤胞移植で妊娠が成立した．

妊娠経過

妊娠成立後，血圧は110～120/50～60 mmHgを推移し，尿路感染は認めず胎児成長も順調であった．その後もAHは認めず経過良好であったが，妊娠30週から自己導尿時に頭痛を自覚するようになり，尿道バルーンを留置し3週間おきに交換した．妊娠31週で頸管長28 mmと短縮傾向にあり，頻回の子宮収縮を認め切迫早産の診断で入院管理を開始した．安静と塩酸リトドリンの持続点滴で子宮収縮は改善した．

経腟分娩では自然陣痛や児娩出時の刺激が，帝王切開術では腹腔内操作や術後疼痛がAHの誘因となる可能性があり，いずれの分娩方式を選択した場合も硬膜外麻酔でAHを予防する必要があることを説明し，本人の希望を考慮して経腟分娩を選択した．麻酔科医，小児科医と事前カンファレンスを行い，AH予防のため硬膜外麻酔を併用したうえで，妊娠37週以降での計画分娩とした．硬膜外麻酔は陣痛の発来または誘発時に硬膜外チューブを留置することとし，使用薬剤はロピバカイン塩酸塩水和物の持続点滴を選択した．子宮収縮薬はオキシトシンを用

い，帝王切開術への移行は産科的適応の場合とした．さらに詳細な取り決め事項を作成し，麻酔科医，小児科医と情報を共有した（表1）．

分娩経過

妊娠37週3日から2日間かけて頸管拡張を行い，陣痛誘発を計画していた．妊娠37週5日に自然陣痛が発来した．分娩経過を図1に示す．陣痛発来後，血圧は5分おきに計測しCTG連続モニタリングを開始した．陣痛間隔5分，子宮口3 cm開大した午前3時にL3/4椎間から頭側に向けて硬膜外カテーテルを留置した．0.1％ロピバカイン塩酸塩水和物を3 mg投与し1.0 mg/minで持続投与開始した．患者は温痛覚障害があり麻酔レベルを評価できないため，AH症状

表1　分娩時の取り決め事項

分娩第一期	・硬膜外麻酔開始 ・頻回の内診（1時間間隔） ・持続血圧測定（5分間隔） ・CTG連続モニタリング ・頻回の導尿（1時間間隔） ・異常発汗や徐脈などAH症状に注意した観察 ・NICU、麻酔科へ適宜、分娩進行を報告
分娩第二期	・怒責回避 ・会陰切開と子宮底圧出法、吸引補助分娩
分娩第三期	・メチルエルゴメトリン使用の回避
AH発症時	・誘因の除去（内診、導尿、陣痛誘発など） ・発作性高血圧があれば速やかに降圧加療開始
緊急帝王切開術の適応	・胎児心拍異常、薬剤抵抗性高血圧など産科的適応に順ずる

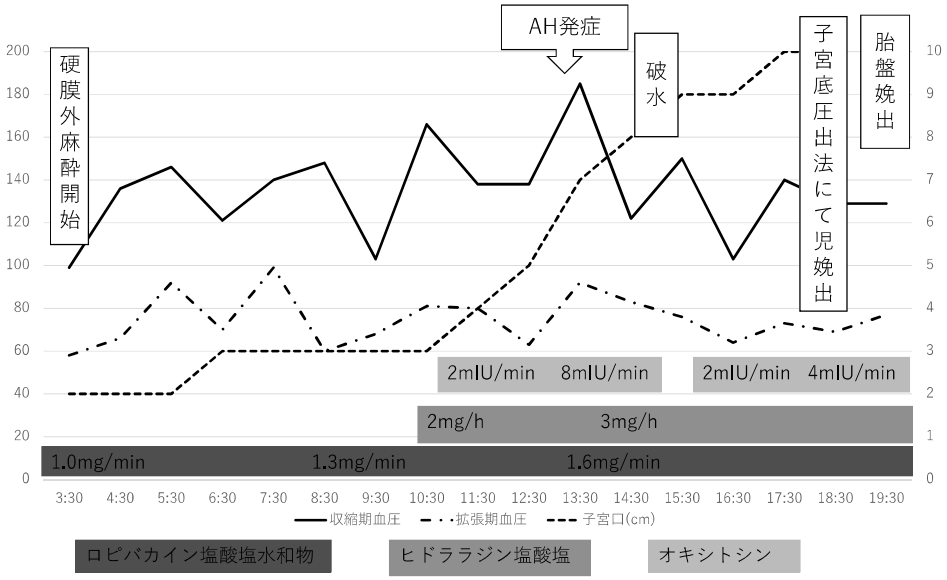


図1　分娩経過

がないことと，異常高血圧がないことを効果判定の指標と考え，胎児への血流を考慮し収縮期血圧120～160 mmHgを目標とした．その後，血圧は90～140/60～90 mmHg台を推移していたが，分娩進行に伴い収縮期血圧150 mmHg以上の高血圧を認めるようになったため，収縮期血圧160 mmHg以上の発作性高血圧が起こる可能性を考慮し，7時50分に0.1％ロピバカイン塩酸塩水和物を1.3 mg/minへ増量した．その後，安定するも11時に発作性に収縮期血圧が160 mmHgまで上昇したため，0.1％ロピバカイン塩酸塩水和物を1.6 mg/minへ増量し，ヒドララジン塩酸塩を2 mg/hで開始した．その後，微弱陣痛となり，血圧が安定したことを確認し11時半からオキシトシン2 mIU/minで陣痛促進を開始した．オキシトシンは30分おきに2 mIU/minずつ増量し，13時にオキシトシンを8 mIU/minまで増量した．増量後，子宮収縮が2－3分間隔，子宮口が6－7 cmまで開大した時点で，AHと思われる収縮期血圧170～200 mmHgの急激な血圧上昇と頭痛を認めた．速やかに陣痛促進を中止し，ヒドララジン塩酸塩を3 mg/hに増量し血圧は安定し頭痛も消失した．自然陣痛で経過観察していたが，再度微弱陣痛となったため，16時半にオキシトシンを2 mIU/minで再開し，4 mIU/minまで増量し有効陣痛を得ることができた．その後は血圧も100～130/50～70 mmHg台と安定しAH症状は認めなかった．子宮口が8 cmまで開大した時点で高位破水し，17時57分に子宮口全開大となった．AHの誘発を回避するため怒責はかけず，18時49分に会陰切開と子宮底圧出法により児を娩出した．当初，吸引分娩を検討していたが軽度の子宮底圧出法にて児娩出が可能であったため施行しなかった．児は2670g，アプガースコア1分値9点，5分値10点の女児で異常を認めなかった．胎盤娩出はスムーズで分娩時出血は771 mlであった．産褥経過は異常なく，産褥6日目に母児ともに退院となった．現在第二子希望で当院生殖医療センターに通院中である．

考　察

National Spinal Cord Injury Statistical Center；NSCISC（2017）によると，米国での脊髄損傷は年間17,000件発症し，新規受傷者の20％が女性である．女性の場合，一過性の脳下垂体性性腺機能低下を認める可能性があるが，そのほとんどで半年以内に月経が再開する[5,6]．月経が再開すれば妊娠は可能で[7]，わが国でも症例報告はまだ少ないが自然妊娠の報告もある[8,9]．

脊髄損傷患者の妊娠分娩管理は，切迫早産や尿路感染，排尿障害，破水や子宮収縮の自覚が困難といった問題点がある[1,2]．非妊娠女性と比較した研究では，脊髄損傷患者では膣内細菌叢が変化しているとの報告があり[10]，早産や低出生体重児の頻度が高い．さらに最も重要な合併症としてAHがある．AHは第5～6胸髄以上の高位脊髄損傷患者の85％に生じ，神経麻痺領域から受ける刺激により引き起こされる発作性の症状である[3,4]．

腹部の内臓を支配する交感神経は第5～6胸髄と第2腰髄から出ており，第5～6胸髄より上位の脊髄損傷では排泄や腹部の温痛覚は失われるが，麻痺領域からの刺激は交感神経を過剰に興奮させる．正常であれば過剰な刺激は中枢から抑制されるが，脊髄損傷患者ではその機能が失われているため，過剰な交感神経の刺激により発作性高血圧を引き起こす．この血圧上昇は大動脈弓や頸動脈洞の圧受容体で感知され，非麻痺領域の血管を拡張させる．それにより頭痛，発汗，顔面紅潮や迷走神経反射による徐脈を引き起こす．重症例では，脳卒中など生命に関わる合併症を引き起こす可能性がある[11-13]．

麻痺領域の刺激，つまり子宮収縮，膀胱の充満，排尿や排便を含む膀胱直腸刺激，手術による腹腔内操作，内診などが誘因となりAHは引き起こされる[4,14]ため，高位脊髄損傷合併妊娠出産はハイリスクである．

分娩方式はVerduynら[15]の報告では，帝王切開が57％（n＝50）で，第6胸髄以上の高位脊髄損傷患者に限ると帝王切開術は25％（n＝

27）である．重症AHによる適応が1例，他は帝王切開術既往，双胎妊娠，臍帯脱出，前期破水といった産科的適応であった．本邦では脊髄損傷合併妊娠の報告は少ないが，森川ら[16]の報告では帝王切開術は43％（n＝23）であった．第5胸髄レベル以上の高位脊髄損傷患者では陣痛がAHの誘因となるため，陣痛発来または誘発時までに硬膜外チューブを挿入し，AHを予防する必要がある[4]．また，外科手術時の腹腔内操作や術後疼痛がAHを引き起こす可能性があり，帝王切開を選択した場合にも硬膜外麻酔が推奨されている[17]．

硬膜外麻酔については，分娩第一期の子宮収縮や頸管拡張による内臓痛はTh10-L1に，分娩第二期の下部軟産道の伸展に伴う体性痛はS2-S4に伝達されるため，麻酔レベルはTh10まで必要である．硬膜外麻酔を用いた経腟分娩の過去の症例報告では，硬膜外カテーテルをTh12-L3間とL3-L5間のいずれかの椎間に1カ所ずつ，合計2本を挿入し管理した報告[8,9,18]とL3－L5のいずれかの椎間へ挿入し，1本で管理した報告[19-21]がある．局所麻酔は0.1-0.2％ロピバカイン[8,18]，0.125％ブピバカイン[19]，1％メピバカイン[9,20]，1-2％リドカイン[21]とさまざまで，フェンタニル1.6-4μg/mlを併用した症例[8,18,19]もあり，一定の見解は得られていない．さらに，温痛覚障害のある脊髄損傷妊婦では麻酔レベルの評価が難しく，血圧が麻酔レベルの評価指標となる[8]．

さらに，硬膜外麻酔のみで適切な降圧が得られない場合は降圧薬を使用するべきであり，調節性の高い持続点滴可能で即効性短時間作用性の薬剤が推奨されている[22]．

本症例では，受傷後に月経再開しており性交渉も可能であったが，子宮内膜症性不妊症で体外受精へ移行した．われわれは，不妊治療の段階から妊娠の成立，分娩方法について事前に詳細に説明し，同意を得た上で治療を開始した．脊髄損傷患者の体外受精による妊娠の報告は少なく，十分なデータを得られなかったため採卵時のAH予防について難渋した．採卵操作がAHの誘因となる可能性は十分に予想され，かつ，本症例の場合，経腟操作の刺激による下肢の不随意運動が出現することからも，何らかの鎮静が必要であると考えた．本症例では日常生活や普段の経腟診察でAH症状を認めないことを考慮し，麻酔科医，本人と相談の上で静脈麻酔を選択した．麻酔科医立会いのもと，静脈麻酔下で十分な鎮静をかけた上で採卵し，AH発症時にはすみやかな原因の除去，つまり採卵の中止と降圧加療を行うこととした．静脈麻酔は1％プロポフォールを用い70μg投与したのち，3μg/minで持続投与した．血圧は90～100/50～60 mmHg台を推移しAHを発症することなく採卵できた．

分娩方式は経腟分娩を選択し，産科的適応がある場合にのみ帝王切開へ切り替える方針とした．分娩時の対応として，あらかじめ他科と連携して取り決め事項を作成した（表1）．陣痛発来時より麻酔科医，小児科医と情報共有を行ったうえで，硬膜外麻酔の併用を開始した．患者は子宮収縮に伴う自覚症状に乏しいため，1時間おきの内診と頻回の血圧測定，CTG連続モニタリング，頭痛，発汗，顔面紅潮といったAHを疑う身体所見に留意して管理を行った．AHはその誘因の除去により症状改善を得られる場合が多く，分娩中のAH発症時には誘因の除去，つまり速やかな陣痛誘発，促進の中止と硬膜外麻酔の評価，ヒドララジンの持続点滴を開始する方針とした．また，手術操作や術後疼痛もAHの誘因となるため，AH発症を緊急帝王切開の適応とはせず，胎児心拍異常，薬剤抵抗性の異常高血圧など産科的適応時のみ緊急帝王切開へ移行することとした．

今回の症例では，合併症として切迫早産と陣痛発来後の早期に硬膜外麻酔を行ったにもかかわらず，AHの合併を認め一時陣痛促進を中止し，降圧剤投与を行う必要があった．切迫早産に関しては妊娠27週までは2週間おき，子宮収縮を自覚した妊娠27週以降は1週間おきに妊婦健診を施行し，子宮頸管長を計測することで切迫早産を早期に発見することができた．さら

に医師と共に腹部触診することで子宮収縮の感覚を指導した．切迫早産の管理入院後は連日CTGモニタリングを実施し，子宮収縮の出現時に助産師と共に腹部を触診し，子宮収縮の感覚を習得することで，微細な変化でも子宮収縮を自覚できるようになった．脊髄損傷合併妊婦の20％で早産を合併するため，妊娠中期よりCTGモニタリングを実施し，CTGの波形と腹部触診により子宮収縮を指導することで，より早期に切迫早産を発見することができると考える[23]．陣痛発来や前期破水の自覚に乏しく，それらを誘因にAH発症する可能性があるため，妊娠37週をめどに計画分娩や管理入院が推奨されている[24]．

AHの合併については，降圧目標を収縮期血圧120～160 mmHgとしたが，結果的にAHを発症した．平常時の収縮期血圧から20％以上の血圧上昇をAHとして対応する[22]ことから，本症例では平常時血圧が110～120/50～60 mmHg前後であったことを考えると目標値をさらに低く設定してもよかったかもしれない．さらに，分娩第一期にAHを発症したことから，分娩第一期のTh10-L1へ伝達される子宮収縮や頸管拡張による内臓痛への麻酔が不十分であった可能性が高い．あらかじめTh12/L1に2本目の硬膜外カテーテルを挿入し，内臓痛に効果のあるフェンタニルを併用することでAHは防げたのではと考える．AHが発症しやすいとされる分娩第二期[25]にはAHが発症しなかったことから，分娩第二期のS2－S4に伝達される下部軟産道の進展に伴う体性痛への麻酔は十分であったと考える．

AHによる発作性高血圧は血圧の変動が激しく，誘因除去のみでも急激な降圧を得られることから，母体低血圧や胎児循環も考慮すると，降圧薬の開始や増量の判断は難しい．本症例では妊娠中の降圧薬として当院では第一選択薬であるヒドララジン塩酸塩を用いた．降圧剤の選択に関しては，報告により一定の見解はないが，より即効性があり短時間作用のニカルジピン塩酸塩を使用したほうがより調節性が高い可能性もあり，今後の検討が必要と考える．

さらに，われわれは陣痛発来を硬膜外麻酔の開始としたが，頸管拡張でも十分にAH発症のリスクがあり，本症例のように頸管拡張のみで自然陣痛発来することも予想されるため，頸管拡張前の硬膜外カテーテル留置について検討することが妥当であったと考える．

さまざまな問題点は残るが，起こりうる合併症をあらかじめ想定し，他科と横断的な連携をはかり，母児共に良好な転帰を迎えることができた．

結　語

今回，高位脊髄損傷合併妊娠女性に対し硬膜外麻酔を併用し，経腟分娩で挙児を得ることができた症例を経験した．脊髄損傷に起こりうる周産期合併症をあらかじめ想定し，他科との事前の連携をはかり，取り決め事項を統一したことで，母児ともに良好な転帰を迎えることができた．硬膜外麻酔を行うことで合併症を軽減し，患者の希望どおり経腟分娩が可能であった．

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症例報告

妊娠中期に進行胃癌と診断しS-1/L-OHP（SOX）による化学療法を施行した1例

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兵庫医科大学産科婦人科学講座
田島　千裕　　浮田　祐司　　松岡　理恵　　上東真理子
加藤　　徹　　原田佳世子　　柴原　浩章

Key words
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SOX therapy
chemotherapy

概要　進行胃癌合併妊娠にSOX（S-1/L-OHP）による化学療法を施行し，生児を得た1例を経験した．症例は34歳，妊娠10週より持続する上腹部症状を主訴に受診し，妊娠23週で低分化型腺癌の診断となった．妊娠26週0日よりSOX療法を1コース施行し，妊娠32週5日に選択的帝王切開術で分娩に至った．分娩後は外来で化学療法を施行したが，帝王切開後約9カ月で永眠された．児は1,572gの女児，Apgar score：8点（1分値），9点（5分値）で出生し，生後54日で退院となり，現在まで発育・発達に異常を認めない．妊娠中の化学療法の母児への影響は未だ不明な点が多い．進行胃癌の化学療法はSP療法が推奨されてきたが，近年はSOX療法に変わりつつある．胃癌合併妊娠において明確な指針は未だ示されておらず，今後も症例毎の対応を要するが，SOX療法の母児への影響を検討する上で貴重な1例と考えられた．

緒言

日本人女性における胃癌の罹患率は，乳癌，大腸癌についで3番目である．年代別ではほとんどが50歳以降に発症し，生殖年齢における胃癌の発症は稀である．悪性腫瘍合併妊娠の頻度は妊婦の0.1％であり，そのうち胃癌合併妊娠は0.03～0.1％と報告される[1]．また胃癌合併妊娠の多くで胃癌に特徴的な胃腸症状が悪阻と類似しているために見過ごされやすく，診断時には進行例が増え予後不良な症例が多いとされる[2]．

進行胃癌では手術不能な症例が多く，化学療法が選択される．しかし，妊娠中に化学療法を施行した場合，母児への影響はいまだ不明な点が多く，子宮内胎児発育不全，子宮内胎児死亡や切迫早産を発症しやすいとの報告もある[3]．進行胃癌に対する化学療法としては，SP療法《フルオロウラシル系（S-1）/白金製剤（CDDP）》が広く推奨されてきた[1) 4) 5)]．その後は2018年の胃癌ガイドラインに示されるように，SP療法からSOX療法《フルオロウラシル系（S-1）/白金製剤（L-OHP）》に移行しつつある[6]．しかし胃癌合併妊娠に対するSP療法の報告は少なく，SOX療法に関しては更に報告は少ない．今回，妊娠中に進行胃癌を認めSOX療法を施行し，生児を得た一例を経験したので報告する．

症例

症例は34歳，4妊1産，2016年2月*H.pylori*胃炎の診断で2回の除菌歴がある．家族歴は父方祖父：骨肉腫，母：高血圧，祖母：2型糖尿病に加え，消化器癌が多数（父：B型劇症肝炎，肝癌，伯父：大腸癌，母方祖父：膵臓癌，母方曽祖父：胃癌（詳細不明））であった．自然妊娠成立後，妊娠10週頃より持続する上腹部症状を認めたため，妊娠23週でA病院を受診した．上部内視鏡検査による生検で低分化型腺癌と診断され，妊娠24週4日に当院上部消化管外科および当科紹介となった．

初診時の血液検査では食事摂取不良によると考えられる電解質異常，軽度の低アルブミン血症を認めた．

兵庫医科大学産科婦人科学講座
〒663-8501　兵庫県西宮市武庫川町1番1号

Department of Obstetrics and Gynecology, Hyogo College of Medicine
1-1 Mukogawa-cho Nishinomiya-city, Hyogo 663-8501, Japan

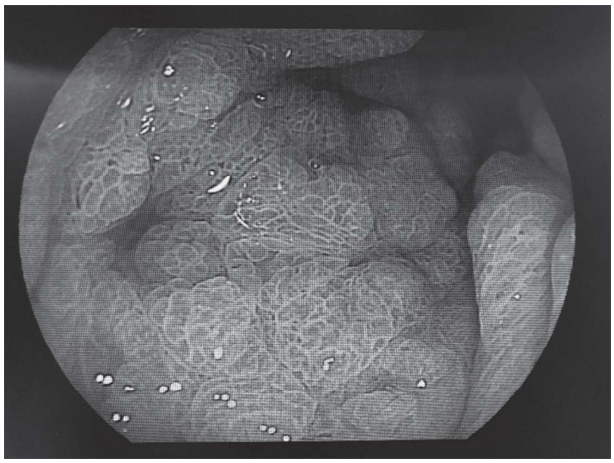


図1　上部消化管内視鏡
ひだの肥厚や蛇行を認める．

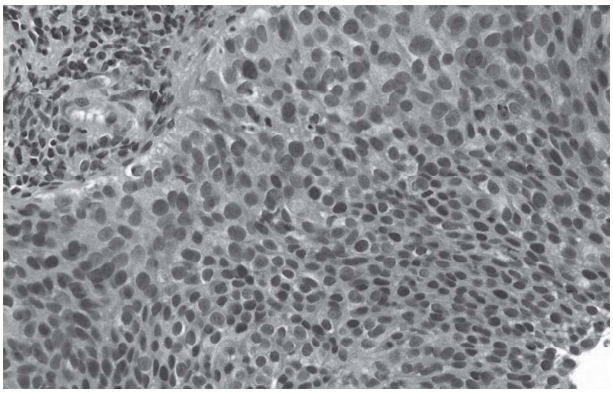


図2　病理組織検査（HE染色，400倍強拡大）
腺腔形成がなく，癌細胞がびまん性に浸潤している．

腫瘍マーカーはCEA 0.9ng/mLと正常，CA19-9 252.5U/mLと著明な上昇を認めた．

妊娠24週5日に上部消化管内視鏡検査を施行した．送気による胃の伸展は不良で，ひだの肥厚や蛇行などの変化を認め，肉眼的分類ではBorrmann 4型，びまん浸潤型であった（図1）．同時に生検を行い，病理組織検査でGroup 5，Adenocarcinoma，por，HER2（-）と診断した（図2）．

胸・腹部骨盤CTでは胃壁は不整に肥厚し，胃周囲リンパ節は多数腫大していた．以上よりStage III b（T4N2M0）の進行胃癌（4型進行胃癌・スキルス胃癌）と診断した．消化器内科・消化器外科・産婦人科・小児科の合同カンファレンスにより，現時点での手術適応は無く，化学療法後に児娩出の方針とし，本人・御家族とも同意された．

従来進行胃癌に対する化学療法ではSP療法が第一選択であったが，最近はSOX療法に移行してきている．そこで，充分なインフォームドコンセントのもと，妊娠26週0日よりSOX療法を1コース（S-1：100mg/body + L-OHP：120mg/body）施行した．身長：158cm，体重：52.9kg-2kg（胎児分）＝50.9kg，体表面積：1.499による数式から，S-1は100mg/bodyとfull doseとした．L-OHPは妊娠中であることを考慮し，CDDPに対する報告と同様に120mg/bodyとdose downし，80％量で投与した．妊娠26週0日から妊娠28週6日までS-1（100mg/day）を21日間経口投与し，妊娠26週0日にL-OHP（120mg/day）を静注した．

その後，母体は抗癌剤による重篤な副作用症状を認めず，児も子宮内胎児発育不全，羊水過多や羊水過小などを認めず経過した．在胎32週5日に選択的帝王切開術で，1,572gの女児，Apgar score：8点（1分値），9点（5分値）で娩出となった．同時に審査腹腔鏡を施行し，低分化型腺癌の腹膜播種病変を確認した．

帝王切開術後14日目からSOX療法を再開し，計8コースを施行したが，上部消化管内視鏡検査，腫瘍マーカーよりProgressive Disease（PD）と診断した．そこで二次化学療法であるパクリタキセル（PTX）＋ラムシルマブ（RAM）療法に変更し，2コース施行後に審査腹腔鏡を施行したが，播種病変の増悪，および腹水の増加を認め，再度PDと診断した．

次に三次化学療法であるニボルマブ（Nivo）に変更し，2コース施行したが，その後Nivoによる特発性血小板減少性紫斑病（Idiopathic Thrombocytopenic Purpura；ITP）ないしは播種性血管内凝固症候群（Disseminated Intravascular Coagulation；DIC）によると推測される血小板低下（1.7万／μL）を認め入院となった．血小板低下および貧血の進行のため連日輸血を行い，ステロイドパルス治療を行ったが，入院後12日目（C/S後約9カ月）に急激な意識障害を認め，永眠された．意識障害の原因としては，頭痛を訴えていたことからくも膜下出血，あるいは癌性髄膜炎の可能性が示唆された．

一方，児は低出生体重のためNICUに入室したが，生後54日で退院となった．退院後は当院小児科外来で発育・発達をフォローしており，2歳9カ月になる現在も発育・発達に異常を認めない．

考察

胃癌の症状としては悪心・嘔吐や上腹部痛等があり，胃癌合併妊娠においても同様の症状を呈する．一方，妊娠初期の悪阻でも同様の症状を呈し，全妊娠の70％以上が悪阻による症状を呈する[3]ことから，妊娠中では胃癌と悪阻の症状を鑑別することは困難である．妊娠中には内視鏡やCTなどの画像検査の施行は躊躇されることも多く，病変の発見が遅れる要因となる．Min JSらは20例の胃癌合併妊娠について報告しているが，16例（80％）はstage III以上で発見され，発見

時の平均週数は31週であり，14例（70％）は妊娠22週以降に発見されている．さらに，発見後1年以上の生存率は20％であった[7]．従って胃癌合併妊娠は発見が遅れやすく，極めて予後不良である．今回の症例においても診断時期は妊娠23週であり，診断後1年以内に永眠されている．

胃癌は分化度により分化型胃癌と未分化型胃癌に大別される．分化型胃癌は*H.pylori*感染による萎縮性胃炎の結果として，腸上皮化生を伴う粘膜萎縮を経て発生し，異型細胞が腺管構造を形成し，限局性に増殖する[8]．一方，未分化型胃癌は低分化型腺癌・非充実型，あるいは印環細胞癌の組織型を呈する．非充実型は癌細胞が著明な繊維形成を伴って散在びまん性に浸潤し，特に繊維性間質量が多い場合はスキルス癌に相当する．印環細胞癌は細胞内粘液貯留が豊富な印環細胞からなり，腺管形成はほとんど認めない．粘膜内で密に増殖し浸潤することで，スキルス胃癌へと進展する．

若年者の胃癌は組織型が未分化型の場合が多く，進展様式は散在びまん性（スキルス型）であり，腫瘍が小さくてもリンパ行性，あるいは漿膜から腹腔内へ播種性に転移するという特徴のため，診断時には内視鏡治療の適応になりにくく，予後不良である[8]．

今回の症例は除菌後約半年での胃癌発症であった．本邦では*H.pylori*感染による胃炎の多くが胃体部に萎縮を伴い，除菌治療後に胃酸分泌が回復することで，逆流性食道炎を来しやすいという特徴がある[9]．本症例における除菌治療完遂後の腹部症状についても逆流性食道炎と診断したことが，内視鏡検査の遅延につながった可能性がある．

胃癌組織内にはエストロゲンレセプター（ER），プロゲステロンレセプター（PgR）などの性ホルモンレセプターや，エストロゲンやプロゲステロンなどの性ホルモンを認める．胃癌培養細胞に女性ホルモンを投与すると胃癌細胞の増殖が促進すること，男性に好発する胃癌と異なりスキルス胃癌は若年女性に好発することから，胃癌増殖と女性ホルモンには密接な関係があると報告されている[10)～12)]．

妊娠中はエストロゲンが上昇する[3]ため，若年女性の妊娠では胃癌の進展が加速する可能性があり，特に30歳未満の患者では胃癌による死亡率が高い傾向にある．また妊娠による免疫抑制状態や妊娠中の血液循環の増加により，腫瘍の急速な成長を促進する．

妊娠中の進行胃癌に対する化学療法の目的は，母体の延命のため可能な限り早期に化学療法を施行することと，胎児の健康な発達と安全を確保するためにできる限り長く妊娠を継続させることにある．胃癌合併妊娠の治療は，胃癌の進行期及び診断時の胎児の週数に依存することが報告されている[10]．すなわち胃癌を妊娠22週以前に診断した場合は，人工妊娠中絶後に治療するという選択がある．妊娠28週以降に診断した場合は，早期に分娩した後に治療を開始することが望ましい．

しかしながら本症例のように，妊娠22週から28週に診断した場合の明確な治療指針はなく，個別化して妊娠管理を行うことになる．この時期に診断された胃癌の治療は，妊娠28週から30週頃まで妊娠を継続し分娩後に化学療法を開始するか，妊娠中に化学療法を施行するか，いずれの報告も存在する[1) 4) 5) 13)～15)]．化学療法直後の児娩出は抗癌剤の胎児移行により骨髄抑制をきたし，血球減少から易感染性を惹起し重症敗血症を発症したという報告もある[13)]．本症例においては通常1クール3週間のうち最後の1週間が休薬期間とされていたが，児娩出時期との兼ね合いを考慮した結果，帝王切開までに約5週間の休薬期間をおいての児娩出とした．充分な休薬期間のため，抗癌剤による児の骨髄抑制の影響が少なかった可能性がある．一方で5週間母体の胃癌治療を停止したことから，癌の進行が加速した可能性も考えられる．従って母体の全身状態や妊娠経過により，症例毎に治療方針について詳細な検討が必要と言える．今回の症例は，診断後1年以内に死亡しており，予後不良であった．

SP療法は国内での第III相試験であるJCOG9912試験，およびSPRITS試験の結果から，HER2陰性胃癌においてもっとも推奨されるレジメン（エビデンスレベルA）である[6)]．またSOX療法もG-SOX試験によりSP療法と同等の有効性が示されており，HER2陰性胃癌の一次治療において推奨されるレジメン（エビデンスレベルB）とされている[6)]．世界的にもL-OHPの胃癌治療への導入が進み，標準的治療薬剤の1つと位置づけられている[16)]．SP療法とSOX療法の最大の違いはCDDPと比較してL-OHPでは腎毒性の副作用の出現率が少ない点である[17)]．大量輸液による水分負荷を必要としないため，外来投与が可能である[6)]．また海外では直腸癌合併妊婦に対して妊娠中にL-OHPを投与し健常児を得た報告が複数存在する[18) 19)]．

本症例では妊娠23週の診断直後から複数の関連科による合同カンファレンスを行った．進行度・組織型より予後不良であり，診断時点での根治術は困難であった．一方で人工妊娠中絶や計画分娩も困難であり，帝王切開までの待機期間中の患者の心理を考慮し，妊娠中の化学療法の施行を選択した．根治ではなく延命を目標にしていることや予後について説明したところ，家族環境を考慮し，患者本人およびご家族も外来治療を強く希望された．患者のQOLを維持できるSOX療法を選択し，母児共に重篤な副作用を認めることなく治療が可能であった．

結論

胃癌合併妊娠に対する化学療法としてSOX療法を施

行した．しかし他に類似報告は少なく非常に限定的である．そのため現状としては妊娠中に化学療法の施行が必要な場合，消化器内科医だけでなく，産科医や小児科医を交えての十分な議論の上，十分なインフォームドコンセントを行うことが重要である．今後も胃癌合併妊娠に対するSOX療法の有効性・安全性に関する知見の集積が必要と言える．

利益相反

今回の論文に関して，開示すべき利益相反状態はありません．

著者役割

田島千裕：筆頭著者
浮田祐司：症例の臨床指導，症例の分析・考察，論文の執筆指導
松岡理恵：症例の臨床指導，症例の分析・考察，論文の構成・執筆に関与
上東真理子：論文の構成・執筆に関与
加藤徹：論文の構成・執筆に関与
原田佳世子：論文の構成・執筆に関与
柴原浩章：論文の構成・執筆に関与

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Advanced gastric cancer diagnosed during the second trimester and treated with chemotherapy with S-1 / L-OHP (SOX) : A case report

Chihiro Tajima, Yuji Ukita, Michie Matsuoka, Mariko Kamihigashi, Toru Kato, Kayoko Harada, Hiroaki Shibahara

Department of Obstetrics and Gynecology, The Hyogo College of Medicine

We report a case of 34-year-old pregnant woman who was diagnosed with poorly differentiated, advanced gastric adenocarcinoma at 23 weeks of gestation, she was initiated on a course of SOX (S-1 / L-OHP). At 32 weeks and 5 days of gestation, she delivery a girl (weight: 1572g; Apgar score of 8 points〔1 minute value〕and 9 points〔5 minute value〕) via selective caesarean section. She underwent chemotherapy at the postpartum outpatient clinic, but died 9 months after the caesarean section. At the time of discharge (54 days after birth), the baby did not show any growth and development abnormalities. The effects of chemotherapy during pregnancy on mothers and infants are still unknown. Previously, SP therapy was recommended as chemotherapy for advanced gastric cancer; however, in the recent years, SOX therapy has been recommended. Thus far, the guidelines pertaining to chemotherapy for gastric cancer during pregnancy have been unclear. Hence, cases need to be treated individually. This case provides insights into the impact of SOX on pregnant mothers and their infants.

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Phase II study of adjuvant chemotherapy with paclitaxel and nedaplatin for uterine cervical cancer with lymph node metastasis

Munetaka Takekuma[1] | Mototsugu Shimokawa[2] | Shin Nishio[3] | Hideo Omi[4] | Tsutomu Tabata[5] | Yuji Takei[6] | Kaei Nasu[7] | Yoshiyuki Takahashi[8] | Shinji Toyota[9] | Yoshikazu Ichikawa[10] | Atsushi Arakawa[11] | Fuminori Ito[12] | Hiroshi Tsubamoto[13] | Taisuke Mori[14] | Yasuyuki Hirashima[1] | Kimihiko Ito[15]

[1]Department of Gynecologic Oncology, Shizuoka Cancer Center, Shizuoka, Japan

[2]Cancer Biostatistics Laboratory, Clinical Research Institute, National Kyusyu Cancer Center, Fukuoka, Japan

[3]Department of Obstetrics and Gynecology, Kurume University School of Medicine, Fukuoka, Japan

[4]Department of Obstetrics and Gynecology, Iwate Medical University Hospital, Iwate, Japan

[5]Department of Obstetrics and Gynecology, Mie University Hospital, Mie, Japan

[6]Department of Obstetrics and Gynecology, Jichi Medical University, Tochigi, Japan

[7]Department of Obstetrics and Gynecology, Oita University Faculty of Medicine, Oita, Japan

[8]Department of Obstetrics and Gynecology, Okinawa Chubu Hospital, Okinawa, Japan

[9]Department of Obstetrics and Gynecology, Nara Prefecture General Medical Center, Nara, Japan

[10]Department of Obstetrics and Gynecology, Japanese Red Cross Shizuoka Hospital, Shizuoka, Japan

[11]Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

[12]Department of Obstetrics and Gynecology, Nara Medical University, Nara, Japan

[13]Department of Obstetrics and Gynecology, Hyogo College of Medicine, Hyogo, Japan

[14]Department of Obstetrics and Gynecology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan

[15]Department of Obstetrics and Gynecology, Kansai Rosai Hospital, Hyogo, Japan

Correspondence
Munetaka Takekuma, Department of Gynecology, Shizuoka Cancer Center, Nagaizumicho, Suntogun, Shizuoka, Japan.
Email: m.takekuma@scchr.jp

The purpose of this phase II trial was to assess the efficacy and toxicity of paclitaxel and nedaplatin (TN) as the initial postoperative adjuvant chemotherapy for uterine cervical cancer with lymph node metastases (LNM). Patients with FIGO stage IB1-IIA2 squamous cell carcinoma of the uterine cervix were enrolled. Histological confirmation of LNM was mandatory. Intravenous paclitaxel at 175 mg/m^2 and nedaplatin at 80 mg/m^2 were administered every 28-day cycle, of which there were 5 cycles after radical hysterectomy. Sixty-two patients were enrolled in the study from November 2011 to July 2015. Their median age was 48.5 years (range 28-64). The median tumor diameter was 37 mm (5-64). Overall, 30 patients (48.4%) had 1 metastatic lymph node, 11 (17.7%) had 2, 3 (4.8%) had 3, 5 (8.1%) had 4, and 13 (21.0%) had 5 or more. With a median follow-up of 45.7 months (range 23.4-69.5), the 2-year relapse-free survival and 2-year overall survival rates were 79.0% (90% CI, 69.0%-86.2%) and 93.5% (95% CI, 83.7%-97.5%), respectively. Almost all adverse

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events were relatively mild. Grade 3-4 adverse events (NCI-CTC ver. 4.0) that occurred in 5% or more of patients were neutropenia (60.7%) and infection (6.6%). The proportion of patients who completed 5 cycles of treatment was 90.3%. Postoperative adjuvant chemotherapy with TN for cervical cancer with LNM was demonstrated to be an effective and feasible treatment. A phase III trial is warranted to compare this with concurrent chemoradiotherapy.

KEYWORDS
cervical cancer, paclitaxel and nedaplatin, phase II study, postoperative adjuvant therapy, systemic chemotherapy

1 | INTRODUCTION

Cervical cancer is the most common gynecological malignancy worldwide, accounting for 7.9% (57 600) of new cancer cases and 7.5% (265 700) of all cancer deaths among females in 2012.[1] The incidence of cervical cancer is higher in developing countries because of the lower availability of cancer screening systems. In developed countries, the majority of cervical cancer patients are diagnosed at an early stage of the disease (FIGO stages I-II).

Patients with early-stage cervical cancer require radical hysterectomy with pelvic lymphadenectomy or definitive radiotherapy (RT)/concurrent chemoradiotherapy (CCRT). In Japan, at more than 80% of institutions, radical hysterectomy is chosen as the primary treatment for patients with stage IB1 and IIA1 tumors.[2] Subsequently, patients with prognostic risk factors for recurrence receive postoperative adjuvant therapy. Peters et al.[3] showed a significant survival advantage associated with the use of CCRT rather than RT alone in patients with high-risk cervical cancer. In the guidelines for cervical cancer of several countries, the pathological findings of lymph node metastasis (LNM) and/or parametrial invasion (PMI) are defined as high-risk prognostic factors, and postoperative CCRT is recommended as adjuvant treatment for these cases.[4-8]

There has recently been much debate regarding the risk-benefit balance of postoperative CCRT for cervical cancer.[9-12] Patients with LNM have been shown to exhibit a greater rate of distant failure than those without this,[9,10] so postoperative adjuvant therapy should not only control local recurrence, but also prevent distant metastasis. Postoperative CCRT would be expected to induce serious toxicities, which could continue throughout the patient's life; this is because the organ in the pelvis targeted by RT has already been subjected to radical surgery.[11,12] In this context, systemic chemotherapy (CT) alone could play an important role as postoperative adjuvant therapy for patients with high-risk cervical cancer.

The combination of paclitaxel plus platinum is standard treatment for patients with advanced/recurrent cervical cancer.[13,14] Nedaplatin (cis-diammine glycolato platinum) was developed as a less nephrotoxic and neurotoxic analog of cisplatin. We showed that the combination of paclitaxel plus nedaplatin (TN) would have favorable antitumor activity and be feasible for advanced/recurrent cervical cancer.[15] Recently, Li et al.[16] showed the efficacy of nanoparticle albumin-bound paclitaxel plus nedaplatin for patients with advanced/recurrent cervical cancer.

We conducted a phase II trial involving the application of postoperative systemic CT alone with the combination of paclitaxel plus nedaplatin to uterine cervical cancer patients with LNM, to evaluate the efficacy and toxicity of this regimen.

2 | MATERIALS AND METHODS

All patients provided written informed consent before enrollment. The trial was registered with the UMIN-Clinical Trials Registry (UMIN000005605) and was conducted in accordance with the Declaration of Helsinki. The trial protocol was approved by the Kansai Clinical Oncology Group (KCOG) Protocol Review Committee and the institutional review board of each participating institution before patient enrollment.

2.1 | Eligibility

Patients who had undergone radical hysterectomy and pelvic lymphadenectomy for FIGO stage IB1, IB2 or IIA of uterine cervix were enrolled in this trial. Histological type included squamous cell carcinoma alone. Histological confirmation of LNM was mandatory. In addition, patients had to have: no residual tumor after surgery; age ranging from 20 to 70; and ECOG performance status score 0-1. Patients were also required to have adequate hematological (absolute neutrophil count [ANC] ≥1500/μL, platelets ≥100 000/μL, hemoglobin ≥9.0 g/dL), renal (creatinine ≤1.5 mg/dL) and hepatic function (bilirubin ≤1.2 mg/dL, sGOT/GPT ≤100 U/L). Patients were excluded from the study if they had para-aortic LNM confirmed histologically, a positive surgical margin or peritoneal metastasis.

2.2 | Treatment

Treatment had to be started within 6 weeks after surgery. Chemotherapy administration was as follows: paclitaxel at 175 mg/m^2

over 3 hour plus nedaplatin at 80 mg/m^2 over 1 hour on day 1. Five cycles of chemotherapy were repeated every 28 days. Patients were premedicated with dexamethasone (20 mg) and ranitidine (50 mg) or famotidine (20 mg) intravenously 30-90 minute prior to infusion. Diphenhydramine (50 mg) was also given orally 30 minute prior to treatment. Chemotherapy was discontinued in cases of progressive disease, unacceptable toxicity or patient's refusal.

All patients were required to have an ANC of more than 1500/μL and a platelet count more than 75 000/μL prior to beginning each cycle. They were removed from the study if their blood count had not recovered by 3 weeks after treatment. Dose modifications were made to paclitaxel or nedaplatin for hematological, gastrointestinal, hepatic or neurologic toxicity, based on the most severe grade of toxicity, using the National Cancer Institute-Common Toxicity Criteria (NCI-CTC) version 4.0. Dose reduction levels of paclitaxel/nedaplatin were 150/70 mg/m^2 (level −1), 135/60 mg/m^2 (level −2) and 110/50 mg/m^2 (level −3). Patients requiring dose reduction to less than level −3 were removed from the study.

2.3 | Follow-up evaluation

Prior to each cycle of treatment, a physical examination, routine hematologic studies and blood chemistry analysis were conducted. Once protocol treatment had ended, the patients were evaluated by pelvic examination, Papanicolaou tests, and an analysis of serum squamous cell carcinoma antigen level at the discretion of the attending physician every 1-3 months in the first 2 years, and every 4-6 months during years 3, 4 and 5. In addition, patients underwent a CT or MRI scan every 6 months in the first 2 years and annually thereafter until 5 years.

2.4 | Statistical methods

The sample size was initially calculated based on the assumption of an expected 2-year relapse-free survival (RFS) rate of 80% and the threshold value of 65%, which was based on previously published data.[15-18] Under these assumptions, 58 patients were required to achieve a 1-sided significance level of 5% with power of 85%. Factoring in a 5% dropout rate, we set a target sample size of 63 patients. The primary endpoint of the current study was 2-year RFS, defined as the interval between the date of entry into the study and the date of the first physical or radiographic evidence of disease recurrence. The secondary endpoints were overall survival (OS), adverse events and rate of completion of protocol treatment. OS was calculated from the date of entry into the study to the date of death or last follow-up visit. RFS and OS were calculated using the Kaplan-Meier method, and their confidence intervals (CI) were estimated by Greenwood's formula. All statistical analyses were performed using SAS 9.4 (SAS Institute, Cary, NC, USA).

3 | RESULTS

3.1 | Patient characteristics

Sixty-two patients were enrolled from 14 institutions between November 2011 and July 2015. All patients were eligible for this study, so data from 62 patients were included in the analysis. The baseline characteristics of the patients are shown in Table 1. Their median age was 48.5 years (range 28-64). Fifty-five patients (88.7%) had a performance status (PS) of 0, while 6 patients (9.7%) had a PS of 1. Median tumor diameter was 37 mm (5-64). Overall, 10 patients (16.1%) had parametrial invasion, 44 (71.0%) had deep stromal invasion and 53 (85.5%) had lymphovascular invasion. The median number of resected lymph nodes was 41 (13-88). In total, 30 patients (48.4%) had 1 metastatic lymph node, 11 (17.7%) had 2, 3 (4.8%) had 3, 5 (8.1%) had 4, and 13 (21.0%) had 5 or more.

3.2 | Feasibility

Fifty-six patients (90.3%) completed the treatment protocol as planned. Of the 6 patients who did not complete it, 2 experienced disease progression, 1 prolonged neutropenia, 1 severe allergic reaction for nedaplatin, 1 vesical perforation that resulted from late morbidity of radical hysterectomy, and 1 refused the treatment. Three

TABLE 1 Patient characteristics (N = 62)

	N (%)
Age	Median 48.5 (Range 28-64)
Performance status	
0	55 (88.7)
1	6 (9.7)
Unknown	1 (1.6)
FIGO stage	
IB1	22 (35.5)
IB2	23 (37.1)
IIA	17 (27.4)
Tumor diameter, mm	Median 37 (Range 5-64)
Number of dissected lymph nodes	Median 41 (Range 13-88)
Number of metastatic lymph nodes	
1	30 (48.4)
2	11 (17.7)
3	3 (4.8)
4	5 (8.1)
5 or more	13 (21.0)
Parametrial invasion, yes	10 (16.1)
Deep stromal invasion, yes	44 (71.0)
Lymphovascular invasion, yes	53 (85.5)
Vaginal invasion, yes	20 (32.3)
Positive surgical margin, yes	0 (0)

patients (4.8%) needed dose reduction up to level −1. Among the total of 292 cycles administered, treatment delay occurred in 25 cycles (8.6%).

3.3 | Adverse events

The adverse events are summarized in Table 2. Thirty-seven patients (60.7%) had grade 3-4 neutropenia, while 1 (1.6%) had grade 3-4 anemia. Febrile neutropenia occurred in 1 patient alone (1.6%). There was no severe thrombocytopenia. With regard to non-hematological toxicities, the most common adverse event was alopecia (all grades, 95.0%). Sensory neuropathy (all grades, 81.7%), myalgia/arthralgia (all grades, 66.7%) and fatigue (all grades, 55.7%) were also common, but there were no grade 3-4 non-hematological adverse events in 5% or more of the patients.

Whether the number of dissected lymph nodes was associated with toxicity in all grades was evaluated. Neutropenia was more common in patients with ≥40 dissected lymph nodes than in those with <40 (87.5% vs 65.5%, $P = .041$); in contrast, thrombocytopenia was rarer in patients with 40 or more dissected lymph nodes than in those with <40 (0% vs 13.8%, $P = .030$). The other toxicities had no significant difference between the 2 groups.

TABLE 2 The reported acute toxicities

	N (%)	
Toxicities	**Any grade**	**G3/4**
Hematological toxicities		
Neutropenia	47 (77.0)	37 (60.7)
Anemia	26 (42.6)	1 (1.6)
Thrombocytopenia	4 (6.6)	0
Febrile neutropenia	1 (1.6)	1 (1.6)
Non-hematological toxicities		
Blood bilirubin increased	2 (3.3)	0
Liver enzyme increased	16 (26.2)	0
Creatinine increased	4 (6.6)	0
Hyperkalemia	4 (6.6)	0
Infection	8 (13.1)	4 (6.6)
Allergy	5 (8.2)	1 (1.6)
Vasculitis	7 (11.5)	0
Anorexia	32 (53.3)	1 (1.6)
Nausea	38 (63.3)	1 (1.6)
Vomiting	6 (10.0)	1 (1.6)
Diarrhea	9 (15.0)	0
Constipation	29 (47.5)	0
Pharyngitis/stomatitis	4 (6.7)	0
Alopecia	57 (95.0)	(grade 2≧) 43 (71.7)
Rash/exanthema	18 (30.0)	1 (1.6)
Edema	11 (18.3)	0
Fatigue	34 (55.7)	1 (1.6)
Sensory neuropathy	49 (81.7)	0
Motor neuropathy	3 (5.0)	0
Myalgia/arthralgia	40 (66.7)	1 (1.6)

3.4 | Survival analysis

The median follow-up duration was 45.7 months (range 23.4-69.5). Follow-up data from all patients were available. Data regarding recurrence and death are shown in Table 3. Among 15 patients with recurrence, 9 patients had locoregional recurrence, 5 had distant recurrence, and 1 had both. All 7 deaths were caused by deterioration of the disease.

Table 4 shows the actual clinical courses of the 15 patients with recurrence. Of 9 patients with locoregional recurrence, 8 were treated with RT or CCRT as salvage therapy and, among them, 3 patients are alive with no evaluable disease and 1 is alive with disease. Among 5 patients with distant recurrence, 3 patients with para-aortic recurrence were treated with RT or CCRT, and 1 patient with liver metastasis underwent surgery as salvage therapy. Among these 5 patients, 3 patients are alive without disease. A total of 53 patients (85.5%) are alive without disease, 2 (3.2%) are alive with disease and 7 (11.3%) have died of disease. The 2-year RFS, 2-year OS and estimated 4-year OS rates were 79.0% (90% CI, 69.0%-86.2%), 93.5% (83.7%-97.5%) and 87.1% (74.5%-93.7%), respectively (Figure 1A,B).

4 | DISCUSSION

We report the positive results of a phase II trial involving the application of postoperative systemic CT alone to uterine cervical cancer patients with lymph node metastases. Adjuvant CT alone with TN after radical hysterectomy was demonstrated to be an effective and feasible treatment.

In the current phase II study, the 2-year RFS, 2-year OS and estimated 4-year OS rates were 79.0%, 93.5% and 87.1%, respectively, which were comparable to those seen in previous reports of adjuvant CCRT for the patients with high-risk factors;[3,17,18] nevertheless, the patients in the current study would have more severe risk factors than those in most previous studies. All patients in the current study had LNM, among which approximately 30% had 2-3 metastases, approximately 20% had 5 or more metastases, and approximately 20% had common iliac lymph node metastases. Peters et al.[3] showed that the estimated 4-year PFS and OS rates for patients receiving CCRT were 80% and 81% in a phase III trial in which

TABLE 3 Recurrence and death (N = 15)

Site	N (%)
Recurrence	15 (24.2)
Locoregional	9 (14.5)
Distant	5 (8.1)
Both	1 (1.6)
Death	7 (11.3)

TABLE 4 Clinical course of patients with recurrence (N = 15)

Site of recurrence	Salvage therapy	Status
Locoregional recurrence		
Vaginal	RT[a]	Alive
Vaginal	CCRT[b]	Alive
Vaginal	CCRT	Alive
Vaginal	CCRT	AWD[c]
Vaginal	RT	Dead
Vaginal	RT	Dead
Intra-pelvic lymph nodes	CCRT	Dead
Intra-pelvic lymph nodes	CT[d]	Dead
Peritoneum in pelvis	RT	Dead
Distant recurrence		
Para-aortic lymph nodes	CCRT	Alive
Para-aortic lymph nodes	CCRT	Alive
Para-aortic lymph nodes	RT	Dead
Liver	Surgery	Alive
Lung and mediastinal lymph nodes	CT	Dead
Both		
Intra-pelvic, para-aortic, and mediastinal lymph nodes	CT	AWD

CCRT, concurrent chemoradiotherapy; RT, radiotherapy.
[a]Radiotherapy alone.
[b]Concurrent chemoradiotherapy.
[c]Alive with disease.
[d]Systemic chemotherapy alone.

approximately 90% of patients had nodal involvement and 3% had common iliac nodal involvement. Sehouli et al.[17] showed that the 2-year PFS was 81.8% and the estimated 5-year OS was 77.4% for patients receiving CCRT. In the study by Sehouli et al., only half of the patients had nodal involvement.

Adjuvant chemotherapy alone with TN after radical hysterectomy was demonstrated to be safe and feasible. In the current study, 90.3% of patients completed the treatment protocol as planned and grade 3-4 gastrointestinal toxicities occurred in only 1 patient. There has recently been much debate regarding the risk-benefit balance of postoperative CCRT for cervical cancer.[9-12] Postoperative CCRT would be expected to induce serious gastrointestinal toxicity, which could continue throughout the patient's life; this is because the organ in the pelvis that would be targeted by RT has already been subjected to radical surgery.[11,12] Takekuma et al.[11,12] report that the level of invasiveness of the surgical procedure might be associated with the toxicities of adjuvant CCRT, which meant that patients with ≥40 dissected lymph nodes had significantly more non-hematological toxicities of adjuvant CCRT than those with <40. They suggested that, for patients undergoing CCRT, the use of this radical surgery to increase the possibility of a permanent cure has to be balanced against the possibility of developing a serious illness in association with the postoperative therapy. In this context, systemic CT alone could play an important role as postoperative adjuvant therapy for patients with high-risk cervical cancer.[12] In the current study, the rates of severe non-hematological toxicity were only 0%-6.6% (Table 2). The evaluation of whether the number of dissected lymph nodes was associated with toxicity in all grades showed that there were significant differences regarding neutropenia and thrombocytopenia between patients with ≥40 dissected lymph nodes and those with <40, the reason for which was unclear. However, non-hematological toxicities did not differ significantly between the 2 groups. These findings suggest that postoperative TN therapy could be undergone safely regardless of the level of invasiveness of the surgical procedure.

In the current study, no severe neurotoxicity or thrombocytopenia occurred, which matched the results of a previous phase II study revealing the efficacy of TN therapy for advanced/recurrent cervical cancer.[15] Currently, paclitaxel combined with carboplatin (TC) is accepted as one of the standard treatment options for cervical cancer, in accordance with the results of the JCOG0505 trial, showing the noninferiority of TC to paclitaxel and cisplatin.[14] Severe neurotoxicity occurred in 4.8% and severe thrombocytopenia occurred in 24.6% of patients of the TC arm in the JCOG0505 trial, indicating

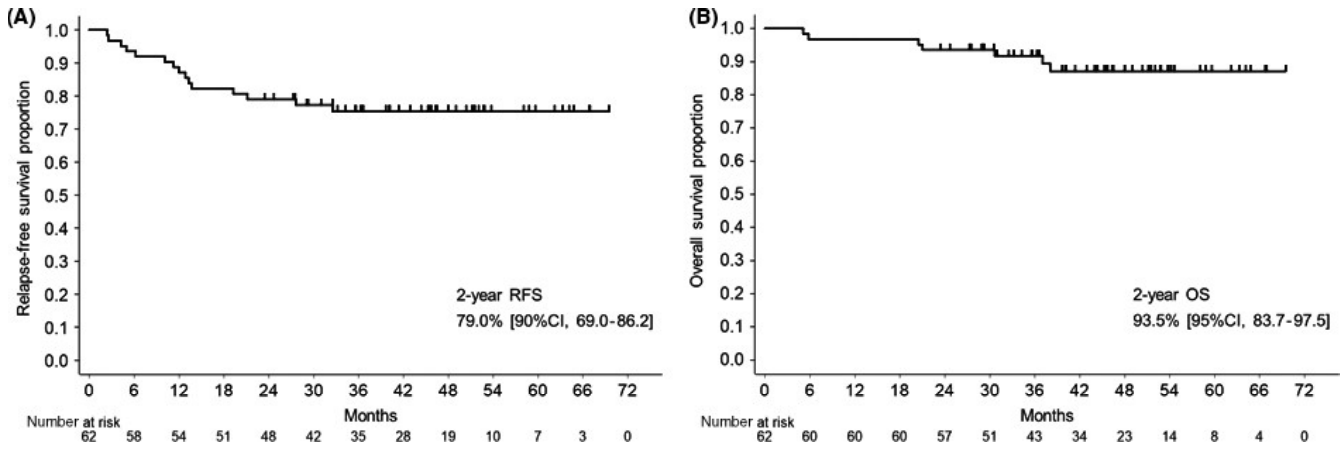


FIGURE 1 Outcomes of eligible patients enrolled in the KCOG-G1101 study. A, 2-year relapse-free survival: 79.0% (90% CI, 69.0%-86.2%). B, 2-year overall survival: 93.5% (83.7%-97.5%)

that these toxicities remain unresolved. A TN therapy could be an alternative to a regimen of postoperative adjuvant CT.

The most beneficial aspect of using systemic CT alone in a postoperative setting is that RT could be utilized for recurrences in the pelvis as salvage therapy if the pelvic field has not yet been irradiated. In the current study, 8 patients with recurrence in the pelvis and 3 patients with para-aortic recurrence were treated with RT or CCRT as salvage therapy; 6 of these are still alive and would be expected to survive for a long time. Another particularly beneficial aspect of systemic CT alone would be that systemic CT could control distant metastasis. The Gynecologic Oncology Group (GOG) in the USA has conducted a randomized phase III trial, the GOG0724 trial (NCT00980954), to test the hypothesis that the addition of further cycles of systemic CT following the completion of CCRT would decrease distant metastasis and improve survival. In contrast, there might be a discouraging aspect of using systemic CT alone associated with local control. In the current study, more intra-pelvic recurrence occurred (16.1%) than in the CCRT arm of Peters' trial (8.7%).[3]

It has been proposed that systemic CT alone could have a survival benefit even without RT. Several retrospective studies on postoperative adjuvant CT alone have also been reported and all of them conclude that systemic CT alone as postoperative adjuvant therapy could obtain similar or better results compared with RT/CCRT.[18-21] Matsuo et al.[22] showed in a multi-institutional retrospective study that systemic chemotherapy might be as effective a postoperative treatment as radiation-based therapy in node-positive stage IB-IIB cervical cancer. In a phase III trial conducted by Curtin et al., patients were randomized to systemic CT alone and systemic CT followed by whole pelvic RT after radical hysterectomy. Although this trial could not achieve a positive result regarding the primary endpoint, they conclude that the patterns of recurrence were statistically similar between the 2 arms, and both regimens were well tolerated.[23]

The current study has a few limitations. First, the follow-up period was short. The expected survival duration of the patients in the current study would be relatively long because this is a study on early-stage disease. A secondary endpoint in the current study is the 5-year OS, for which the results will be reported at a later date. Second, the late adverse events could not be reported. The standard postoperative adjuvant therapy is RT or CCRT in which severe late adverse events have occurred, and so compared with this standard treatment, data of the late adverse event of CT alone would be important. Third, in the current study, no restrictions were placed on the surgical procedure performed; namely, radical hysterectomy or lymphadenectomy. The results of postoperative adjuvant therapy could be affected by the outcome of the surgery performed before the study.

In summary, adjuvant CT alone with TN after radical hysterectomy for patients with high-risk early-stage cervical cancer was demonstrated to be an effective and feasible treatment. We suggest that a prospective randomized study be conducted with the aim of testing the noninferiority of systemic CT alone to CCRT as optimal adjuvant therapy for patients with factors placing them at high risk for recurrence.

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ORCID

Munetaka Takekuma http://orcid.org/0000-0002-0807-1845
Shin Nishio http://orcid.org/0000-0003-2526-630X

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Effects of leuprorelin for the treatment of recurrent gynecological cancer by assessment including self-administered quality-of-life questionnaire

Hiroshi Tsubamoto, Tomoko Ueda, Kayo Inoue, Roze Isono-Nakata, Shinichiro Saeki, Yu Kato and Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan

Abstract

Aim: To investigate the effects of leuprorelin using a self-administered quality-of-life (QOL) questionnaire in patients with recurrent gynecological cancer.

Methods: Records of patients who received 3.75 mg leuprorelin every 4 weeks for the treatment of recurrent gynecological cancer were retrospectively reviewed. The physical domain of the QOL questionnaire, Care Notebook, was used to assess physical symptoms. Symptom deterioration was defined as a ≥10-point increase in baseline score; otherwise, symptoms were defined as controlled. Radiological and serological responses were evaluated according to the 2011 Gynecological Cancer Intergroup criteria.

Results: From 2007 to 2015, 25 patients were administered leuprorelin for the treatment of epithelial ovarian cancer, granulosa cell tumor, endometrial cancer, endometrial stromal sarcoma and clear cell cervical cancer (in 13, 3, 6, 2 and 1 patients, respectively). Twenty patients had received a median of three lines (range 1–12 lines) of chemotherapy. Ten patients had progressive disease during their previous round of chemotherapy. Twenty patients completed the questionnaire every 4 weeks. Following leuprorelin treatment for 8 weeks, the symptom and disease control rates were 65% (13/20) and 44% (11/25), respectively. Two patients, one each with granulosa cell tumor and endometrial cancer, had stable disease at 6 months. Among the 20 patients who completed the QOL questionnaire, symptom control and disease control at 8 weeks showed a significant correlation (P = 0.016).

Conclusion: Leuprorelin had minimal anticancer activity. The physical domain of the QOL questionnaire could be used to assess effects of hormonal treatment.

Key words: gynecological cancer, hormonal treatment, quality of life.

Introduction

Patients with late-stage cancer are administered chemotherapy to improve cancer-related symptoms and to maintain their quality of life (QOL). However, continuing chemotherapy may reduce QOL due to adverse events. These patients therefore require less toxic treatment.

Overexpression of receptors for luteinizing hormone–releasing hormone has been reported in ovarian and uterine neoplasms, with preclinical studies reporting that gonadotropin-releasing hormone (GnRH) analogs inhibited proliferation of these tumors.[1] GnRH agonists have been used traditionally to treat patients with relapsed epithelial ovarian cancer (EOC), granulosa cell tumor (GCT), endometrial

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Correspondence: Dr Hiroshi Tsubamoto, Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan. Email: tsuba@hyo-med.ac.jp

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cancer (EC) and low-grade endometrial stromal sarcoma (ESS).[2,3] A randomized clinical trial of patients with EOC who experienced disease progression during or relapsed shortly after platinum-paclitaxel treatment showed similar survival during treatment with leuprorelin or treosulfan.[4] Of 15 patients with GCT, 6 (40%) patients demonstrated partial response (PR) after treatment with GnRH agonists.[5]

Symptom relief is important during palliative cancer treatment. However, to our knowledge, no previous reports have evaluated QOL during treatment of recurrent gynecological cancer with GnRH analogs. We administered the self-administered QOL questionnaire, Care Notebook, in our daily oncologic practice starting in 2007. This retrospective study evaluated the effects of leuprorelin by radiological, serological and the QOL questionnaire assessment in patients with recurrent gynecological cancer.

Methods

This retrospective study included patients who received leuprorelin for recurrent gynecological cancer during the 2007–2015 period. Patients were subcutaneously administered 3.75 mg leuprorelin every 4 weeks until they experienced disease progression, unacceptable toxicity or until the patient discontinued for any other reasons. The physical symptoms were evaluated by measuring the physical domain score on the self-administered Care Notebook QOL questionnaire at baseline and every 4 weeks after treatment with leuprorelin (http://www.care-notebook.com/en/index.html).[6] Care Notebook is composed of 10 items that assess physical well-being (Table 1), six items that assess mental well-being and eight items that assess life well-being. Each item was scored from 0 (no complaints) to 10 points (worst complaint). Care Notebook included open-ended questions, such as 'Please tell us how we might help you improve your health and life', which could be helpful for the health-care team members responsible for the care of the patient. The physical domain score was calculated as the sum of points from 10 items, which ranged from 0 (no symptoms) to 100 points (worst symptoms). A difference of 10 points was considered clinically significant.[7] Symptom improvement was defined as a ≥10-point reduction in baseline score, whereas symptom deterioration was defined as a ≥10-point increase in baseline score or inability to administer the QOL questionnaire due to disease progression. Symptom stabilization was defined as neither symptom improvement nor deterioration. Physical symptom control was defined as the sum of symptom improvement and stabilization. Response was evaluated according to the 2011 Gynecological Cancer Intergroup (GCIG) criteria, which incorporated response evaluation criteria in solid tumors (RECIST), version 1.1, and serum cancer antigen 125 concentration.[8] Disease progression in patients with GCT was defined as a twofold or more increase in estradiol concentration compared with baseline. The disease control was defined as the sum of complete response (CR), PR and stable disease (SD). The physical symptom control rate (p-SCR) and disease control rate (DCR) were the percentages of patients who achieved physical symptom control and disease control at 8 weeks, respectively. The ratio of progression free survival (PFS) with leuprorelin to that with previous chemotherapy was calculated for patients with PFS longer than 8 weeks after leuprorelin administration. Overall survival (OS) was defined as the time from the first leuprorelin administration to death. All statistical analyses were performed using XLSTAT 2014 (Addinsoft). Collection of patient data was approved by the institutional review board at Hyogo College of Medicine.

Table 1 The questionnaire of physical well-being (English version)

Did you have any of these problems? Please circle the number that best applies to you, using 10 for the worst condition.														
Q1	Pain?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q2	Shortness of breath?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q3	Nausea?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q4	Loss of appetite?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q5	Trouble sleeping?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q6	Constipation	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q7	Diarrhea	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q8	Abdominal bloating?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q9	Fatigue	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all
Q10	Physical weakness?	Severe	10	9	8	7	6	5	4	3	2	1	0	None at all

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Results

During the 2007–2015 period, 25 patients were enrolled (Tables 2,3). Their median age was 62 years (range 26–82 years). Five patients had no previous history of chemotherapy, whereas the other 20 had received a median of three lines of previous chemotherapy (range 1–12 lines). Ten patients had progressive disease during previous chemotherapy. Of the 20 patients administered the QOL questionnaire, four (20%) showed improvement and nine (45%) showed stabilization of physical symptom at 8 weeks, making the p-SCR 65% (Table 4). According to 2011 GCIG criteria, no patient achieved CR or PR, but 11 (44%) of the 25 patients had SD at 8 weeks, making the DCR 44%. Among the 20 patients administered the QOL questionnaire, the symptom control assessed by the physical domain of QOL questionnaire and the radiologically and serologically evaluated disease control at 8 weeks was significantly associated ($P = 0.016$).

A representative case of a correlation among radiological, serological and the QOL questionnaire assessment is shown in Figure 1. Patient 14 had recurrent GCT during anastrozole treatment. Soon after secondary debulking surgery, she had pelvic, liver and lymph node metastases, and was treated with leuprorelin. Patient 13 was diagnosed with recurrent low-grade serous (LGS) ovarian carcinoma after fertility preservation treatment. She had SD for 5 months of leuprorelin treatment. Then, she agreed to undergo debulking surgery. Two other patients, one with GCT (patient 16) and one with EC (patient 21), showed SD at 6 months, making the 6 month PFS rate 8% (2/24 evaluable patients). Of the eight patients with high-grade serous ovarian cancer, none had PFS of over 3 months. The anticancer activity of leuprorelin and prior chemotherapy was compared in 19 patients who received prior chemotherapy. Of the six patients with PFS on leuprorelin for longer than 2 months, four (patients 7, 11, 20 and 21) had an equal or longer PFS on leuprorelin than on the previous chemotherapy. Thus, leuprorelin showed favorable PFS in 21% (4/19) of patients treated with more than three lines of chemotherapy. The OS of patients with ovarian cancer was 13 months (95% confidence interval [CI] 7.7–18 months) and the OS of patients with EC was 9 months (95% CI 3–15 months).

Table 2 Summary of patient characteristics

No. of total patients	25
Age median, range	62 (26—82)
Types of cancer	
Ovarian	16 (64%)
Uterine	9 (36%)
Prior treatment	
Chemo	19 (76%)
Hormonal treatment	5 (20%)
Naïve	4 (16%)
Response during prior chemo	
Progression	10 (53%)
Stable disease	9 (47%)
CR or PR	0 (0%)
Recommended to discontinue chemo	9 (36%)
Chemo holiday	11 (44%)

Chemo, chemotherapy; CR, complete response; PR, partial response.

Discussion

Assessing the efficacy of leuprorelin using a QOL questionnaire showed symptom control in 65% of patients with recurrent gynecological cancer. Because most patients with gynecological cancer have undergone primary surgery, including bilateral salpingo-oophorectomy, the toxicities of leuprorelin were minimal. However, one of the two patients who had undergone fertility-preserving surgery showed mild climacteric symptoms. The antitumor activity of leuprorelin was evaluated by measuring PFS at 6 months and by comparison with prior chemotherapy. Two patients, one each with GCT and EC, showed PFS of 22 and 6 months, respectively. Patient 13, with LGS ovarian cancer, had SD for 5 months and then underwent debulking surgery, suggesting that PFS in this patient may have been longer than 6 months. Two patients each with EOC and EC experienced equal or longer PFS on leuprorelin than on previous chemotherapy. These four patients had each received more than three lines of previous chemotherapy, suggesting that previous chemotherapy had low antitumor activity.

According to the recent National Comprehensive Cancer Network clinical practice guidelines for the treatment of ovarian neoplasms,[9] leuprorelin is considered acceptable for the treatment of EOC, especially for LGS and grade 1 endometrioid carcinomas as well as GCT. A single institutional study found that maintenance hormonal treatment resulted in longer median PFS than observation alone in patients with stage II–IV LGS carcinoma who had undergone primary surgery and chemotherapy (hazard ratio 0.44; $P < 0.001$).[10] Of the 70 patients in that study who received hormonal treatment, 39 (56%) were

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Table 3 Characteristics and responses of individual patients

Case	Types of cancer	Age	Histology	Lines of prior chemo	Response of prior chemo	PFS of prior chemo (months)	Previous hormonal Tx	After treatment of leuprorelin				
								p-QOL score at 2 months	2011 GCIG criteria at 2 months	PFS (months)	OS (months)	Status
1	EOC	62	HGS	1	SD	8	—	Stabilized	PD	1	23	DOD
2		56	HGS	2	PD	8	—	Stabilized	PD	2	12	DOD
3		63	HGS	2	PD	5	—	Deteriorated	PD	2	13	DOD
4		48	HGS	3	PD	3	—	Deteriorated	PD	1	10	DOD
5		65	HGS	4	PD	2	Letrozole	—	PD	2	5	DOD
6		71	HGS	4	PD	4	—	Improved	SD	3	5	DOD
7		54	HGS	6	SD	3	—	Stabilized	SD	3	30	DOD
8		60	HGS	8	PD	2	—	Improved	PD	2	9	DOD
9		29	Endometrioid	2	SD	5	—	Improved	PD	2	6	DOD
10		55	Endometrioid	5	SD	6	—	Deteriorated	PD	<1	<1	DOD
11		57	Endometrioid	12	SD	3	—	—	SD	5	8	DOD
12		51	Mixed	5	SD	9	—	Deteriorated	PD	2	15	DOD
13		26	LGS	3	SD	43	—	—	SD	5†	101	AWD
14	GCT	81	Adult type	0	—	—	—	Stabilized	SD	4	6	DOD
15		64	Adult type	0	—	—	—	Improved	SD	4	27	DOD
16		31	Adult type	0	—	—	—	—	SD	22	74	AWD
17	EC	76	G2	0	—	—	MPA	Stabilized	PD	2	3	DOD
18		57	G2	2	PD	7	MPA	Stabilized	PD	1	9	DOD
19		70	G2	3	PD	3	—	Deteriorated	PD	2	3	DOD
20		69	G2	4	SD	4	—	Stabilized	SD	4	22	DOD
21		70	G2	5	SD	5	—	Stabilized	SD	6	12	DOD
22		62	Serous	3	PD	2	—	Deteriorated	PD	2	4	DOD
23	ESS	62	LG	1	—	9	MPA	—	SD	5	60	DOD
24		35	LG	3	PD	4	MPA	Deteriorated	PD	<1	<1	DOD
25	CX	82	Clear cell	0	—	—	—	Stabilized	SD	4	28	DOD

†Patient 13 underwent debulking surgery after 5 months of treatment with leuprorelin. Patients 13 and 16 did not undergo bilateral oophorectomy before treatment with leuprorelin. Patient 13 had mild climacteric syndrome. 2011 GCIG criteria, the 2011 criteria of the Gynecological Cancer Intergroup; Chemo, chemotherapy; CX, cervical cancer; EC, endometrial cancer; EOC, epithelial ovarian cancer; ESS, endometrial stromal sarcoma; GCT, granulosa cell tumor; HGS, high-grade serous; LG, low grade; LGS, low-grade serous; MPA, medroxyprogesterone acetate; p-QOL, physical domain of quality of life; PD, progressive disease; PFS, progression free survival; SD, stable disease; Tx, treatment.

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Table 4 Efficacy of leuprorelin in each type of gynecologic cancer

Types of cancer	Number	p-SCR at 2 months	DCR at 2 months	PFS at 6 months
EOC	13	60% (6/10)	31% (4/13)	0% (0/12)
GCT	3	100% (2/2)	100% (3/3)	33% (1/3)
EC	6	67% (4/6)	33% (2/6)	17% (1/6)
ESS	2	0% (0/1)	50% (1/2)	0% (0/2)
CX	1	100% (1/1)	100% (1/1)	0% (0/1)
Total	25	65% (13/20)	44% (11/25)	8% (2/24)

CX, cervical cancer; DCR, disease control rate; EC, endometrial cancer; EOC, epithelial ovarian cancer; ESS, endometrial stromal sarcoma; GCT, granulosa cell tumor; p-QOL, physical domain of quality of life; p-SCR, physical symptom control rate; PFS, progression free survival.

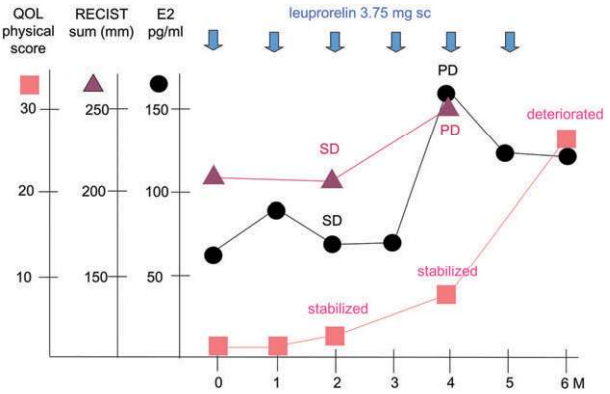


Figure 1 Representative case of correlation among radiological, serological and quality-of-life (QOL) questionnaire assessment (Case 14). QOL physical score was calculated using physical domain of self-administered QOL questionnaire Care Notebook. A 10-point increase in QOL physical score was defined as 'deteriorated'. In patients with granulosa cell tumor (GCT), disease progression was defined as a twofold or more increase in estradiol concentration compared with baseline. E2, estradiol concentration; PD, progressive disease; RECIST, response evaluation criteria in solid tumors; SD, stable disease.

treated with letrozole and seven (10%) with leuprorelin. In another retrospective study for the treatment of relapsed high-grade ovarian cancer, the 3-month PFS rate was 56% among 54 patients treated with letrozole and 65% among 43 patients treated with tamoxifen.[11] A literature review of individual cases and small case series reported that 40% of patients with GCT responded to GnRH analogs and that the response rate to hormone therapy, including GnRH analogs, aromatase inhibitors and progestins, was 71%.[5] Another study, however, involving patients enrolled in the nationwide network and registry of histopathology in the Netherlands, reported a response rate to hormonal therapy of 18%.[12] The disease in patient 16 showed progression after leuprorelin treatment for 22 months, followed by treatment with letrozole for 45 months.

National Comprehensive Cancer Network clinical practice guidelines for patients with uterine neoplasm regard GnRH analogs as acceptable for the treatment of recurrent ESS (category 2B).[13] To the best of our knowledge, however, no patients with recurrent ESS who had undergone bilateral salpingo-oophorectomy experienced CR or PR following treatment with GnRH analog alone. In contrast, aromatase inhibitors have induced CR or PR in several patients.[14] Currently, we do not propose leuprorelin but propose letrozole for the treatment of recurrent ESS.

Leuprorelin was first administered to our patient with platinum-resistant ovarian cancer (PROC) in 2007, and a prospective clinical trial (UMIN000001 561) for the treatment of recurrent gynecological cancer started in 2009. We administered leuprorelin instead of letrozole, because only a small number of cases treated with letrozole were reported before 2009. A randomized phase 3 study using leuprorelin for the treatment of PROC was reported in 2002,[4] while aromatase inhibitors including letrozole for the treatment of ovarian cancer were reported in phase 2 trials.[11] In GCT, response to GnRH agonists has been reported since 1989,[15] while response to letrozole was first reported in 2009.[16] Leuprorelin showed a response rate of 28% in endometrial cancer in 1996.[17] In 1998, leuprorelin was reported to be effective for treatment of patients with low-grade ESS,[18] while five case reports using letrozole were published before 2009.[19]

In this study, symptom control was evaluated by measuring the physical domain of QOL questionnaire, Care Notebook. There is no consensus regarding the appropriate QOL questionnaire for measuring patient-reported outcomes in patients with gynecological cancer. A translated version of the QOL questionnaire following the standard forward- and back-translation procedure may sound somewhat

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unnatural, and contains some questions about unfamiliar customs. Care Notebook was developed in Japan, which provided an advantage for use in our daily practice. Each question is simple and based on a 0–10-point linear analog scale that can be easily completed by patients. Our oncologic outpatients filled out the questionnaire during the waiting time in the hospital, which helped physicians to better understand the symptoms and healthcare needs of patients and to improve communication. Two randomized control trials found that the use of a QOL questionnaire in daily oncologic practice improved patients' QOL.[20,21]

QOL assessment of hormonal treatment of gynecological cancer was previously reported in two clinical trials. In a phase 3 randomized study using the European Organization for Research and Treatment of Cancer (EORTC) QOL questionnaire (QLQ-C30 and QLQ-OV-28), chemotherapy deteriorated social functioning of patients with PROC at 8 weeks compared with tamoxifen, while other domains including physical symptoms showed no differences.[22] In a phase 2 study on anastrozole, baseline QOL was measured in 45 patients with PROC by using EORTC QLQ-C30.[23] The symptom control and disease control were significantly associated, results that are consistent with those of this study. Scoring the physical domain of the QOL questionnaire could be useful to evaluate effects of hormonal treatment.

This study had several limitations, including its small sample size and the use of a single QOL questionnaire. To the best of our knowledge, however, this study is the first report to assess QOL in patients with recurrent gynecological cancer who were treated with GnRH analogs. Because of its minimal toxicity and low cost, leuprorelin might be a treatment option for patients with recurrent gynecological cancer, and QOL questionnaire could be useful to evaluate the effects of hormonal treatment.

Disclosure

None declared.

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Multi-institutional phase II study of neoadjuvant irinotecan and nedaplatin followed by radical hysterectomy and the adjuvant chemotherapy for locally advanced, bulky uterine cervical cancer: A Kansai Clinical Oncology Group study (KCOG-G1201)

Taisuke Mori[1], Hiroshi Makino[2], Tomoharu Okubo[3], Yoichiro Fujiwara[4], Morio Sawada[1], Haruo Kuroboshi[1], Hiroshi Tsubamoto[5], Homare Murakoshi[6], Takashi Motohashi[7], Jo Kitawaki[1] and Kimihiko Ito[8]

[1]*Department of Obstetrics and Gynecology, Kyoto Prefectural University of Medicine, Graduate School of Medical Science,* [3]*Department of Obstetrics and Gynecology, Kyoto First Red Cross Hospital,* [4]*Department of Obstetrics and Gynecology, Kyoto City Hospital, Kyoto,* [2]*Department of Obstetrics and Gynecology, Gifu University, Gifu,* [5]*Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya,* [6]*Department of Obstetrics and Gynecology, Chibune General Hospital, Osaka,* [7]*Department of Obstetrics and Gynecology, Kuwana City Medical Center, Kuwana and* [8]*Department of Obstetrics and Gynecology, Kansai Rosai Hospital, Amagasaki, Japan*

Abstract

Aim: A multi-institutional phase II trial was conducted to determine the efficacy and toxicity of neoadjuvant chemotherapy with irinotecan and nedaplatin followed by radical hysterectomy and adjuvant chemotherapy for locally advanced, bulky stage IB2-IIB cervical cancer.

Methods: Patients with International Federation of Gynecology and Obstetrics (FIGO) stage IB2-II, bulky type (>4 cm in diameter) squamous cell carcinoma of the uterine cervix were enrolled. Irinotecan (60 mg/m^2) was administered intravenously on days 1 and 8 and nedaplatin (80 mg/m^2) was also administered on day 1 of every 21-day cycle. After two cycles of chemotherapy, a radical hysterectomy was performed. Until 6 weeks after the surgery, three to five cycles of the regimen were added as adjuvant chemotherapy. The primary endpoint was the 2-year relapse-free survival rate. The response rates and toxicities were evaluated as secondary endpoints.

Results: Thirty-two patients from seven institutions were enrolled in this study. The median age was 48 years (range 25–75 years). The average follow-up period was 37.8 months (15–71 months). Twenty-three patients completed the regimen as planned. The objective response rate (complete response + partial response) for the neoadjuvant chemotherapy regimen was 81.2%. The 2-year and 5-year relapse-free-survival rates were 87.5% and 78.8%, respectively. The incidence of grade 3/4 neutropenia was 6.3% and 34.4% during neoadjuvant and adjuvant treatment, respectively. All other toxicities were well tolerated.

Conclusion: Our treatment showed efficacy and tolerability for patients with locally advanced, bulky stage IB2-IIB cervical cancer. This suggests that treatment has the potential to improve the prognosis compared to concurrent chemo-radiotherapy.

Key words: adjuvant chemotherapy, cervical cancer, irinotecan, nedaplatin, neoadjuvant chemotherapy.

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Correspondence: Dr Taisuke Mori, Department of Obstetrics and Gynecology, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan. Email: moriman@koto.kpu-m.ac.jp

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Introduction

Uterine cervical cancer is one of the most common gynecological malignancies and leading cause of cancer-related deaths in women all over the world.[1] Although the standard treatment of cervical cancer has been a radical hysterectomy or cisplatin-based concurrent chemo-radiotherapy (CCRT), there is still controversy regarding optimal treatment approaches for locally advanced, bulky cervical cancer. Many studies have demonstrated that tumor volume could be one of pivotal unfavorable prognostic factors for patients with locally advanced cervical cancer regardless of therapeutic strategies, including surgery and radiation therapy (RT).[2–4] For example, Landoni *et al*. reported that the 5-year survival rate in patients with stage IB2-IIA cervical cancer with a tumor diameter greater than 4 cm after surgery or radiation was 70–72%, which was worse than those with a tumor diameterless than 4 cm (87–90%).[5] Thus, novel strategies for treating bulky cervical cancer tumors are needed.

There has also been much debate regarding the risk–benefit balance of surgical interventions compared with CCRT. The merit of CCRT or RT is that it is less invasive and can be done relatively safety for elderly patients and patients with severe complications. Patients can avoid urinary and rectal dysfunctions, which can occur with radical surgery. On the other hand, the advantages of surgery are as follows: First, a pathological diagnosis using specimens from surgery allows for a precise evaluation regarding the risk for recurrence, which can help optimize individualized treatment. Second, ovarian transposition to preserve ovarian function and fertility could be beneficial for patients; however, pelvic RT or CCRT would invariably lead to ovarian failure in premenopausal women.[6,7] Recently, the incidence of younger patients with cervical cancer who desire fertility preservation has increased and novel surgical methods, including trachelectomy, have been developed.[8] Therefore, the National Comprehensive Cancer Network (NCCN) guidelines recently added a new fertility-sparing algorithm for treatment of locally advanced cervical cancer.[9] Thus, the surgical intervention as well as RT should be reevaluated to fully understand the individualized treatment corresponding to each patient.

Neoadjuvant chemotherapy (NAC) followed by surgery is one option for locally advanced cervical cancer. First, studies show that NAC can reduce tumor size, control distant micro-metastasis and help patients undergo surgery with more ease.[10–12] Second, NAC can reserve the functions of ovaries and the vagina. A recent meta-analysis showed that the early response to NAC could predict survival in cervical cancer patients[13]; however, an ideal regimen for NAC has not still been established.

Tumor volume is known as one of the postoperative recurrence risk factors. Therefore, it is common for patients with bulky tumors to be considered for adjuvant therapy after surgery. Postoperative irradiation sometimes induces serious lifelong debilitating toxicities, including intestinal obstruction and pelvic lymphedema. In addition, the capability of postoperative irradiation to control distant recurrence might be poorer. Therefore, systematic adjuvant chemotherapy could be alternative to RT to prevent from local and distant recurrence.

The combination of NAC regimen with irinotecan and nedaplatin has been conducted in a phase I Japanese Gynecologic Oncology Group (JGOG) 1063.[14] This treatment was effective and well tolerated for Japanese patients with stage IB2-IIB cervical cancer. In this current study, we conducted a phase II trial involving NAC with irinotecan and nedaplatin, followed by radical hysterectomy and systematic adjuvant chemotherapy for locally advanced, bulky stage IB2-IIB cervical cancer to see if this treatment option helps improve the survival of patients.

Methods

Patient selection

Patients were treated in accordance with the protocol G-1201 of the Kansai Clinical Oncology Group (KCOG). This protocol was approved from the KCOG protocol review committee and institutional review board in each hospital. Written informed consent was obtained from all patients before the enrollment.

Patients who were pathologically diagnosed with FIGO stages IB2, IIA2 and IIB cervical cancer were enrolled. Eligibility criteria were as follows: (i) histologically confirmed invasive squamous cell carcinoma of the uterine cervix; (ii) FIGO stage system (1994 version): stage IB2 and IIA, measurable lesions greater than 4 cm in greatest diameter on direct measurement or magnetic resonance imaging (MRI), or stage IIB measurable lesions greater than 4 cm in greatest diameter on direct measurement or MRI; (iii) primary tumors that can be directly measured or measured on MRI; (iv) no previous treatment; (v) an age of 20–75 years at enrollment; (vi) Eastern Cooperative

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Oncology Group performance status (PS) of 0 or 1; (vii) extended hysterectomy is feasible; (viii) preserved function of major organs (bone marrow, heart, liver and kidney) and lab values within specified ranges, including a neutrophil count greater than 2000/μL, a platelet count greater than 100 000/mm^3, a hemoglobin level greater than 9.0 g/dL (values after blood transfusion are accepted), levels of aspartate aminotransferase and alanine aminotransferase less than 100 IU/L, a total bilirubin level less than 1.5 mg/dL, a serum creatinine level less than 1.5 mg/dL, creatinine clearance greater than 60 mL/min, normal electrocardiogram or electrocardiographic changes; and (ix) written informed consent for voluntary participation in this study. The exclusion criteria were as follows: (i) distinct evidence of infectious disease; (ii) serious concurrent disease (cardiac disease, uncontrolled diabetes mellitus, malignant hypertension and a bleeding tendency); (iii) active double cancer; (iv) interstitial pneumonia or pulmonary fibrosis; (v) body fluid retention requiring treatment; (vi) unstable angina, a history of myocardial infarction within 6 months before enrollment or serious arrhythmias requiring treatment; (vii) contraindications for irinotecan or nedaplatin; (viii) diarrhea; (ix) intestinal paralysis or ileus; (x) pregnant women, or women who want to become pregnant; (xi) a history of serious drug hypersensitivity or drug allergy; (xii) Hepatitis B antigen positive; and (xiii) patients considered unsuitable by their attending physicians for study-related reasons.

Treatment regimen

A combined regimen consisting of irinotecan (60 mg/m^2) and nedaplatin (80 mg/m^2) was used. Irinotecan was administered as intravenous infusion on days 1 and 8 and nedaplatin was also administered on day 1 of every 21-day cycle. After two cycles of chemotherapy, an abdominal radical hysterectomy was then performed. Until 6 weeks after the surgery, three to five cycles of the same regimen were added as adjuvant chemotherapy. Adjuvant treatment was also allowed to select radiotherapy including CCRT by pathological risk factors. Chemotherapy was discontinued if progressive, unacceptable toxicities occurred and if the patient refused treatment.

If the neutrophil count was less than 1500/μL and/or the platelet count was less than 50 000/μL on day 1 of any cycle, treatment was delayed/skipped for 1 week. The dose reduction levels of irinotecan/nedaplatin were 50/70 mg/m^2 (level 1) and 40/60 mg/m^2 (level 2). Patients requiring dose reduction to less than level 2 were removed from the study. If the blood count had not recovered by 3 weeks after treatment, the patient was excluded from the study.

Patient characteristics

Thirty-two patients from seven institutions were enrolled in this study between May 2012 and January 2017. Patient characteristics are shown in Table 1. The median age was 48 years (range 25–75 years). Twenty-nine patients (90.6%) had a PS of 0, while three (9.4%) patients had a PS of 1. Nine patients had FIGO stage IB2 (28.1%), four had IIA2 (12.5%) and 19 had IIB (59.4%). The median tumor diameter was 5.4 cm (4.0–7.4 cm). Thirteen patients had keratinizing type squamous cell carcinoma (40.6%) and 19 had nonkeratinizing type (59.4%).

Table 1 Patients characteristics (n = 32)

Characteristics	n (%)
Age (years), median (range)	48 (25–75)
Performance status, n (%)	
0	29 (90.6%)
1	3 (9.4%)
FIGO stage, n (%)	
IB2	9 (28.1%)
IIA2	4 (12.5%)
IIB	19 (59.4%)
Tumor diameter (mm), median (range)	54 (40–70)
Histological type (SCC), n (%)	
Keratinizing type	13 (40.6%)
Nonkeratinizing type	19 (59.4%)
Surgery, n (%)	
Radical hysterectomy	31 (96.9%)
Not conducted	1 (3.1%)
Lymph node metastasis (n = 31), n (%)	
Positive	15 (48.4%)
Negative	16 (51.6%)
Positive lymph invasion (n = 31), n (%)	
Positive	15 (48.4%)
Negative	16 (51.6%)
Vascular space invasion (n = 31), n (%)	
Positive	15 (48.4%)
Negative	16 (51.6%)
Response to NAC, n (%)	
Pathological CR	2 (6.2%)
CR	7 (21.9%)
PR	17 (53.1%)
SD	5 (15.7%)
PD	1 (3.1%)

CR, complete response; NAC, neoadjuvant chemotherapy; PD, progressive disease; PR, partial response; SCC, squamous cell carcinoma; SD, stable disease.

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Evaluation and follow-up

Responses were evaluated according to the Response Evaluation Criteria in Solid Tumors guidelines (RECIST version. 1.1). Briefly, we used MRI for all patients enrolled before and after treatment, and measurement of response was based on the longest perpendicular diameters of the lesions following treatment. Complete response (CR) was defined as the complete disappearance of all measurable disease. Partial response (PR) was defined as more than 30% reduction in the sum of the products of the perpendicular diameters of the measurable lesions. Progressive disease (PD) was defined as more than a 25% increase in the sum of the products of the perpendicular diameters of the measurable lesions or the appearance of any new lesions. Stable disease (SD) was defined as disease that did not qualify as either response or progression. Toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria version 4.0. After treatment, patients were assessed every 1–3 months for the first 2 years and every 4–6 months for the third, fourth and fifth year. In addition, patients underwent a computed tomography or MRI scan every 6 months for the first 2 years and annually thereafter for 5 years. Relapse-free survival (RFS) was defined as the time from the date of starting treatment to the first appearance of PD. Overall survival (OS) was defined as the time from the date of the starting treatment to the date of death or last follow-up visit. The primary endpoint was the 2-year RFS. The secondary endpoints were the response rate to the chemotherapy, the 2-year/5-year OS rates and toxicities.

Statistical methods

The RFS and OS analyses were performed by the Kaplan–Meier method, and all causes of death were included in the survival calculation. The log-rank test was used to determine whether two survival curves were significantly different. All statistical analyses were performed using JMP Pro version 11 software (SAS Institute Inc.).

Results

Response to NAC regimen

All patients were able to complete the NAC regimen before the surgery; however, one (3.1%) patient could not undergo a radical hysterectomy since the tumor lesion was considered unresectable. Therefore, a radical hysterectomy was performed in 31 patients (96.9%) and the pathological surgical margins were negative in all cases. Lymph node metastasis was pathologically diagnosed using specimens from the radial hysterectomy. Positive lymph node metastasis was seen in 15 (48.4%) patients and was negative in 16 (51.6%) patients. Positive lymph and vascular space invasion were seen in 15 (48.4%) patients but absent in 16 (51.6%) patients (Table 1).

The response to the NAC regimen is summarized in Table 1 and is also represented as waterfall plots in Fig. 1. A CR was obtained in nine (28.1%) patients, two (6.9%) of them had pathological CR and PR was obtained in 17 (53.1%) patients. Namely, the objective response (CR + PR) was 81.2%. On the other hand, SD was observed in five (15.7%) patients and PD was in one (3.1%) patient. Responders were defined as patients who had a CR or PR due to the NAC regimen, while nonresponders were defined as SD or PD.

Feasibility

Twenty-three (71.9%) patients completed the treatment (Table 2). Unfortunately, the protocol was discontinued for nine patients who were switched to cisplatin-based CCRT (28.1%) because of positive lymph node metastasis after surgery (seven patients) and because of a short distance to a resection margin (one patient). Surgery was not performed in the other case. Of the 15 patients with positive lymph node metastasis, the remaining eight patients received adjuvant chemotherapy consisting of a combination of irinotecan with nedaplatin according to the protocol schedule. The regimen for six of 26 responders

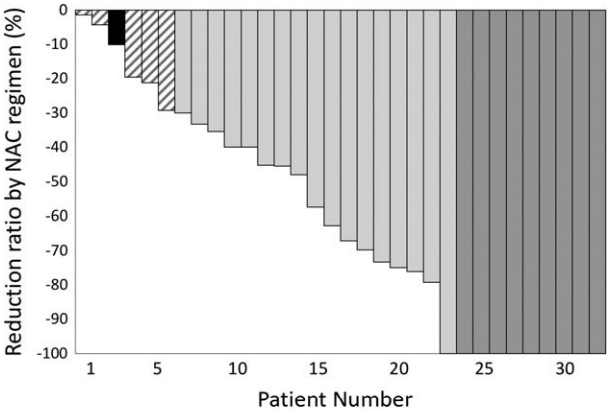


Figure 1 The response to the NAC regimen was represented as waterfall plots. (■) CR; (□) PR; (▨) SD; (▩), PD. CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

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Table 2 Protocol completed/withdrawn cases (n = 32)

Response	Completed	Withdrawn	Total
Responder (CR + PR)	20 (62.5%)	6 (18.8%)	26 (81.3%)
Nonresponder (SD + PD)	3 (9.4%)	3 (9.4%)	6 (18.7%)
Total	23 (71.9%)	9 (28.1%)	32 (100%)
Chi-square test		P = 0.18	–
The protocol was discontinued		n (%)	
Due to positive lymph node metastasis after surgery	7 (21.9%)	–	–
Shortage of the distance to the resection margin	1 (3.1%)	–	–
A case considered as unresectable by radical hysterectomy	1 (3.1%)	–	–

CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

(CR + PR) and three of the nonresponders (SD + PD) (18.8% and 9.4%, respectively) was discontinued and these patients then instead received cisplatin-based CCRT. There was no significant association between response to the NAC regimen and the protocol status (P = 0.18). Among the total 154 cycles administered, treatment delay occurred in nine cycles (5.8%). None of cases required decrease in the protocol-scheduled chemotherapy or reduction in the dose. Overall six (26.0%) cases were delayed for 1 week because of neutrophil count of less than 1500/μL.

Adverse events

Toxicities higher than grade 3 are shown in Table 3. Grades 4 and 3 neutropenia were seen in two and 11 patients, respectively (6.3% and 34.4%), grade 3 diarrhea and vomiting was seen in one (3.1%) patient and vertigo was also seen in one (3.1%) patient during the NAC regimen. On the other hand, grade 4 and 3 neutropenia were seen in two and 11 patients (8.7% and 47.8%, respectively) and grade 3 fatigue was seen in one (4.3%) case during the adjuvant treatment.

Survival

Of the 32 patients, all including the patients who discontinued the therapy were followed up for an average period of 37.8 months (15–71 months). Of the 23 patients who completed the treatment protocol as planned, three (13.0%) patients had recurrence. The recurrence was seen in the intrapelvic location in all three patients and RT was then performed. Of the nine patients who discontinued the therapy and switched to CCRT, three (33.3%) patients had recurrence, two had loco-regional recurrence and one had local and distant recurrence (para-aortic and supraclavicular lymph node metastasis). The 2-year RFS and estimated 5-year RFS rates were 87.5% and 78.8%, respectively (Fig. 2). As shown in Fig. 3, responders to the NAC regimen had a better prognosis compared to the nonresponders (2-year RFS: 94.7% vs 50.0%, respectively; 5-year PFS: 94.7% vs 33.3%, respectively; P = 0.0002).

Discussion

The 2-year and 5-year RFS rates of this study were 87.5% and 78.8%, respectively. In addition, the 2-year and 5-year OS rates were 95.7% and 89.7%, respectively. All patients enrolled in this study had bulky tumors longer than 4 cm. Previous studies have showed that a large tumor size has a detrimental effect on prognosis in patients with locally advanced

Table 3 Grade >3 toxicities by use of the National Cancer Institute Common Toxicity Criteria version 4.0

Neoadjuvant chemotherapy (n = 32)	n (%)	Adjuvant (n = 23)	n (%)
Grade 4 neutropenia	2 (6.3%)	Grade 4 neutropenia	2 (8.7%)
Grade 3 neutropenia	11 (34.4%)	Grade 3 neutropenia	11 (47.8%)
Grade 2 neutropenia	7 (21.9%)	Grade 2 neutropenia	6 (26.1%)
Grade 3 diarrhea	1 (3.1%)	Grade 3 fatigue	1 (4.3%)
Grade 3 vomiting	1 (3.1%)	Grade 2 vomiting	2 (8.7%)
Grade 2 vomiting	4 (12.5%)	–	–
Grade 3 vertigo	1 (3.1%)	–	–

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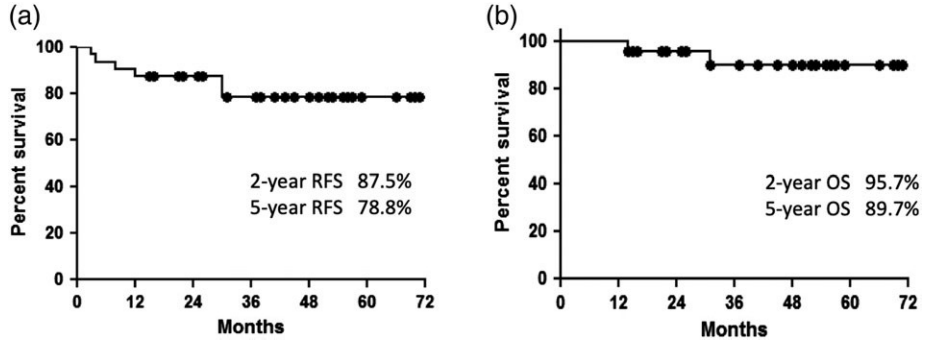


Figure 2 Outcomes of eligible patients enrolled in the Kansai Clinical Oncology Group-1201 study. (a) Relapse-free survival in patients with complete protocol (n = 23). Two-year relapse-free survival: 87.5%; 5-year relapse-free survival: 78.8%. (b) Overall survival in patients with complete protocol (n = 23). Two-year overall survival: 95.7%; 5-year overall survival: 89.7%.

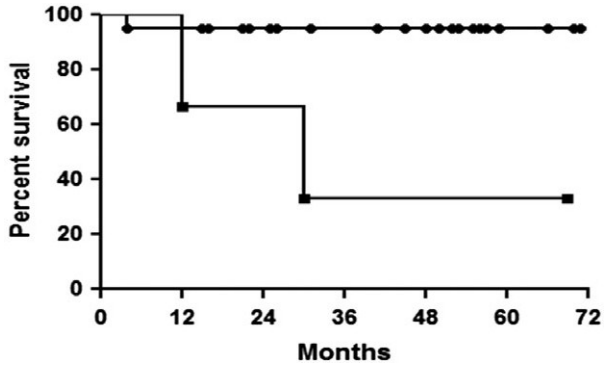


Figure 3 Outcomes of eligible patients enrolled in the Kansai Clinical Oncology Group-1201 study. Relapse-free survival in cases with responder (n = 20) and nonresponders (n = 3). (–●–) Responder (CR + PR); (–■–) nonresponder (SD + PD). CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease.

cervical cancer.[5] Previous studies also showed that the 5-year survival rate in patients with a tumor diameter longer than 4 cm was 49–63%, whereas the 5-year survival rate in patients with smaller tumors was 67–90%.[15–18] Our findings contrast with these previous studies since those with locally advanced, bulky stage IB2-IIB cervical cancer showed more preferable survival rates.

In the Unites States, the NCCN and National Cancer Institute guidelines have recommended CCRT for patients with locally advanced IB2-IIB cervical cancer.[9] According to Quinn *et al*., the 2-year and 5-year OS for patients receiving CCRT for FIGO stage IB2-IIB cervical cancer was 81.5–83.1% and 66.9–70.5%, respectively.[2] On the other hand, a recent retrospective meta-analysis from the Unites States using the Surveillance, Epidemiology and End Results program that examined 10 933 cases with stage IB-IIB cervical cancer recently reported that 23% of patients with tumors greater than 4 cm, 20% of patients with stage IIB and 55% of patients with positive lymph node metastasis received surgery plus RT.[19] These results suggest that surgical intervention could be a viable option for patients with locally advanced stage IB2-IIB cervical cancer in even the United States.

In Japan and other countries, a radical hysterectomy is generally performed for patients with locally advanced cervical cancer. NAC is one approach that utilizes chemotherapy before surgery to improve the outcomes in this patient population and has been reported in many previous studies; however, there is still controversy regarding the optimal regimen of locally advanced, bulky cervical cancer. A meta-analysis of 872 patients from five trials regarding NAC studies showed that NAC followed by surgery significantly improved the prognosis, including OS and PFS, compared with surgery alone.[20] The analysis also demonstrated that shorter intervals (<14 days) and more intensive cisplatin doses (25 mg/m^2 per week) were associated with survival benefits. Based on this background, irinotecan and nedaplatin were selected for the NAC regimen in this study. This combination regimen has been already reported in two other Japanese groups. Yamaguchi *et al*. reported on the efficacy and tolerance of the same NAC regimen followed by hysterectomy for stage IB2-IIB cervical cancer.[21] Although they included patients with tumors less than 4 cm in size, their response rate (CR + PR) was 75.8%. Abou-Taleb *et al*. also reported on the irinotecan and nedaplatin NAC regimen; they

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found the response rate to be 75.0%.[22] These results were also comparable to our findings (81.8%). Regarding the survival rate, Yamaguchi *et al*. showed that the 2-year PFS rate was 73.8%,[21] which appeared to be a poorer prognosis than that found in our results (87.5%). In their study, the additional postoperative treatment was not regulated and 30 out of 66 patients (45.5%) did not receive any postoperative treatment although most patients had large tumors. Therefore, this suggests that postoperative treatment would have useful for those patients with locally advanced, bulky stage IB2-IIB cervical cancer. On the other hand, Abou-Taleb *et al*. concluded that there were no significant differences in survival rates, including PFS and OS, between NAC followed by surgery and primary radiotherapy.[22] However, the study by Abou-Taleb *et al*. had a similar limitation to Yamaguchi *et al*. since adjuvant chemotherapy was not regulated in the NAC protocol.

Concurrent chemo-radiotherapy is also recommended as postoperative adjuvant treatment for patients with high risk factors for recurrence.[23–25] However, there has been much debate regarding the risk–benefit balance of postoperative CCRT for patients with cervical cancer.[26–29] First, patients who receive postoperative CCRT have exhibited a greater rate of distant metastasis. It is crucial for postoperative adjuvant therapy to not only control local recurrence but also prevent distant metastasis. Second, postoperative CCRT could induce serious toxicities. For example, Nakamura *et al*. suggested that lymphedema was significantly more common in patients who received RT or CCRT after radical hysterectomy.[30] Other investigators also showed postoperative obstruction was significantly more frequent in patients who received RT after surgery.[31] In this context, systematic chemotherapy might play a critical role as postoperative adjuvant therapy for patients with high-risk cervical cancer.[29]

A recent meta-analysis that included 825 patients with locally advanced cervical cancer recently showed that responder had favorable prognosis according to the 3-year and 5-year OS.[13] In the current study, the RFS was 94.7% at 2 years and at 5 years. However, for the nonresponders the RFS was 50% and 33.3% at 2 years and 5 years, respectively. These results suggest that the early response to the NAC regimen might predict the outcome for patients with locally advanced cervical cancer.

The treatment used in our study was well tolerated since severe (grade 3/4) adverse events did not frequently occur. However, our study has some limitations. First, nine of 32 patients discontinued the treatment and were switched to CCRT, since seven patients developed lymph node metastasis after surgery, one was found to have a short distance to the resection margin and one was considered unresectable. Our sample size was quite small and therefore a study with a larger cohort could help verify our results. Second, the follow-up period was somewhat limited. The primary endpoint in this study was the 2-year RFS which was quite higher than expected. Also, the late adverse events could not be examined. Therefore, longer-term survival rates and the late toxicities should be further investigated.

In conclusion, neoadjuvant irinotecan and nedaplatin followed by radical hysterectomy and adjuvant chemotherapy showed efficacy and tolerability for patients with locally advanced, bulky stage IB2-IIB cervical cancer. This strategy might be one therapeutic option to preserve ovarian functional in young patients who express a desire for pregnancy. Our findings suggest that neoadjuvant irinotecan and nedaplatin followed by radical hysterectomy and adjuvant chemotherapy has the potential to improve the prognosis compared the CCRT.

Disclosure

None declared.

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子宮内膜症に対する外来診療の実態調査

笠原恭子[1)]，辻　祥子[1)]，樋口明日香[1)]，森宗愛菜[1)]
吉野芙美[1)]，木村文則[1)]，村上　節[1)]，柴原浩章[2)]
1）滋賀医科大学産科学婦人科学教室
2）兵庫医科大学産科婦人科学教室

An actual condition survey on outpatient care of endometriosis

Kyoko KASAHARA[1)], Shoko TSUJI[1)], Asuka HIGUCHI[1)], Aina MORIMUNE[1)]
Fumi YOSHINO[1)], Fuminori KIMURA[1)], Takashi MURAKAMI[1)] and Hiroaki SHIBAHARA[2)]
1) Department of Obstetrics and Gynecology, Shiga University of Medical Science
2) Department of Obstetrics and Gynecology, Hyogo College of Medicine

緒　言

子宮内膜症の患者数は，増加傾向にあるといわれている[1)]．内膜症の手術療法は，疼痛の緩和，妊孕性改善のいずれにも有効であるが再発の問題があり，若年女性のpoly-surgeryを避けるためには，臨床子宮内膜症を含む内膜症の外来管理が重要である．

このような背景のもとに，生殖内分泌・女性ヘルスケア研究部会ではグループスタディとして，近畿地区で一般的に行われている子宮内膜症の外来診療の実態を明らかにすることを目的とした調査を行った．

対象と方法

第138回近畿産科婦人科学会学術集会の会場で学会参加者を対象にアンケート調査を行った．学会参加者は約900人，回答者総数は116名，勤務先は無床診療所20名，有床診療所13名，大学病院27名，大学以外の病院53名，複数回答・無回答が3名で，回答者の29％が診療所，71％が病院勤務であった．専門は産婦人科全般：55名，生殖・内分泌：18名，腫瘍：15.5名，女性ヘルスケア：12.5名，周産期：12名，研修中：2名，無回答1名であった（2つ選択した回答は0.5人ずつに分けて集計）．

結　果

1）子宮内膜症患者を1週間で何人外来診療しているか（図1）

専門領域がさまざまな回答者のほとんどが，少人数ずつながらも内膜症患者を診察している（図1A）．11人以上の割合は大学病院・無床診療所で比較的多く，手術症例・不妊症例を表している可能性がある（図1B）．

2）問診表にある項目

月経痛，月経量，下腹痛は回答者の100％，91％，73％に選択されていた．性交痛・排便痛は37％，29％に選択されており，内膜症を疑った場合，個別に質問されていることが多いと思われる．

3）どのような検査を行うか（図2）

①月経困難症患者の初診時（A）

基本的な診察手法が多く選択されていた．

②月経困難症患者で低用量エストロゲン・プロゲスチン配合薬（LEP）の使用を考慮する際の検査（B）

身長・体重，血圧測定が初診時より多く，血栓症リスクが意識されていると思われた．また，OC/LEPガイドラインではとくに推奨されていないが[2)]，半数以上の回答者が肝機能検査，凝固機能検査を挙げていた．

4）臨床子宮内膜症に対する治療を選択する上で考慮する項目（図3）

チョコレート嚢胞や挙児希望は予想通り上位であったが，提示した項目の全てが過半数の回

答者に選択され，この疾患が患者の状態を総合的に判断しながら診療されていることが確認された．

5）使用している薬物（図4）

機能性月経困難症（A），臨床子宮内膜症による月経困難症（卵巣チョコレート嚢胞なし）(B)

どちらも最も用いられているのはLEP，2番目が非ステロイド性抗炎症薬（NSAIDs）であった．ジエノゲストは機能性月経困難症の4番目，臨床子宮内膜症の3番目であった．複数回答可であったので，ホルモン療法と，NSAIDs・漢方薬の併用も多いと推測される．

施設別で検討したが，診療所で（A）経口避妊薬（OC），（B）レボノルゲストレル放出子宮内システム（LNG-IUS）がやや多かった以外は，ほとんど差がなかった．

6）臨床子宮内膜症による月経困難症（卵巣チョコレート嚢胞なし）に行うホルモン療法，使用頻度の高い順（図5）

20歳代ではLEPが圧倒的な1位であった（図5A）．一方40歳代ではジエノゲストが第1選択で，使用頻度2番目ではLNG-IUSが僅差で多く，LEPとジエノゲストが同数であった（図5B）．

7）卵巣チョコレート嚢胞で手術を勧めるのは大きさが何cm以上の場合か

①挙児希望あり／将来の挙児希望はあるが現在はなし／将来にわたって挙児希望なし，②月経困難症あり／なし，③40歳未満／以上で12通りに分類し，50歳以上を加えて13通りの患者背景を設定し，【　】cm以上に記入する欄と，「大きさにかかわらず」に○をする欄を用意した．そこから得られた結果の主なものを述べる．

・どの群でも，おおむね4～6 cmで手術が勧められていたが，病院では主に4 cm以上，診療所では5～6 cm以上で手術適応と判断されることが多かった．病院の方が手術適応とするサイズが小さい傾向があることが示され，診療所から紹介された卵巣チョコレート嚢胞の多くが手術対象になっていると推測された．
・「挙児希望あり」と「50歳以上」で，他の群に比べて「大きさにかかわらず手術を勧める」という回答が多かった．また，全般的に40歳以上は40歳未満よりも，小さくても手術を勧める傾向があったが，「挙児希望あり＋月経困難症あり」では，年齢による対応の差が少なかった．具体的な治療目標があると手術が

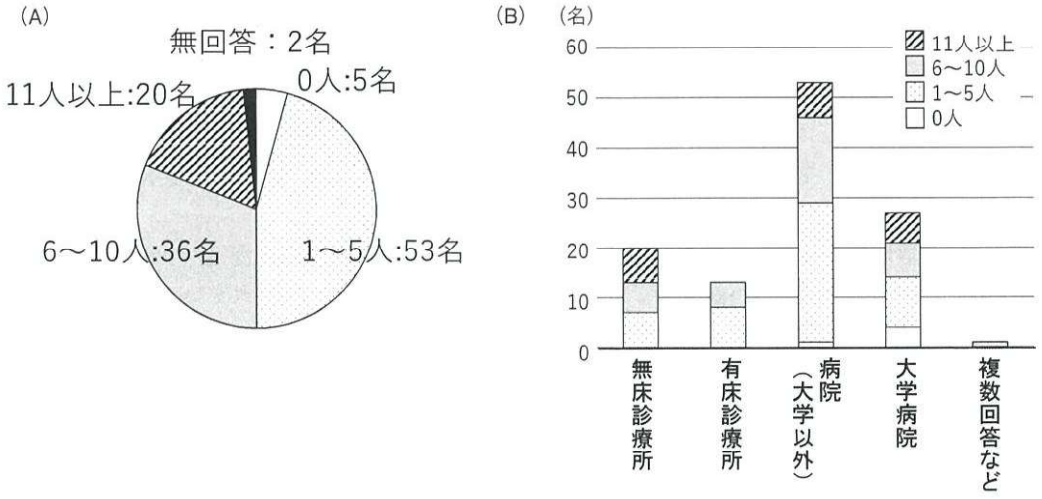


図1　子宮内膜症患者を1週間で何人外来診療しているか．
（A）全体
（B）勤務先別

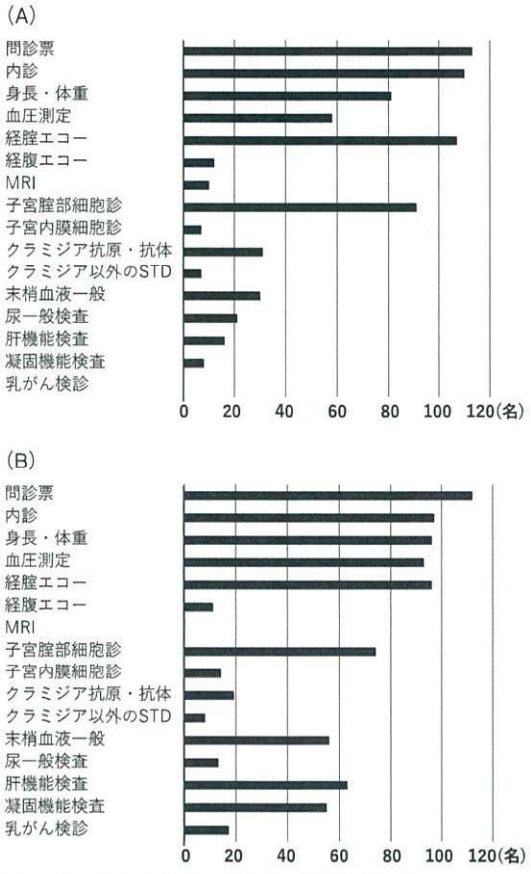


図2　どのような検査を行うか（複数回答可）.
（A）月経困難症患者の初診時
（B）月経困難症患者に対しLEPの使用を考慮する際

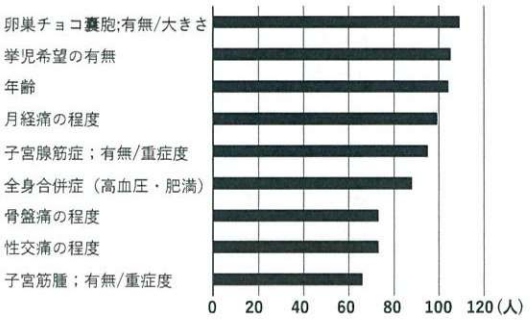


図3　臨床子宮内膜症に対する治療を選択する上で考慮する項目（複数回答可）

積極的に行われやすく，加齢による癌化リスク上昇は考慮されやすいようである.

考　察

機能性月経困難症，臨床子宮内膜症による月経困難症の，いずれの場合も治療の第1選択はLEPであった．臨床子宮内膜症のLEP，ジエノゲストは予想通りだが，機能性月経困難症で臨床子宮内膜症とほとんど同程度にホルモン療法が行われているという実態は興味深い．使用経験の蓄積によってホルモン療法の効果が，医師・患者双方によく知られるようになり，広く普及している様子が伺えた.

機能性月経困難症に対して約半数の回答者がジエノゲストを使用しており，対症療法やLEPでは鎮痛効果が得られない場合や，血栓症リスクの高い40歳以上や肥満症例では選択肢の1つになっていると思われる．また，若年女性の月経困難症の多くは機能性であるが，近年，その一部が子宮内膜症の潜在症例であることが指摘されており，思春期内膜症として注目されている[3,4]．LEPなどホルモン療法の積極的な選択は，将来の不妊リスクの軽減にむすびつく可能性があり有用と思われる.

臨床子宮内膜症による月経困難症（チョコレート嚢胞がなし）のホルモン療法として，20歳代ではLEPが圧倒的に多く使用されていた．一方，40歳代ではジエノゲストが最も多く使われていたが，20歳代よりも選択肢が分散する傾向にあり，患者のニーズの多様性が伺われた．ジエノゲストは病巣への直接作用があり，慢性深部痛に対する効果が高い．また，肝機能，脂質代謝や凝固能へも影響が少ないため，血栓症リスクからLEPが使いにくい症例にも投与できるという利点がある．にもかかわらず，40歳代のジエノゲストが20歳代のLEPのように圧倒的な1位にならなかったのは，しばしば見られる不定期な不正性器出血が関係している可能性がある．30歳代までにLEPを経験した患者にとって，月経開始時期が分かるLEPは利便性から手放せないのかもしれない.

また，40歳代でもLEPが臨床子宮内膜症治療の主な選択肢の1つになっていることが明らか

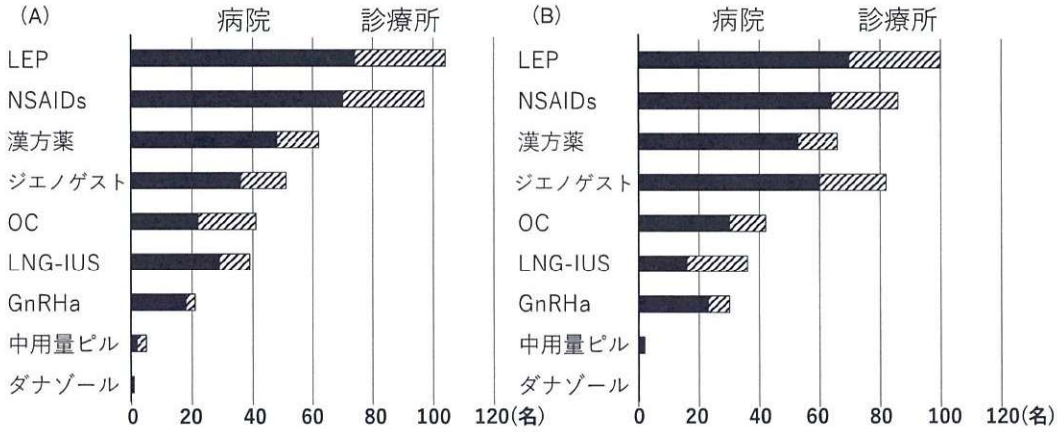


図4　使用している薬物（複数回答可）
（A）機能性月経困難症
（B）臨床子宮内膜症による月経困難症（卵巣チョコレート嚢胞なし）

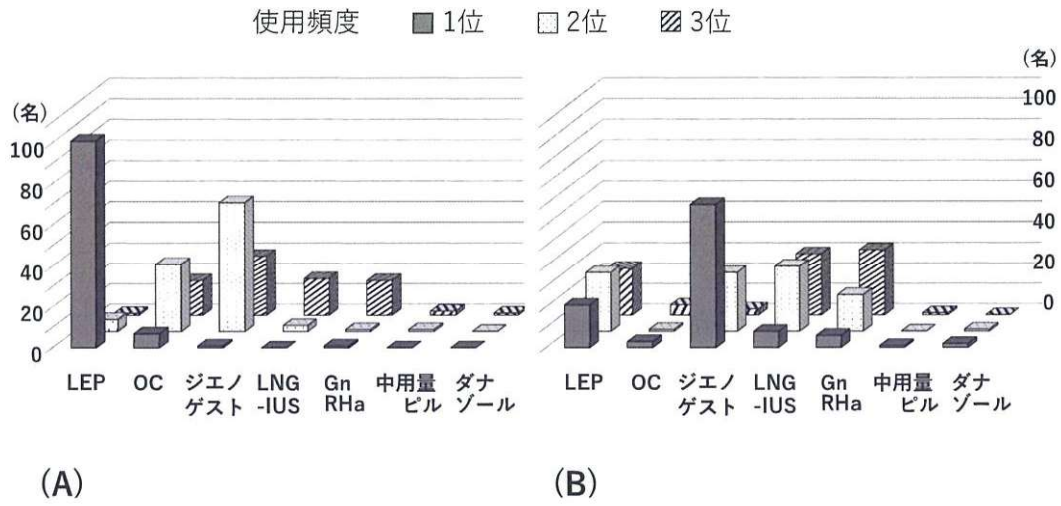


図5　臨床子宮内膜症による月経困難症（卵巣チョコレート嚢胞なし）に対するホルモン療法
（A）20歳代　（B）40歳代

になった．40歳以上は一般に心筋梗塞などの心血管系の障害が発生しやすくなる年代であり，LEPは加齢以外に心血管系障害を高める臨床所見を認めない場合のみ慎重投与とされている[2]．また，深部静脈血栓症の発症リスクは服用開始後3カ月以内が最も多くその後減少するが，4週間以上の休薬期間をおいて服用再開すると，初回投与と同様の発症リスクになる[2]．LEPの有益性に着目して，20～30歳代からLEPを内服し，心血管系障害のリスクとなる合併症・喫煙がなければ40歳を超えてもLEPを継続投与するというストラテジーが，一般的に行われていることが調査結果から推測された．

しかしながら，加齢とともに個々の症例の心血管系障害のリスクも潜在的に変化する可能性がある．子宮内膜症の受療者数は増えてお

り，疾患の啓発や治療法の進歩によってLEPによる長期管理例は今後も増加すると予測される．OC/LEPのガイドラインではLEPの副効用に着目して，心血管系リスクのない40歳代のLEP使用に対して許容的なコメントが記載されている[2]．しかし基本に立ち返れば，使いなれてきたLEPであっても血栓症リスクに注意する必要があるだろう．

結　語

以前は日本人患者にはホルモン療法が好まれないなどといわれていたが，今回のアンケート調査で機能性月経困難症・臨床子宮内膜症に対して積極的にホルモン療法が行われていることが確認された．また，臨床子宮内膜症に対して，20歳代だけでなく40歳代でもLEPは主な選択肢の1つになっていることが示された．40歳代のLEP投与は，おそらく20～30歳代から開始された継続投与例と推測され，内膜症の長期管理の一端が垣間見える．40歳代では新規投与は避け，継続投与であっても潜在的に血栓症リスクが高くなっていることを医師も患者も認識し，心血管系障害を高める臨床所見を見落とさないことが重要である．

最近臨床現場に広まりつつあるLEPの長期投与や，子宮筋腫で承認されたGnRHアンタゴニストなど，新しい治療戦略が加わりつつある分野であり，私たちの子宮内膜症診療は今後も変化していくと思われる．既存の薬剤の長所と問題点をよく理解して使いこなしながら，より進歩した治療戦略を取り入れていかなければならない．

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Original Article

Phase I study on pegylated liposomal doxorubicin in combination with docetaxel for patients with platinum-resistant or partially platinum-sensitive epithelial ovarian cancer: The Kansai Clinical Oncology Group study

Kensuke Hori, Kimihiko Ito, Kentaro Kuritani[1], Shiho Kuji[2], Naoto Furukawa[3], Hiroshi Tsubamoto[4], Atsushi Arakawa[5]

Department of Obstetrics and Gynecology, Kansai Rosai Hospital, Amagasaki, [4]Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, [1]Department of Obstetrics and Gynecology, Osaka Rosai Hospital, Sakai, [2]Department of Gynecology, Shizuoka Cancer Center, Nagaizumichou, Shizuoka, [3]Department of Obstetrics and Gynecology, Nara Prefectural Seiwa Medical Center, Ikoma-Gun, [5]Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Aichi, Japan

For correspondence: Dr. Kensuke Hori, Department of Obstetrics and Gynecology, Kansai

ABSTRACT

Context: In platinum-resistant ovarian cancer, single-agent chemotherapy is recommended for the reduction of adverse events. However, in clinical practice, some patients can tolerate drug-specific adverse events.

Aims: We assessed the safety of pegylated liposomal doxorubicin (PEG-LD) and docetaxel regimen in the first cycle of ovarian cancer.

Settings and Design: We performed a phase I study to evaluate the combination therapy of PEG-LD and docetaxel.

Materials and Methods: We recruited five patients with recurrent ovarian cancer within 12 months of first-line platinum-based chemotherapy. All patients had measurable disease severity. PEG-LD and docetaxel were intravenously administered on day 1 and every 21 days using three dose levels: 25 mg/m^2 PEG-LD and 50 mg/m^2 docetaxel; 30 mg/m^2 PEG-LD and 50 mg/m^2 docetaxel; and 30 mg/m^2 PEG-LD and 60 mg/m^2 docetaxel.

Statistical Analysis Used: We defined the maximum tolerated dose of the combination therapy based on the modified Fibonacci method.

Results: Five patients were enrolled in this study. The median treatment-free interval was 5.5 months. Two dose-limiting toxicities (Grade 4 neutropenia) were observed in two patients. One complete response, one partial response, one stable disease, and two progressive disease cases were observed. The overall response rate was 2/5, and the disease control rate was 3/5. The median overall survival was 7.4 months.

Conclusions: We determined that 25 mg/m^2 of PEG-LD and 50 mg/m^2 of docetaxel were safe and effective doses. This preliminary efficacy and safety data should be further investigated in a Phase II trial.

KEY WORDS: Docetaxel, ovarian cancer, pegylated liposomal doxorubicin, platinum resistance, platinum sensitivity, therapeutic dose

INTRODUCTION

Combined chemotherapy using taxane and platinum is currently the standard initial treatment for recurrent ovarian cancer. The time to recurrence after the completion of initial chemotherapy, i. e., treatment-free interval (TFI) can be correlated with the response rate (RR). In general, TFI for drug susceptibility is <1 month, whereas TFI of 6 months indicates drug resistance. In addition, cases with TFI of ≤12 months were less sensitive to chemotherapy compared with those with TFI of ≥12 months.[1]

The addition of platinum-based regimen to standard chemotherapy has been recommended

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Rosai Hospital, Inabasou, Amagasaki, Hyogo, Japan. E-mail: hori-ken@kanrou.net

for the relapse of platinum-sensitive ovarian cancer. In cases of platinum resistance, no significant difference between a multidrug and single-agent regimen was observed in prolonging life or RR in past trials. In addition, single-agent chemotherapy is the treatment of choice when aiming for the reduction of adverse events. The selection of an agent for platinum-resistant cases is dependent on the absence of cross-resistance to the initial treatment.[2-4] However, in clinical practice, some patients can tolerate drug-specific adverse events. The agents recommended for platinum-resistant epithelial ovarian cancer relapse are paclitaxel, docetaxel, irinotecan, pegylated liposomal doxorubicin (PEG-LD), topotecan, gemcitabine, and oral etoposide in the United States and Japan.[5-14]

The addition of PEG-LD to standard chemotherapy is recommended by the British Ovarian Cancer Treatment Guidelines not only for platinum-resistant cases but also for those with intermediate sensitivity. PEG-LD is an anthracycline agent that selectively targets the tumor organizational liposome. Conventional doxorubicin is no longer used for chemotherapy because of its alopecia effect and toxicity to the heart, digestive organs, and blood.[15,16] Previous clinical trials have reported that PEG-LD has no cross-tolerance with other chemotherapeutic agents for recurrent epithelial ovarian cancer. In addition, PEG-LD is not significantly different from paclitaxel, topotecan, and gemcitabine in terms of overall survival (OS) and progression-free survival (PFS). Furthermore, the management of adverse events, such as hand–foot syndrome (HFS) and stomatitis, is relatively easy. The toxicity profile of PEG-LD does not overlap with that of other agents that are currently used in Japan.[16,17]

In other countries, PEG-LD has been identified as the standard therapeutic agent for recurrent platinum-resistant epithelial ovarian cancer; however, its use in Japan was only authorized in April 2009. The recommended dose (RD) of PEG-LD is 50 mg/m^2 given intravenously every 4 weeks. However, HFS (Grades 1–3), which was the main adverse event, occurred in 48%.[18] In the only retrospective study available for PEG-LD, the effective dose was found to be 30–40 mg/m^2. Moreover, compared with the 50 mg/m^2 dose, the 40 mg/m^2 dose had similar OS and PFS but fewer adverse events.[18] Since the year 2000, few clinical trials comparing the anticancer spectrum and toxicity profile of combination therapy with PEG-LD and docetaxel have been conducted, mainly for breast cancer. Kouroussis *et al.* reported that the maximum tolerated dose (MTD) was 35 mg/m^2/week for docetaxel and 14 mg/m^2/week for PEG-LD in a Phase I study of patients with solid tumor. The combination therapeutic overall response in that study was reported to be 22.7%.[20,21] Recently, Sparano *et al*. performed a Phase III clinical trial on 751 patients with progressing breast cancer. They reported that a combination therapy of 60 mg/m^2 docetaxel and 30 mg/m^2 PLD→PEG-LD given every 3 weeks improved the time to progression (TTP) and RR compared with 75 mg/m^2 docetaxel given as a single agent every 3 weeks.[22,23]

On the basis of the hypothesis that the toxicity profile and anticancer spectrum of docetaxel differ from those of PEG-LD, we aimed to perform a Phase I trial on the efficacy of combination therapy with PLD and docetaxel, compared with single-agent PEG-LD regimen, for patients with platinum-resistant ovarian cancer in Japan.

MATERIALS AND METHODS

Study protocol, ethics committee approval, and informed consent

For the purpose of evaluating the combination therapy of PEG-LD and docetaxel, the member institutions of the Kansai Clinical Oncology Group (KCOG) assigned patients with recurrent epithelial ovarian and peritoneal carcinomas between January 2010 and March 2011 into the KCOG protocol (KCOG-G0901). Approval for this study was granted by the Local Ethics Committees of KCOG, Kansai Rosai Hospital, Shizuoka Cancer Center, Nara Medical University, Hyogo Medical College, and Nagoya Municipal University. All patients who participated in this study provided written informed consent after a thorough explanation of the contents of the study and provision of other explanatory documents.

Patient eligibility

All patients were required to meet the following criteria: (1) histologic or cytologic diagnosis of Mullerian carcinoma (epithelial ovarian carcinoma, fallopian tube carcinoma, or primary peritoneal carcinoma); (2) recurrence within 12 months after the final course of the first-line platinum-based chemotherapy (including recurrence identified by tumor markers); and (3) ECOG performance status of 0–2. To be eligible to receive first-line platinum-based chemotherapy, patients had to be at least 20 years old and not older than 75 years old at the time of enrollment. In addition, patients who met the following criteria were included: hemoglobin >9.0 g/dL, white blood cell count 3000–12,000/mm^3, absolute neutrophil count (ANC) ≥1500/mm^3, platelet count ≥100,000/mm^3, aspartate transaminase/alanine transaminase ≤2.5 times the upper limit of normal (ULN), alkaline phosphatase ≤2.5 times ULN, bilirubin <1.2 mg/dL, serum creatinine ≤1.5 times ULN, cardiac function with left ventricular ejection fraction (LVEF) ≥50% and electrocardiogram within normal limits, and no symptoms requiring treatment. The patients were expected to survive for at least 3 months and should not have received chemotherapy for more than 4 weeks.

The principal investigator or subinvestigator excluded the following patients: (1) patients with severe complications or active systemic infection that may affect the administration

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and proper assessment of the drugs; (2) patients with multiple synchronous and metachronous cancers within 5 years of disease-free interval, with the exceptions of basal and squamous cell carcinoma, carcinoma *in situ*, and intramucosal carcinoma-like lesions, which are curable by local treatment; (3) patients with angina or myocardial infarction within the last 90 days (day 1 was defined as the day before enrollment); (4) patients who had symptomatic brain metastases that required the administration of steroids or anti-inflammatory agents; (5) patients who have received bone marrow transplant, hematopoietic stem cell transplant, or high-dose chemotherapy; (6) patients who have received prior chemotherapy consisting of anthracycline, including PLD, or docetaxel; (7) patients with a history of hypersensitivity to the components of anthracycline, taxane, or liposomal doxorubicin (mPEG-DSPE, hydrogenated lecithin, cholesterol, ammonium sulfate, histidine, sucrose, hydrochloric acid, and sodium hydroxide); and (8) patients who were or suspected to be pregnant or lactating.

Treatment

The patients were required to undergo medical history interview, physical examination, and laboratory tests within 14 days of starting the treatment. Toxicity assessment using the Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v. 3.0) and complete blood count were obtained weekly. Tumor measurements and tumor markers, when applicable, were examined after one or two cycles of chemotherapy. Adverse events were reported and recorded as they occurred.

On day 1 of a 21-day cycle, PEG-LD and docetaxel were given in combination as an intravenous infusion for 1 h. This study arm was created to assess the toxicity of each sequential dose setting [Table 1]. This design began with the treatment of the first patient at a Level 1 dose. If the next three patients did not experience (DLT), dose escalation continued. Additional patients were enrolled at the same dose level on the basis of the classic modified Fibonacci scheme.[24]

DLT was defined as (1) Grade 4 neutropenia lasting longer than 7 days; (2) Grade 4 thrombocytopenia; (3) any Grade 3 or Grade 4 nonhematologic toxicity, except nausea, emesis, anorexia, alopecia, anaphylaxis, and infusion reaction; (4) serum bilirubin of ≥3.0 mg/dL; or (5) any episode of febrile neutropenia.

Treatment was delayed by 1 week for patients with ANC ≤1500 cells/mm^3, at least Grade 2 stomatitis, at least Grade 2 HFS, or platelet count ≤100,000 cells/mm^3. Both PEG-LD and docetaxel doses were reduced by 25% if a patient's nadir values for ANC were <500 cells/mm^3 and for platelet count were <25,000 cells/mm^3 as well as for those with Grade 3/4 nonhematologic toxicities, except for fatigue, nausea, emesis, anorexia, alopecia, hypokalemia, hyponatremia, and infusion reaction. PEG-LD dose was reduced by 25% for Grade 3 palmar-plantar erythrodysesthesia (PPE) and Grade 3/4 stomatitis. Therapy was discontinued in cases of voluntary patient withdrawal, development of clinical congestive heart failure (LVEF of <45% or a decrease of 20% or more from pretreatment value), two dose reductions, cumulative anthracycline level of ≥500 mg/m^2 with PEG-LD, prolonged Grade 2 infusion reaction within 24 h of administration, or disease progression or at the discretion of the treating physician.

Table 1: Accelerated dose escalation schema

Level	PLD (mg/m^2, day 1)	DXT (mg/m^2, day 1)
0	25	50
1	30	50
2	30	60

PLD=Pegylated liposomal doxorubicin, DXT=Docetaxel

Response criteria

Response assessment was based on guidelines set forth in the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. For the assessment of safety and toxicity, we used CTCAE (version 3.0). The primary objective was to determine RD of PEG-LD in combination with docetaxel. MTD was used to determine RDs of PEG-LD and docetaxel. The secondary objective was to evaluate the best overall response (RR) and safety of PEG-LD in combination with docetaxel.

RESULTS

Six patients were enrolled in the study between January 2010 and March 2011. The baseline characteristics, dose levels, total number of cycles, and OS are listed in Table 2. One patient, designated as patient 5, dropped out because of an improper regimen administered by the treating physician. Two patients who were enrolled at dose Level 1 experienced DLTs, such as Grade 4 neutropenia, whereas three patients who were enrolled at dose Level 0 did not experience DLT. MTD for this study arm was dose Level 0.

Table 3 presents the severity of hematologic toxicities for all patients in the entire study arm. There were no Grade 2–4 thrombocytopenia and Grade 3–4 anemia observed in any patient. Table 4 shows the most common nonhematologic toxicities for each patient. Grade 2 toxicities were uncommon, and no Grade 3–4 toxicities occurred. The antitumor response of patients in this study arm is shown in Tables 2 and 5. One complete response was documented in a patient who was enrolled at dose Level 1. One partial response was documented in a patient who was enrolled at dose Level 0. RR was 2/5 (40%), the disease control rate was 3/5 (60%), and the median OS time was 7.4 months (range, 4.5–15.4 months). After experiencing DLT and dropping out of this study, two patients who were enrolled at dose Level 1 continued to undergo treatment at reduced doses for nine or four cycles.

DISCUSSION

This small preliminary Phase I study evaluated the safety profile and MTD of combination therapy with PEG-LD and

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Table 2: Patient characteristics, dose level, total cycles, and overall survival

Patient number	Level	Age (years)	Histology	TFI (months)	PS	Measurable disease	Nonmeasurable disease	DLT	Total cycles	Overall survival (months)
1	1	61	Serious	9.1	0	None	CA125	Neutropenia G4	9	15.4 AWD
2	1	47	Serious	3.7	2	None	Pleural effusion	Neutropenia G4	4	14.0 DOD
3	0	71	SSPC	1.0	0	Peritoneal dissemination	Ascites	None	7	7.4 DOD
4	0	53	Serious	11.0	0	Peritoneal dissemination	Ascites	None	3	4.5 DOD
6	0	66	High-grade adenocarcinoma	5.5	2	Peritoneal dissemination	Ascites	None	7	6.2 AWD

One patient enrolled as "number 5" dropped out because of improper regimen by the treating physician. Two patients enrolled at dose Level 1 experienced DLT (Grade 4 neutropenia). Three patients enrolled at dose Level 0 experienced no DLT. The maximum tolerance dose for this study arm was dose Level 0. No Grade 2–4 thrombocytopenia was observed in any patient. No Grade 3–4 hemoglobin decrease was observed in any patient. AWD=Alive with disease, DOD=Death of disease, SSPC=Serous surface papillary carcinoma, TFI=Treatment-free interval, PS=Platinum-sensitive, DLT=Dose-limiting toxicity

Table 3: The worse grade of hematological toxicities for each patient

Patient number	Level	Neutropenia	Hemoglobin decreased	Thrombocytopenia
1	1	4	2	-
2	1	4	-	1
3	0	4	1	-
4	0	3	1	-
6	0	3	2	1

Grade 2 toxicities were uncommon, and no Grade 3–4 toxicities occurred

docetaxel administered every 21 days to patients with recurrent epithelial ovarian and peritoneal carcinomas. We demonstrated that this combination regimen was feasible with minimal hematologic and nonhematologic toxicities. Grade 4 neutropenia was considered DLT. MTD was 25 mg/m^2 PEG-LD, followed by 50 mg/m^2 docetaxel on day 1 and every 21 days thereafter.

Studies on the combination of PEG-LD and docetaxel for other malignancies, including breast cancer, have been published since 2000. In 2003, Fracasso *et al*. reported their Phase I study on advanced malignancy in 22 patients who were enrolled in two treatment arms. In their accelerated, dose escalation trial, Arm A comprised PEG-LD followed by docetaxel on days 1 and 15 every 28 days, whereas Arm B comprised docetaxel followed by PEG-LD on days 1 and 15 every 28 days.[20] Patients enrolled in that study had malignancies other than ovarian cancer, whereas all patients in our study had only ovarian cancer. Our study was conducted in only one arm, which was PEG-LD followed by docetaxel; this is because myelosuppression when doxorubicin is followed by paclitaxel is less severe compared with that when paclitaxel is followed by doxorubicin. Moreover, paclitaxel may increase the concentration of unchanged doxorubicin.

Recently, Sparano *et al*. reported a Phase III trial in which they compared docetaxel (75 mg/m^2) given as a single agent every 3 weeks with a combination of docetaxel (60 mg/m^2) and PEG-LD (30 mg/m^2) given every 3 weeks. Their results indicated that the addition of PEG-LD to docetaxel was superior to docetaxel alone in terms of TTP and RR. The percentage of Grade 3/4 neutropenia in patients who received combination treatment and docetaxel alone was 66% and 65%, respectively. Grade 3/4 PPEs occurred in 24% patients enrolled in this trial. On the basis of previous studies, we concluded that the toxicities were acceptable.[22,23]

Since the toxicity profiles and anticancer spectrum of PEG-LD and docetaxel are considerably distinct, only Grade 4 neutropenia was observed in the trial reported by Sparano *et al*.; however, all patients continued to experience other Grade 1–2 hematologic and nonhematologic toxicities. The low incidence of adverse events observed in our study may be related to the dosage of PLD. RD of PLD alone for patients with ovarian cancer is limited to 50 mg/m^2 on day 1 and every 28 days thereafter. Grade 1–3 PPE was the main consequence of PEG-LD toxicity and occurred in 48% patients. In a Japanese Phase II trial, PPEs occurred in 78.4% patients treated with 50 mg/m^2 PEG-LD every 28 days. PPEs caused delayed PEG-LD treatment in 40.8% patients, and 4.1% patients stopped participating in the trial.[19] The efficacy of PEG-LD at a dose of 30–40 mg/m^2 every 28 days was identified in previous retrospective studies. In particular, there was no significant difference between the 40 and 50 mg/m^2 PEG-LD doses in terms of RR, PFS, and OS as well as DLT.[18] Our findings, coupled with those of previous studies, demonstrated that if PEG-LD was administered at a dose of 10 mg/m^2 per week, the efficiency of prolonging the survival is improved and toxicity is reduced.

To the best of our knowledge, this is the only Phase I trial that utilized the day 1 schedule of the administration of this combination therapy every 21 days to treat relapse of epithelial ovarian carcinoma. Sparano *et al*. conducted their Phase I study of PEG-LD and docetaxel in patients with advanced breast carcinoma using a 21-day schedule. In that study, MTD was 30 mg/m^2 PEG-LD and 60 mg/m^2 docetaxel. At these MTDs, 60% patients experienced Grade 3–4 neutropenia and 13% experienced Grade 3–4 stomatitis and PPEs; DLT of this regimen was febrile neutropenia. With the addition of granulocyte-colony-stimulating factor, MTD was 30 mg/m^2 PEG-LD and 75 mg/m^2 docetaxel every 28 days. Of 29 patients who received this regimen, 55% showed response to the

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Table 4: The worse grade of nonhematological toxicities for each patient

Patient number	PPE	Stomatitis	Nausea	Emesis	Diarrhea	Anorexia	Fatigue	Alopecia	Motory neuropathy	Sensory neuropathy
1	1	1	-		-	1	-	-	-	-
2	1	1	-		-	1	1	1	-	-
3	-	2	1	1	2	1	1	-	-	1
4	-	-	-	1	-	-	-	-	-	-
6	-	1	-	-	-	1	-	1	-	1

One CR was documented in a patient enrolled at dose Level 1. One PR was documented in a patient administered at dose Level 0. Response rate was 40%, and disease control rate was 60%. The median of overall survival was 7.4 months–range, 4.5–15.4 months). Two patients enrolled at dose Level 1 continued to be administered with reducing doses for 9 and 4 cycles after experiencing DLT and dropping out this study. PPE=Palmar-plantar erythrodysesthesia, CR=Complete response, DLT=Dose-limiting toxicity, PR=Partial response

Table 5: Response to treatment for each cycle

Patient number	Dose level	Number of cycles								
		1	2	3	4	5	6	7	8	9
1	1	PD*	-	SD	SD	SD	SD	SD	SD	SD
2	1	-	CR	-	CR					
3	0	-	-	SD	-	SD	-	PD		
4	0	-	-	PD						
6	0	-	SD	-	PR in	-	PR	-		

*PD (new lesion appeared at a neck lymph node). CR=Complete response, PR=Partial response, SD=Stable disease, PD=Progressive disease

treatment. Previous reports that used various other schedules have shown serious myelosuppression, mucositis, and skin toxicities.[22,23] MTD of our study ameliorated the hematologic and nonhematologic complications observed in these previous studies.

CONCLUSION

The administration of 25 mg/m^2 PEG-LD, followed by 50 mg/m^2 docetaxel, on day 1 and every 21 days thereafter in patients with platinum-resistant or partially platinum-sensitive epithelial ovarian cancer appeared to be a well-tolerated regimen with acceptable toxicities. RR was 40%, and the disease control rate was 60%. The median OS time was 7.4 months (range, 4.5–15.4 months). These results, coupled with those of other studies, warrant Phase II trials of this treatment combination in patients with platinum-resistant epithelial ovarian carcinoma. Based on the findings of our study, new molecular targeted drugs such as bevacizumab, veliparib, and olaparib may be effective in treating gynecological cancer. To confirm this, our group has initiated a Phase II study of cisplatin-based chemotherapy using gemcitabine and bevacizumab (KCOG-G-1601 trial; UMIN000023097).

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Conflicts of interest
There are no conflicts of interest.

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S-1, Oxaliplatin, Nab-paclitaxel and Itraconazole for Conversion Surgery for Advanced or Recurrent Gastric Cancer

MIYUKI SAWASAKI[1], HIROSHI TSUBAMOTO[1], YOSHIHIKO NAKAMOTO[2], AYAKO KAKUNO[3] and TAKASHI SONODA[1]

[1]*Department of Medical Oncology, Meiwa Hospital, Nishinomiya, Japan;*
[2]*Department of Surgery, Meiwa Hospital, Nishinomiya, Japan;*
[3]*Department of Pathology, Meiwa Hospital, Nishinomiya, Japan*

Abstract. *Aim: To evaluate the efficacy of chemotherapy with itraconazole for advanced or recurrent gastric cancer. Patients and Methods: Patients with human epidermal growth factor receptor 2 (HER2) negative unresectable gastric cancer referred to our hospital were included. The regimen comprised 160 mg/m^2 nab-paclitaxel i.v. and 100 mg/m^2 oxaliplatin i.v. on day 1, 60 mg/m^2 S-1 orally on days 1-3, and 400 mg itraconazole orally on days −2 to 2, repeated every 2 weeks for 6-8 cycles. Results: Twenty-three patients aged 40-80 years (median age=68 years) were enrolled, of whom 21 had stomach cancer and two gastroesophageal junction cancer. Regarding stage, two, one, and 20 patients had stage IIIA, IIIB, and IV, respectively. Among patients with liver metastases, 2/10 had simultaneous lung metastases. Nine patients had peritoneal dissemination, and five patients with stage IV disease developed recurrence after primary surgery followed by adjuvant S-1. The other 18 patients had no history of surgery or chemotherapy. The response rate was 70% (complete response in two; partial response in 14). Among 12 patients (67%) who underwent conversion surgery, R0 resection was conducted in eight, and no residual tumour was observed in two. For the population overall, the median overall survival was 24 months (95% confidence intervaI=21 months-not reached) and the 1-year overall survival rate was 95% (95% confidence intervaI=67-98%). Grade 3/4 neutropenia and grade 2 peripheral sensory neuropathy occurred in five (22%) and six (26%) patients, respectively, while no patient developed grade 3/4 thrombocytopenia. Conclusion: Chemotherapy with itraconazole is promising for patients with unresectable gastric cancer.*

Correspondence to: Hiroshi Tsubamoto, Department of Medical Oncology, Meiwa Hospital, Kaminaruo-cho 4-31, Nishinomiya City, Hyogo, 663-8186, Japan. Tel: +81 798471767, e-mail: tsuba@hyo-med.ac.jp

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Gastric cancer remains an important cancer worldwide, and metastatic and unresectable gastric cancer still has poor prognosis. Worldwide in 2018, more than 1,000,000 new cases and approximately 783,000 deaths were reported, making gastric cancer the fifth most frequently diagnosed cancer and the third leading cause of cancer death (1). The key cytotoxic drugs for gastric cancer include 5-fluorouracil (FU) and platinum. In Japan, S-1 plus cisplatin has been the standard first-line treatment for human epidermal growth factor receptor 2 (HER2)-negative advanced gastric cancer (AGC) since 2008 based on the report by Koizumi *et al.* that patients with AGC treated with the S-1 plus cisplatin regimen had better overall survival (OS) than those treated with S-1 alone (2). However, the treatment results remain unsatisfactory, with the median OS in AGC ranging from only 8 to 16 months (3-8). Recent studies showed that the combination regimens of 5-FU, platinum, and taxane are effective for AGC (9-11).

Itraconazole is a common antifungal agent. In 1999, Okumura *et al.* reported the reversal of P-glycoprotein (P-gp, also known as multidrug resistance-1 or ATP-binding cassette sub-family B member 1), mediated resistance of cancer cells by itraconazole (12). In 2007, Chong *et al.* reported that itraconazole inhibited angiogenesis. Since then, preclinical and clinical studies, including our previous investigations, have shown that itraconazole has anticancer activity (13, 14). Itraconazole inhibited the proliferation of gastric cancer cells and cancer-associated fibroblasts *in vitro*, and combination chemotherapy with 5-FU appeared to lead to favourable outcomes (15, 16).

Surgical resection of initially unresectable disease after chemotherapy yields longer survival. We have reported the potential survival benefit of conversion surgery following combination chemotherapy with itraconazole among patients with initially unresectable pancreatic cancer (17). Furthermore, several reports showed promising results of conversion surgery of initially unresectable gastric cancer (18, 19). Therefore, we aimed to investigate the efficacy of combination

therapy with S-1, oxaliplatin, nab-paclitaxel and itraconazole for advanced or recurrent gastric cancer. The dose and schedule of the regimen were determined according to previous clinical studies (10, 17, 20-23).

Patients and Methods

Study design and patients. This phase II trial enrolled patients with unresectable gastric cancer who were diagnosed at a tertiary hospital and referred to our clinic. Patients were enrolled between June 2015 and December 2017 according to the following eligibility criteria: Pathologically diagnosed adenocarcinoma of HER2-negative gastric or gastroesophageal cancer, age 20-80 years, Eastern Cooperative Oncology Group Performance Status score of 0 or 1, and adequate organ function. Tumour staging was according to the Union for International Cancer Control pTNM staging guidelines, eighth edition (24).

This study was approved by the Institutional Review Board of Meiwa Hospital and was registered at UMIN Clinical Trials Registry (UMIN000021340). All patients provided written informed consent prior to participation.

Treatment regimen and assessments. The regimen comprised 160 mg/m^2 nab-paclitaxel *i.v.* on day 1, 100 mg/m^2 oxaliplatin *i.v.* on day 1, 60 mg/m^2 S-1 orally on days 1-3, and 400 mg itraconazole orally on days −2 to 2, repeated every 2 weeks for 6-8 cycles. The clinical response to chemotherapy was evaluated according to the Response Evaluation Criteria in Solid Tumor (RECIST), version 1.1 (25), and the eligibility for conversion surgery was decided by a multidisciplinary treatment team.

The primary endpoint was OS, defined as the time from the first itraconazole administration to death. The secondary endpoints were clinical and pathological response rate, rate of R0 resection (no visible residual tumour) after conversion surgery, and adverse events. Adverse events during the first concurrent administration of itraconazole and chemotherapy were graded according to the National Cancer Institute Common Toxicity Criteria, version 4.0 (26).

Statistical analysis. OS was analysed using the Kaplan–Meier method. The target sample size of 23 was calculated as follows. The threshold value of 50% OS at 1 year was based on previously published data of SPIRITS (2). An expected 1-year OS rate of 80% was assumed by previous clinical trials treating patients with combination chemotherapy and itraconazole, in which hazard ratios of 0.194 and 0.27 were reported for lung and ovarian cancer, respectively (22, 27). Under these assumptions, 21 patients were required to achieve a one-sided significance level of 5% with a power of 90%. Factoring in a 10% dropout rate, we set a target sample size of 23 patients. All statistical analyses were performed using XLSTAT 2014 (Addinsoft, Paris, France).

Results

Patient characteristics. In total, 23 patients with a median age of 68 years (range=40-80 years) were treated with combination chemotherapy and itraconazole at our hospital. Of them, 15 (65%) were male and 8 (35%) female. The patient characteristics are summarized in Table I. Most primary tumours (n=21, 91%) were located in the stomach and most patients (n=18, 78%) had stage IV disease, and five (22%) patients developed recurrence after adjuvant S-1 therapy. All the patients had distant metastases.

Table I. *Patient characteristics (June 2015-Dec 2017).*

	Value
Patients, n	
Total	23
Age	
Median (range), years	68 (40-80)
Gender, n (%)	
Male	15 (65%)
Female	8 (35%)
Location of primary tumour, n (%)	
Stomach	21 (91%)
Gastro-oesophageal junction	2 (9%)
Histology, n (%)	
Poor	7 (30%)
Tub1	3 (13%)
Tub2	7 (30%)
Sig	3 (13%)
Unknown	3 (14%)
Stage, n (%)	
IV	18 (78%)
Recurrence	5 (22%)
Location of metastases or dissemination	
Liver	11 (48%)
Peritoneum	9 (39%)
Lung	2 (9%)
Bone	2 (9%)

Toxicities and compliance. The adverse events during treatment are shown in Table II. One patient developed febrile neutropenia in the seventh cycle but recovered without hospitalization. One patient had grade 4 gastric haemorrhage, but not fatal.

Compliance with the regimen is shown in Table III. In total, 13 (57%) patients received eight cycles or more. Of them, 20% dose reduction was conducted in six (35%) patients due to grade 2 peripheral sensory neuropathy. However, no treatment delays occurred. The median number of treatment cycles among those who underwent conversion surgery (n=12) and those who did not (n=11) was 7 (range=5-10 cycles) and 10 (range=6-14 cycles), respectively.

Efficacy. The overall response rate was 70%, as evaluated according to the RECIST 1.1 criteria (Table IV). The median OS of the overall population was 24 months (95% confidence interval=21 months-not reached), and the 1-year OS rate was 95% (95% confidence interval of 67-98%), which met the expected 1-year OS rate of this trial.

Table II. *Adverse events ≤8 cycles in 23 patients according to National Cancer Institute Common Toxicity Criteria, version 4.0 (26).*

Toxicity	Adverse event	Grade 3	Grade 4	Grade 3/4
Hematological	Leukopenia	3	1	4 (17%)
	Neutropenia	2	2	4 (17%)
	Anemia	0	0	0
	Thrombocytopenia	0	0	0
		Grade 2	Grade 3	Grade 4	Grade 2-4
Non-hematological	T-Bil	0	0	0	0
	AST	0	0	0	0
	ALT	0	0	0	0
	Creatinine	0	0	0	0
	Peripheral neuropathy	6	0	0	6 (26%)
	Gastric hemorrhage	0	0	1	1 (4%)

T-Bil: Total bilirubin; AST: aspartate aminotransferase; ALT: alanine aminotransferase.

Table III. *Compliance with the regimen.*

		Conversion surgery		
Cycles completed	No. of patients	Yes	No	Total
>8	1	7	8	35%
8	3	2	5	22%
7	3	1	4	17%
6	3	1	4	17%
5	1	0	1	4%
4	1	0	1	4%

Table IV. *Efficacy of the regimen.*

Response	Response rate
CR	2 (9%)
PR	14 (61%)
SD	3 (13%)
PD	4 (17%)
CR+PR	16 (70%)

CR: Complete response; PR: partial response; SD: stable disease; PD: progressive disease.

In total, 12 (52%) patients underwent conversion surgery; their operative and pathological outcomes are shown in Table V. Among these 12 patients, eight (67%) patients obtained R0 resection, and two (9%) achieved pathological complete response. The OS was significantly different between those who did and did not undergo conversion surgery (31 *vs.* 22 months; log-rank p=0.033; Figure 1).

Table V. *Operative and pathological outcome of 12 patients.*

	Number of patients
Type of surgery	
Total gastrectomy	8
Subtotal gastrectomy	4
Lymph node dissection	
D0	1
D1	3
D2	7
D3	1
yTNM	
yT	
Ia	2
1b	1
2	0
3	2
4a	5
4b	2
yN	
N0	6
N1	3
N2	2
N3	1
yM	
M0	9
M1	3
Resection margin	
R0	8
R1	3
R2	1
Pathological effect	
Grade 1a	6
Grade 1b	1
Grade 2a	2
Grade 2b	1
Grade 3 (pCR)	2

pCR: Pathological complete response.

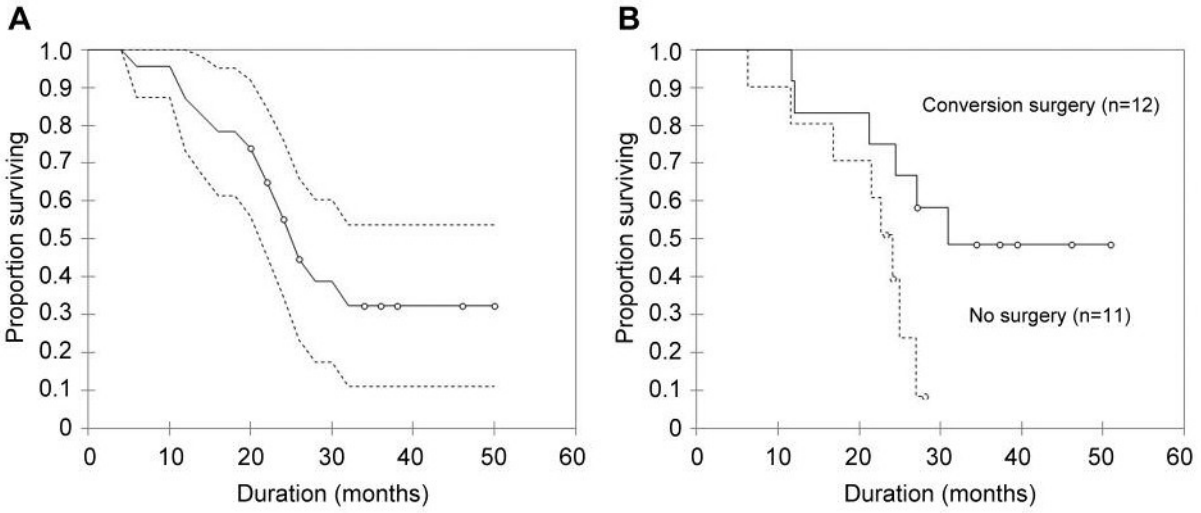


Figure 1. *Kaplan–Meier analysis of overall survival (OS) after induction of chemotherapy with itraconazole. A: OS of the overall population. The 1-year OS was 95% (95% confidence interval=67-98%), and the median OS was 24 months (95% confidence intervaI=21 months-not reached). B: OS according to conversion surgery. The median OS was significantly longer among patients who underwent conversion surgery than those who did not [31 months (95% confidence intervaI=24 months- not reached) versus 22 months (95% confidence intervaI=15-25 months; log-rank p=0.033].*

Discussion

Unresectable AGC has an unfavourable prognosis; however, previous studies have shown a survival benefit in those who underwent conversion surgery following chemotherapy (18, 19, 28-31). Sohda *et al*. reported that surgical intervention was an independent prognostic factor in AGC (32). The key to achieving conversion surgery is administration of an effective chemotherapy regimen. In the study by Ohnuma *et al*., 27% of patients with AGC who had peritoneal metastasis treated with the combination of docetaxel, cisplatin, and S-1 achieved down-staging and underwent curative surgery, resulting in an excellent median OS of 28 months (9). Oxaliplatin has lower risk of renal function than cisplatin, and oxaliplatin-based chemotherapy has been found to be particularly effective for patients with AGC intolerant to cisplatin (33). Sato *et al*. reported that a chemotherapy regimen comprising docetaxel, oxaliplatin, and S-1 for unresectable AGC achieved a median OS of 19.6 months (10). Specifically, the regimen comprised docetaxel at 50 mg/m^2 and oxaliplatin at 100 mg/m^2 on day 8, with S-1 at 80 mg/m^2 on days 1-14 of a 21-day cycle (10).

Nab-paclitaxel has shown to improve the migration of simultaneously administered anticancer drugs. Von Hoff *et al*. reported that compared with gemcitabine alone, the intratumoural concentration of gemcitabine in human pancreatic cancer xenografts increased by 2.8-fold in those treated with gemcitabine plus nab-paclitaxel (34). Yardley *et al*. also reported that nab-paclitaxel achieved a 33% higher tumoural uptake relative to paclitaxel (35). Moreover, Watson *et al*. reported that the combination chemotherapy of nab-paclitaxel, oxaliplatin, and 5-FU was an effective and promising regimen for resectable gastric cancer (11).

Previous studies showed that anti-angiogenic agents, such as bevacizumab, ramucirumab, sunitinib, regorafenib, and apatinib, were effective in gastric cancer (36). Kim *et al*. reported that docetaxel, capecitabine, and cisplatin in combination with bevacizumab improved OS to 38.6 months (37). There have been various investigations on the anti-angiogenic effect of itraconazole, and several clinical trials have shown that the addition of itraconazole to cytotoxic regimens yields a survival advantage in lung, ovarian, breast, pancreatic, and biliary tract cancer (14). In our previous studies, patients with various types of advanced cancer were treated with a regimen including nab-paclitaxel, oxaliplatin, S-1, and itraconazole (17, 20-23). The current regimen was determined according to these previous studies. The median OS of patients who received standard S-1 plus cisplatin regimens without conversion surgery was 13 months (2). In the current trial, a favourable median OS of 24 months (95% confidence intervaI=21 months-not reached) was achieved. There was a significant difference in OS between patients who did and did not undergo conversion surgery (31 *vs*. 22 months) but even patients who did not undergo conversion surgery had favourable survival outcomes.

Aside from its anti-angiogenesis properties, itraconazole also has anticancer activities, including reversing chemoresistance mediated by P-glycoprotein, modulating the signal transduction pathways of hedgehog, and mechanistic targeting of rapamycin and WNT/β-catenin in cancer cells (14). Although the primary

mechanism for the anticancer activity of itraconazole is still unclear, it has been reported to interfere with cholesterol transport (38-40). An *in vitro* study using a Min mouse model of familial adenomatous polyposis showed that itraconazole significantly suppressed intestinal polyp formation. Furthermore, the effects of itraconazole on transcriptional activities may be exerted partly through inhibition of intracellular cholesterol trafficking (41).

The chemotherapy regimen comprising nab-paclitaxel, oxaliplatin, S-1, and itraconazole is effective for conversion surgery for initially unresectable AGC. The regimen yielded a high response rate of 70% and a conversion surgery rate of 52% with acceptable toxicities. Furthermore, it has a survival advantage, with a median OS of 24 months. Collectively, these findings indicate the potential of this chemotherapy regimen for patients with unresectable AGC.

Conflicts of Interest

All Authors have no conflicts to declare.

Authors' Contributions

HT and TS conceived the study; MS and HT analysed the data and drafted the article; YN collect the data and helped in designing the study; AK revised the article for important intellectual content. All Authors read and approved the final article.

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Case Reports

Adolescent bladder endometriosis initially diagnosed as premenarchal disease: a case report and review of the literature

A. Fujishiro[1], H. Tsubamoto[1], Y. Higuchi[2], A. Kanematsu[2], H. Shibahara[1]

[1] Department of Obstetrics and Gynecology; [2] Department of Urology, Hyogo College of Medicine, Nishinomiya-shi, Hyogo (Japan)

Summary

Only a few cases of premenarcheal endometriosis have been reported in the literature, and bladder endometriosis accounts for 1% of all cases of endometriosis. The authors report the case of an 11-year-old girl who presented with macrohematuria due to bladder endometriosis without apparent menarchal discharge from vagina, and was initially diagnosed as premenarcheal endometriosis. MRI revealed that the lesion penetrated the bladder musculature and infiltrated the anterior wall of the lower uterine segment. She underwent transurethral biopsy and hemostasis, which revealed bladder endometriosis. After three months, she again presented with macrohematuria without exacerbation of bladder endometriosis. Periodic macrohematuria occurred after her basal body temperature dropped. Transrectal ultrasound revealed a fistula in the bladder and the internal cervical os. This is the first report of bladder endometriosis without menarchal discharge, which should be kept in mind when considering differential diagnosis of adolescent macrohematuria without apparent menarche.

Key words: Endometriosis; Bladder; Premenarche; Adolecent.

Introduction

Endometriosis is a chronic gynecologic disorder related to the presence of endometrial glandular and stromal tissue outside of the uterine cavity. It is often associated with dysmenorrhea, dyspareunia, chronic pelvic pain, and infertility. Endometriosis affects approximately 10% of women of reproductive age, which leads to infertility in 30% [1]. It is unusual in adolescents, and only a few cases of endometriosis occurred prior to menarche [2, 3]. Endometriosis of the urinary system is a rare condition, representing 1.2% of all endometriosis cases, of which 84% are in the bladder [4]. The usual site of bladder endometriosis is the dome of the bladder, and 23% of the patients underwent cesarean section [5]. The authors report a case of bladder endometriosis that presented with periodic macrohematuria without menstruation. Informed consent was obtained from the child's parent.

Case Report

An 11-year-old premenarcheal girl presented to a pediatrician because of macroscopic hematuria. She had mild developmental disorder, but her family medical history was unremarkable. Ultrasound examination revealed an elevated lesion, 15 mm in diameter, in the posterior wall of the bladder, and she was referred to a urologist in the present hospital. MRI revealed that the lesion penetrated the bladder musculature and infiltrated the anterior wall of the lower uterine segment (Figure 1), with no abnormality detected in the uterus, vagina, or ovary. In addition, the endocervical gland was developed appropriately for her age, and the authors identified endometrial thickness as physiological change. The preoperative diagnosis was bladder malignancy, with invasion of the anterior wall of the lower uterine segment. Cystoscopy showed that the elevated lesion was continuous to the bladder mucosa in the bladder trigone. No obvious abnormality was noted in either the ureteral orifice or the urethra (Figure 2). Transurethral biopsy and hemostasis were performed. The biopsy specimen revealed bladder endometriosis (Figure 3). The cervix and the vagina were normal. Resistance was felt upon the insertion of the uterine probe into the cervical canal. Three months later, she presented with macrohematuria, but cystoscopy and MRI did not reveal any exacerbation of the bladder endometriosis. Her serum estradiol level was 42 pg/ml, and she presented with macrohematuria after her basal body temperature dropped. However, because of the absence of vaginal bleeding, as in menstruation, transrectal ultrasound was performed. The bladder was invaginated into the internal os, and menstrual blood presumably passed through the fistula between the lower uterine segment and the bladder (Figure 4). The patient has been followed for future repair surgery.

Discussion

Many theories have been proposed to explain the pathophysiology of endometriosis, including retrograde menstruation, metaplasia of the multipotential coelomic epithelium, and embryonic Müllerian rests. Recently, Brosens *et al.* reported that neonatal uterine bleeding

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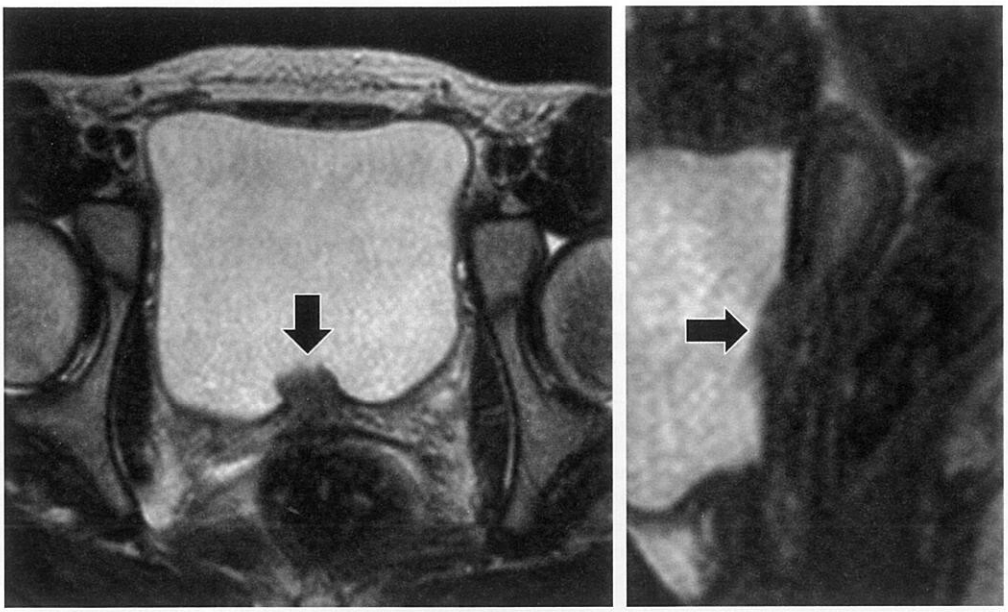


Figure 1. — Magnetic resonance imaging of the intravesical tumor.
(A) Axial, T2-weighted image. (B) Sagittal, T2-weighted image. Arrow indicates a poorly marginated tumor in the lower uterine segment.

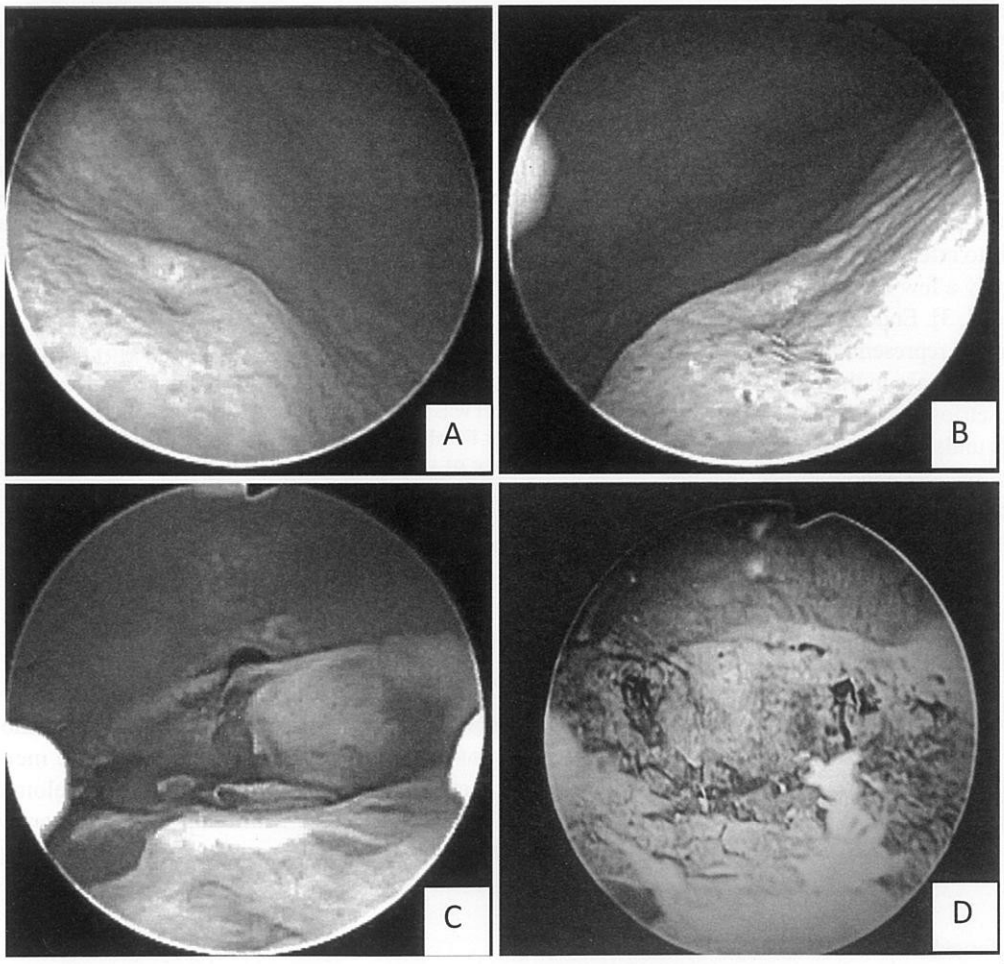


Figure 2. — Cystoscope.
(A) Right orifice. (B) Left orifice. (C) Bleeding from the elevated lesion in the bladder trigone. (D) After biopsy and hemostasis.

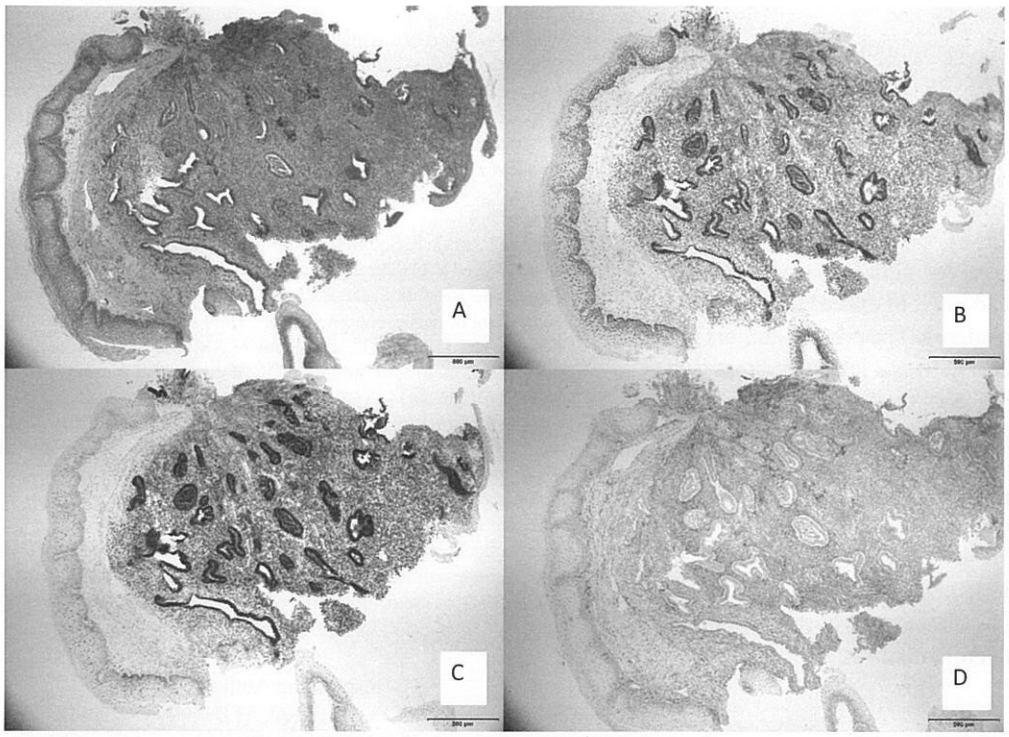


Figure 3. — Pathology of specimen obtained by transurethral resection.
(A) Endometrial glandular cells with squamous metaplasia can be seen under the mucosal epithelium of the bladder (hematoxylin and eosin). Immunohistochemistry shows the endometrial glands stained with both (B) estrogen receptors and (C) progesterone receptors. (D) The endometrial-like stroma is immunopositive for CD10. Bar = 500 nm.

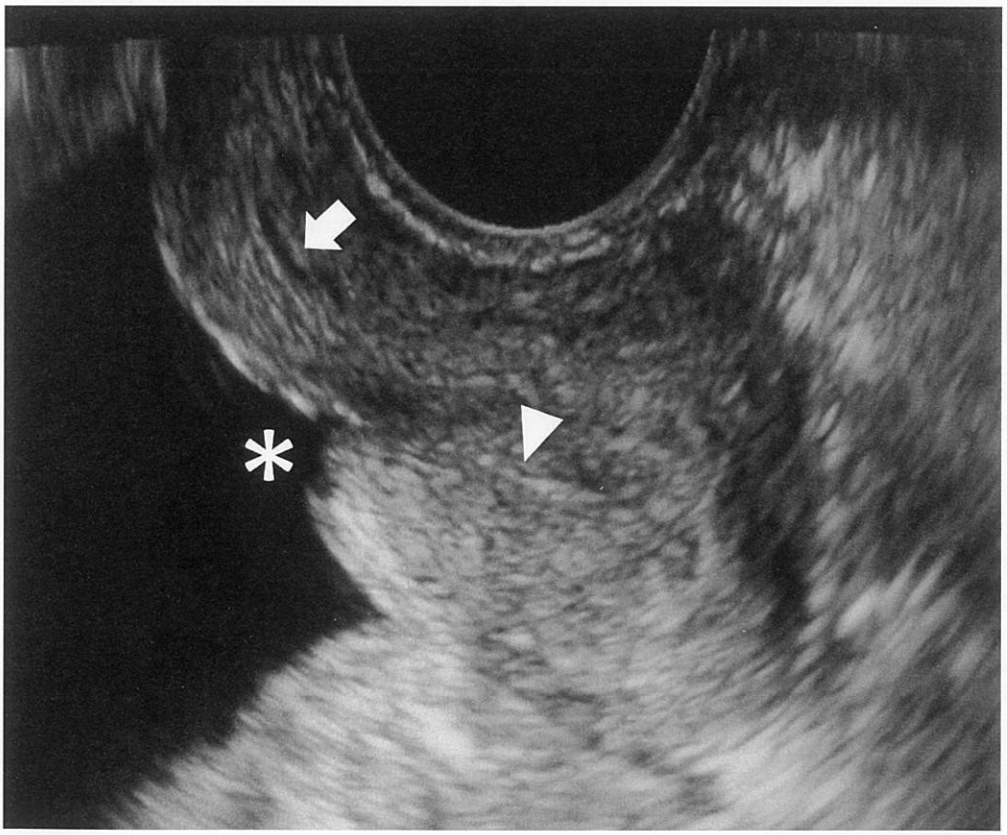


Figure 4. — Fistula between the bladder and the lower uterine segment.
Arrow indicates the cervical canal, arrowhead indicates the endometrium, and asterisk indicates the invaginated bladder toward the lower uterine segment.

caused pelvic endometriosis [6].

The present patient had menses, in the form of macrohematuria, which implicated occlusion of the cervical canal. She might have had congenital cervical dystrophy or congenital vesicouterine fistula. In the literature, however, congenital vesicouterine fistula is rare and generally associated with genital tract abnormalities derived from the Müllerian ducts or urogenital sinus [7, 8].

The use of oral contraceptives pills or progestins to reduce macrohematuria and to prevent exacerbation of bladder endometriosis was considered. The present authors plan to excise the remaining endometriosis lesion and repair the uterus and bladder after the growth of the uterus [9]. The use of OC or progestins was delayed to allow the growth of the uterus.

There are a few case reports of pelvic pain before menarche that were confirmed to be lesions consistent with endometriosis on laparoscopic evaluation [2]. In this case, the girl was referred to the clinic because of macrohematuria; thus, endometriosis must be considered in the differential diagnosis of adolescent macrohematuria.

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Corresponding Author:
A. FUJISHIRO, M.D.
Department of Obstetrics and Gynecology
Hyogo College of Medicine
1-1 Mukogawa-cho, Nishinomiya-shi
Hyogo 663-8501 (Japan)
e-mail: akiko149@hotmail.com

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Case report

Pazopanib treatment of a platinum-resistant recurrence of a high-grade Sertoli cell tumor and assessment of the treatment response by FDG-PET/CT: A case report

Kayo Inoue[a,*], Hiroshi Tsubamoto[a], Keiko Ishida-Nisigami[b], Yoshitaka Torii[a], Seiichi Hirota[a]

[a] Hyogo College of Medicine, Department of Obstetrics and Gynecology, Japan
[b] Hyogo College of Medicine, Department of Clinical Pathology, Japan

ABSTRACT

Ovarian Sertoli cell tumors (SCTs) are rare sex cord tumors (Oliva et al., 2005). The standard treatment for high-grade SCTs is surgery followed by platinum-based chemotherapy. Although platinum-based chemotherapy is also an option for recurrent SCTs (Sigismondi et al., 2012), there is no established chemotherapy regimen for platinum-resistant recurrent SCTs. The effectiveness of pazopanib in treating epithelial ovarian cancer has recently been reported (du Bois et al., 2014; Pignata et al., 2015). In the case described herein, pazopanib was used to treat the platinum-resistant recurrence of a high-grade Sertoli cell tumor, and the response was evaluated by 18F-fluoro-deoxyglucose positron emission tomography (FDG-PET)-computed tomography (CT). Written informed consent to reporting the case was obtained from the patient.

1. Case report

A 32-year-old, gravida 1 woman with Marfan syndrome was referred to our institution owing to rapidly growing pelvic tumors. Imaging results suggested disseminated ovarian cancer. Laparotomy revealed two solid yellow tumors in the ovaries (12 cm and 8 cm in diameter, respectively) and widespread bulky disseminations mainly in the Douglas pouch and the peritoneum under the right diaphragm. Debulking procedures including bilateral salpingo-oophorectomy were performed, but most of the disseminated lesions remained. The pathology report was delayed because of difficulty in making a final diagnosis. The patient underwent 2 cycles of adjuvant chemotherapy with paclitaxel and carboplatin, but the disease progressed. The final diagnosis was made by the central pathology review board and was high-grade SCT. The Ki-67 index was 40%, and immunohistochemical staining of CD99 was negative (Fig. 1A–C). The chemotherapy regimen was immediately changed to bleomycin, etoposide, and cisplatin (BEP). After 5 cycles of BEP, CT showed a complete response. Second-look surgery revealed no macroscopic disease, and pathological examination of multiple biopsies and material obtained from peritoneal stripping of the right diaphragm showed no active microscopic residual tumors. Two additional cycles of BEP were administered.

The patient remained disease-free for the next 5 months, at which point she complained of abnormal genital bleeding, a dark-colored umbilicus, and upper abdominal pain requiring non-steroidal anti-inflammatory drugs (NSAIDs). Transvaginal ultrasonography revealed bulky masses and ascites in the pelvis and umbilicus indicative of a metastatic tumor. FDG-PET/CT findings suggested tumor recurrence in the pelvis, the umbilicus, and the peritoneum under the right diaphragm. Endometrial biopsy revealed metastases of the SCT in the endometrial cavity (Fig. 1D).

Owing to limited treatment options, and after discussion with the patient and her family, pazopanib was chosen for disease control. Pazopanib (800 mg) was administered orally to the patient. One month of pazopanib treatment relieved the upper abdominal pain, eliminating the need for NSAIDs. The skin lesion in the umbilical tumor had mostly disappeared 1 month after initiating treatment (Fig. 2), and genital bleeding had stopped. The largest tumor was located under the right diaphragm. As visualized by FDG-PET/CT, the tumor diameter was 7 cm before treatment and 5 cm 1 month after treatment began; the maximum standardized uptake value (SUVmax) at these times was 10.53 and 5.41, respectively (Fig. 3A, B). The diameter of the umbilical tumor was 3 cm and 2 cm in FDG-PET/CT scans before and 1 month after treatment initiation, respectively, and the SUVmax at these times was 9.55 and 4.51, respectively. The mass in the pelvis also decreased in size (Fig. 3C, D).

The side effects of pazopanib were mild appetite loss and changes in hair color. There were no grade 2 or higher hematologic or non-

* Corresponding author.
E-mail address: ka-inoue@hyo-med.ac.jp (K. Inoue).

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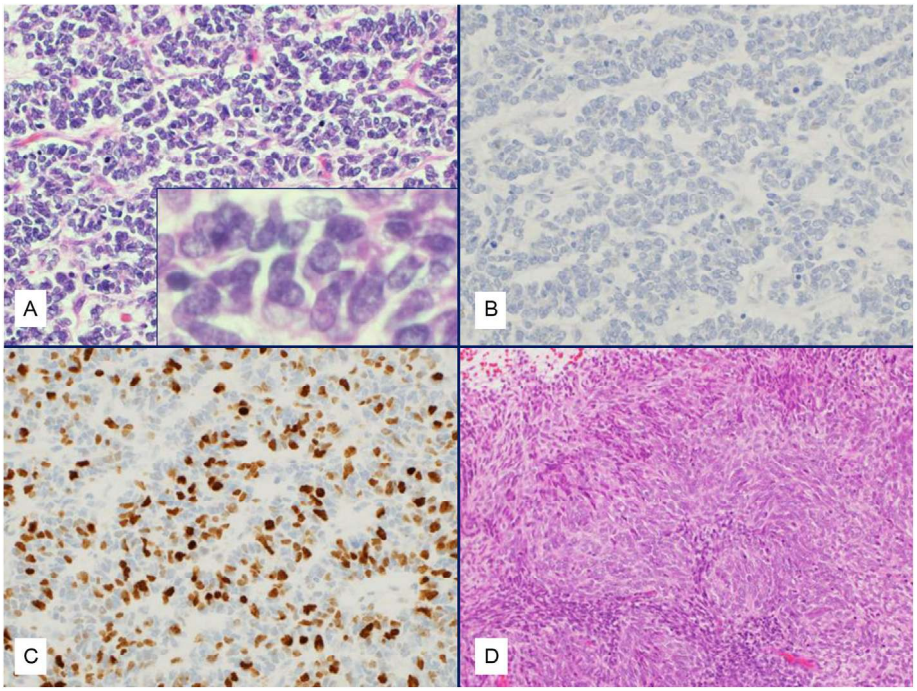


Fig. 1. Microscopic findings of the primary ovarian Sertoli cell tumor (SCT) (A–C). A: Hematoxylin and eosin (H&E) staining, magnification ×20 and ×40 (inset). B: Immunohistochemical staining of CD99 was negative. Magnification ×20. C: Immunohistochemical staining of Ki-67; 40% of the cells were positive. Magnification ×20. D: Microscopic examination of the endometrial biopsy at the time of recurrence shows metastasis of the SCT. H&E, magnification, ×20.

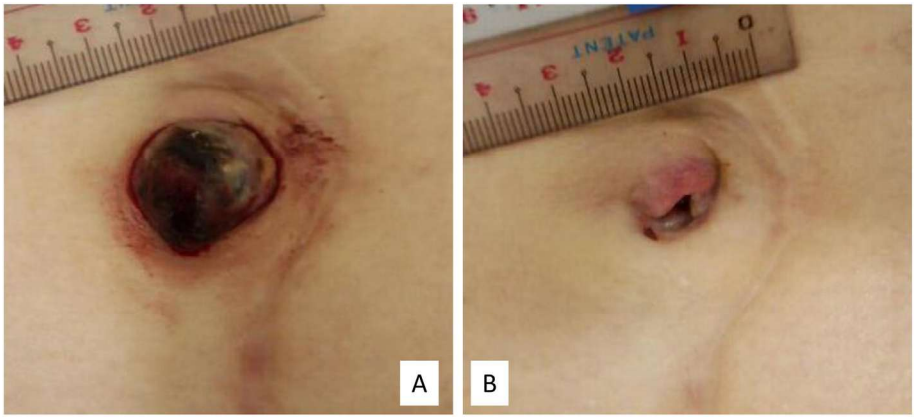


Fig. 2. Gross images obtained before (A) and 1 month after (B) the initiation of pazopanib treatment.

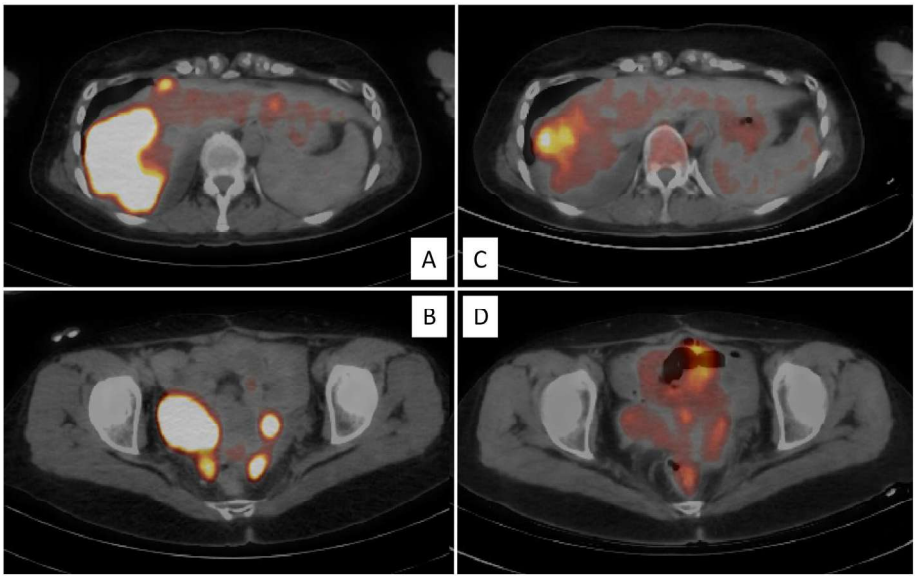


Fig. 3. A: 18F-fluoro-deoxyglucose positron emission tomography-computed tomography images before (A, C) and 1 month after (B, D) the initiation of pazopanib treatment. A: Before treatment, the largest tumor, which was under the right diaphragm, had an SUVmax of 10.53 and a diameter of 7 cm. B: After treatment, this tumor shrank to 5 cm, and the SUVmax decreased to 5.41. C: The image shows the tumor in the pelvis near the uterus and in the Douglas pouch before treatment. D: After treatment, this tumor had decreased in size.

hematologic toxicities, as assessed according to the Common Terminology Criteria for Adverse Events (version 4.0). A slight rise in blood pressure to 135 mmHg (systolic) was well controlled by administration of an antihypertension drug. CT performed 3 months after initiating pazopanib treatment showed stable disease, as evaluated according to the Response Evaluation Criteria in Solid Tumors (version 1.1). Pazopanib was given for another 2 months, and it was discontinued because of oozing bleeding from the umbilical tumor. The patient received best supportive care for 3 months, and then died of disease.

2. Discussion

SCT is rare sex cord tumor, and high-grade SCT is even more rare. The SCT in this case was highly malignant based on both its pathology and clinical characteristics. Although ovarian SCTs are usually unilateral (Oliva et al., 2005), the SCT in this case was bilateral. Robust mitotic activity is associated with the recurrence of SCTs (Oliva et al., 2005), and the Ki-67 index in this case was 40% (Fig. 1C), which predicted an adverse outcome. Sertoli cells express CD99, but in this case, immunohistochemical staining of CD99 was negative (Fig. 1B), perhaps because expression is related to the degree of differentiation (Gordon et al., 1998). Although BEP chemotherapy was an effective treatment for the initial disease, the patient relapsed within 6 months after BEP treatment. Platinum-based chemotherapy is also an option for recurrent SCTs (Sigismondi, Gynecol Oncol, 2012), there is no established chemotherapy regimen for platinum-resistant recurrent SCTs.

Pazopanib, an oral tyrosine kinase inhibitor, exhibits anti-angiogenic effects by inhibiting signaling by the vascular endothelial receptor, the platelet-derived growth factor receptor, and c-kit. Several studies have demonstrated the efficacy of pazopanib in treating epitherial ovarian cancer (EOC). In a single arm phase 2 trial of recurrent EOC, pazopanib monotherapy yielded serological response in 28% patients (Friedlander et al., 2010). Combination chemotherapy with pazopanib and low dose oral cyclophosphamide demonstrated antitumor activity, which was considered as synergistic anti-angiogenic effect by pazopanib and metronomic chemotherapy (Dinkic et al., 2017). In a randomized phase 2 MITO-11 trial of platinum-resistant EOC, combination with pazopanib and weekly paclitaxel showed 3 month improvement of progression free survival (PFS) over weekly paclitaxel alone (hazard ratio [HR], 0.42; p = 0·0002) (Pignata et al., 2015). In a randomized phase 3 AGO-OVAR16 trial of EOC, maintenance pazopanib following the first-line chemotherapy significantly improved PFS over placebo (HR, 0.77; p = 0.0021) (du Bois et al., 2014). In East Asian patients, however, maintenance pazopanib showed a disadvantage in overall survival (Kim et al., 2018).

However, pazopanib treatment of ovarian SCTs has not been assessed previously. Bevacizumab administration is a treatment option for recurrent granulosa cell tumors according to the guidelines of the National Comprehensive Cancer Network and it might have been one of the treatment options in this case. However, recurrent tumors in the Douglas pouch have a high risk of gastrointestinal perforation in administration of bevacizumab (Cannistra et al., 2007). Therefore, pazopanib was chosen for treatment.

In our case, the patient's symptoms disappeared 1 month after initiating pazopanib treatment. Response assessment to anti-angiogenic therapy by FDG/PET-CT has been performed in tumors other than SCTs (Kayani et al., 2011, Farnebo et al., 2014, Prior et al., 2009;27). The PAZPET-1 trial is evaluating the use of 18F-fluciclatide as a biomarker in platinum-resistant ovarian cancers treated by weekly administration of pazopanib and paclitaxel (PAZPET-1 http:~). In our case, FDG-PET/CT was used to monitor the response to pazopanib in platinum-resistant SCT metastases. The imaging results for the largest tumor showed a 48% reduction in the SUVmax and a 40% reduction in tumor size (diameter of the major axis) after 1 month of pazopanib treatment. This finding suggests that early assessment of the treatment response to pazopanib may predict the effectiveness of the treatment (Young et al., 1999).

3. Conclusion

This is the first report of the effectiveness of pazopanib in treating a platinum-resistant recurrence of a high-grade SCT and of treatment evaluation by FDG-PET/CT. Pazopanib may be a viable treatment option for platinum-resistant high-grade SCTs that recur and metastasize.

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BMC Cancer

CASE REPORT Open Access

Itraconazole treatment of primary malignant melanoma of the vagina evaluated using positron emission tomography and tissue cDNA microarray: a case report

Kayo Inoue[1], Hiroshi Tsubamoto[1,2*], Roze Isono-Nakata[1], Kazuko Sakata[1] and Nami Nakagomi[3]

Abstract

Background: Primary malignant melanoma of the vagina is extremely rare, with a poorer prognosis than cutaneous malignant melanoma. Previous studies have explored the repurposing of itraconazole, a common oral anti-fungal agent, for the treatment of various cancers. Here, we describe a patient with metastatic, unresectable vaginal malignant melanoma treated with 200 mg oral itraconazole twice a day in a clinical window-of-opportunity trial.

Case presentation: A 64-year-old Japanese woman with vaginal and inguinal tumours was referred to our institution. On the basis of an initial diagnosis of vaginal cancer metastatic to the inguinal lymph nodes, we treated her with itraconazole in a clinical trial until the biopsy and imaging study results were obtained. During this period, biopsies were performed three times, and ^{18}F-fluoro-deoxyglucose positron emission tomography (FDG/PET)–computed tomography (CT) was performed twice. Biopsy results confirmed the diagnosis of primary malignant melanoma of the vagina. Imaging studies revealed metastases to multiple sites, including the brain, for which she underwent gamma-knife radiosurgery. During the window period before nivolumab initiation, the patient received itraconazole for 30 days. Within a week of itraconazole initiation, pain in the inguinal nodes was ameliorated. PET–CT on days 6 and 30 showed a reduction in tumour size and FDG uptake, respectively. The biopsied specimens obtained on days 1, 13, and 30 were subjected to cDNA microarray analysis, which revealed a 100-fold downregulation in the transcription of four genes: *STATH*, *EEF1A2*, *TTR*, and *CDH2*. After 12 weeks of nivolumab administration, she developed progressive disease and grade 3 immune-related hepatitis. Discontinuation of nivolumab resulted in the occurrence of left pelvic and inguinal pain. Following re-challenge with itraconazole, the patient has not reported any pain for 4 months.

Conclusion: The findings of this case suggest that itraconazole is a potential effective treatment option for primary malignant melanoma of the vagina. Moreover, we identified potential itraconazole target genes, which could help elucidate the mechanism underlying this disease and potentially aid in the development of new therapeutic agents.

Keywords: Melanoma, Vaginal neoplasm, Itraconazole, Repurposing, Off-use

* Correspondence: tsuba@hyo-med.ac.jp
[1]Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan
[2]Department of Medical Oncology, Meiwa Hospital, Nishinomiya, Hyogo 663-8186, Japan
Full list of author information is available at the end of the article

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Background

Primary malignant melanoma of the vagina is extremely rare and accounts for approximately 5% of vaginal cancers [1]. The prognosis of this condition is very poor compared to the prognosis of malignant melanoma arising from the skin because of the advanced clinical stage at presentation, and a recent report showed that the vagina being the primary site of melanoma is itself an independent prognostic factor [2]. The current standard treatment for unresectable or recurrent melanoma is immune checkpoint inhibitors, while RAF/MEK inhibitors are also used in symptomatic patients with *BRAF* mutation [3]. However, a second-line treatment regimen has not been established, and the development of novel treatments is warranted.

Itraconazole, a common oral anti-fungal agent, exerts antitumor activity by modulating the signal transduction pathways in cancer cells and cancer-associated fibroblasts, and by inhibiting angiogenesis [4]. Several clinical trials that investigated the repurposing of itraconazole as an anticancer agent against various types of cancers have yielded promising results [5–11]. Liang et al. suggested that itraconazole may inhibit melanoma growth by blocking Hedgehog, Wnt, and phosphatidylinositol 3-kinase-mammalian target of rapamycin (PI3K/mTOR) pathways, and showed that itraconazole prolonged survival in an in vivo xenograft mouse model of melanoma [12]. However, to our knowledge, the anti-cancer effect of itraconazole on melanoma has not been investigated in humans. Moreover, vaginal melanoma is a rare condition that is genetically and molecularly different from cutaneous melanoma [13], and it is not known whether itraconazole is effective against vaginal melanoma.

In the present report, we describe a case of metastatic, unresectable vaginal malignant melanoma treated with a 30-day course of oral itraconazole in a clinical window-of-opportunity trial, and an early clinical response was obtained. To the best of our knowledge, this is the first report of a case in which itraconazole was used to treat primary malignant melanoma of the vagina in a human patient, and in which its response and potential targets were evaluated by ^{18}F-fluoro-deoxyglucose positron emission tomography (FDG/PET)–computed tomography (CT) and tissue cDNA microarray, respectively.

Case presentation

A 64-year-old, multiparous Japanese woman was referred to our institution with a 1-year history of abnormal vaginal bleeding and a 2-month history of abnormal vaginal bleeding and left groin pain. Her medical and family history was unremarkable, and she was not taking any medication. A gynaecologic examination revealed a raised, dark-red necrotic lesion, 5 cm in diameter, located on the left lateral wall of the proximal vagina, and a non-pigmented thick tumour in the lower two-thirds of the posterior vagina. The cervix and fornix appeared normal. Her left groin was reddish and swollen, and enlarged inguinal lymph nodes were palpable with tenderness. Her left lower abdomen was oedematous. We performed a biopsy of the vaginal tumour, and arrived at a tentative diagnosis of unresectable vaginal cancer with metastasis to the inguinal lymph nodes.

The patient was informed about a window-of-opportunity clinical trial registered in the University Hospital Medical Information Network (UMIN 000018388), which included oral itraconazole therapy and tumour biopsies before and after itraconazole treatment. Informed consent was obtained from the patient for inclusion in the trial. Thus, on the first day of referral, she was started on 200 mg itraconazole orally twice a day, and we planned to continue this treatment regimen until the biopsy results were obtained and imaging studies were performed.

On day 1 of itraconazole treatment, her serum lactic dehydrogenase was slightly elevated (252 U/L) and squamous cell carcinoma antigen was normal (1.1 ng/mL). Her complete blood count and liver function were normal.

On day 6, FDG/PET–CT findings suggested a vaginal tumour and multiple metastases to the left inguinal, pelvic, and para-aortic lymph nodes, and right foot (Fig. 1a-c). The largest total diameter of the aggregated inguinal lymph nodes was 50 mm, and its maximum standard uptake value (SUVmax) was 19.8.

On day 7, she reported relief from her left inguinal pain. Physical examination revealed that her left inguinal nodes had shrunk and that the oedema had improved.

On day 9, microscopic evaluation of the biopsy sample confirmed the diagnosis of vaginal malignant melanoma, with positive immunohistochemical staining for HMB-45, S-100, and Melan A (Fig. 2).

On day 13, pelvic magnetic resonance imaging (MRI) revealed a thickened vaginal wall (Fig. 1b). We subsequently performed a second vaginal biopsy on the same day. Head MRI performed on day 14 revealed brain metastases. Each of the two brain tumours measured 1 cm in diameter, and the patient experienced no headache or neurological symptoms. On day 23, she underwent gamma-knife radiosurgery for brain metastases.

FDG/PET–CT performed on day 30 showed that the diameter of aggregated inguinal lymph nodes decreased to 38 mm, with an SUVmax of 14.6 (Table 1). Overall tumour response indicated stable disease according to the response evaluation criteria in solid tumours (RECIST) version 1.1 [14]. Genetic testing of the tumour yielded negative results for *BRAF* mutation, and immunohistochemical staining of programmed death-ligand 1 (PD-L1) on day 30 yielded a 20% positivity rate. Therefore,

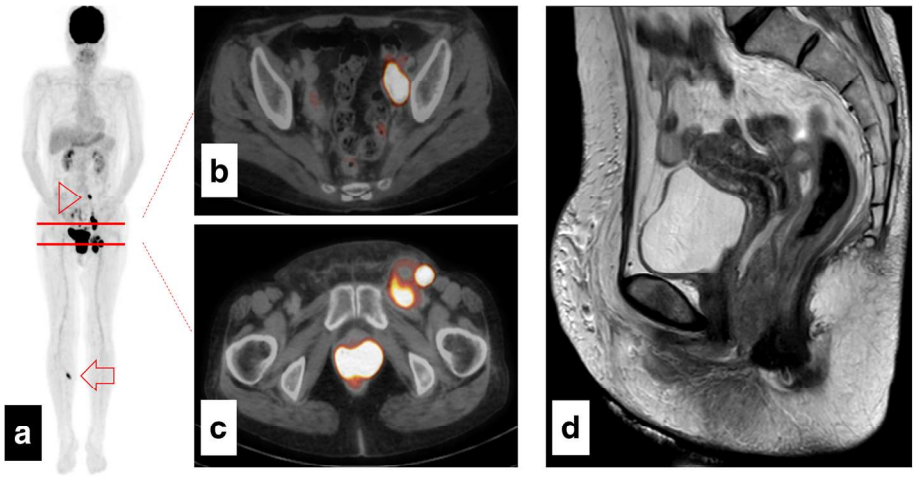


Fig. 1 Images from ^{18}F-fluoro-deoxyglucose positron emission tomography–computed tomography performed 5 days after the initiation of itraconazole (itraconazole) treatment (**a**, **b**, **c**). A magnetic resonance image acquired 12 days after the initiation of itraconazole treatment (**d**). **a** The arrowhead shows paraaortic lymph node metastases, and the arrow shows metastases to the right foot. **b** Pelvic lymph node metastases. **c** Left inguinal lymph node metastases. **d** Thickening of the lower two-thirds of the posterior vaginal wall

nivolumab was chosen for systemic treatment. Itraconazole was discontinued on day 30. A third vaginal biopsy was performed on day 30.

The biopsied specimens obtained on days 1, 13, and 30 were subjected to cDNA microarray analysis. Ribonucleic acid (RNA) was isolated from the specimens and subjected to cDNA microarray analysis using SurePrint G3 Human GE 8x60K v3 Microarray kit (Agilent Technologies, Tokyo, Japan). Altered gene expression was calculated as log2 (messenger RNA [mRNA] levels in itraconazole-treated tissues versus the mRNA level before itraconazole treatment). Genes down-regulated to < 1/32 of baseline levels after 13 days of itraconazole administration are listed in Table 2.

During itraconazole treatment, the patient experienced no side effects and remained pain-free. Itraconazole also relieved her genital bleeding. In the 5 days between stopping itraconazole and starting nivolumab, her left

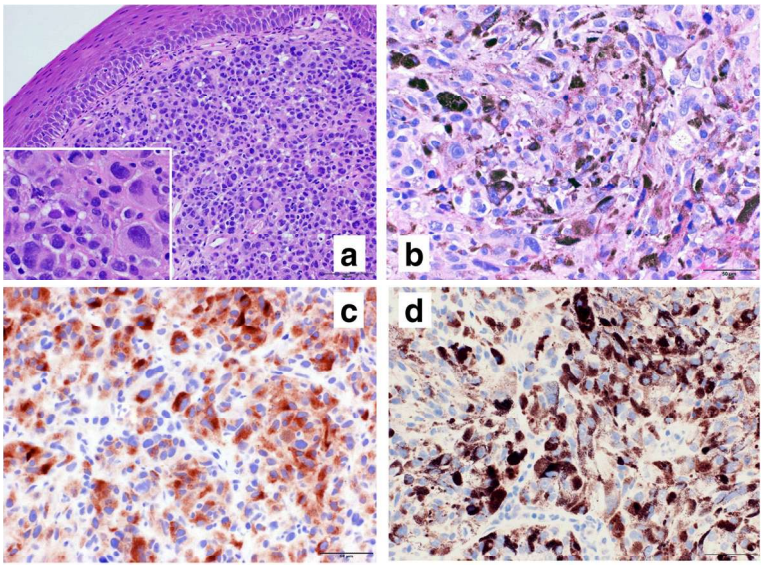


Fig. 2 Histological findings of the vaginal malignant melanoma biopsied on the first day (before itraconazole treatment). **a** and **b** Haematoxylin and eosin staining. **a** Nested tumour cells can be observed under the normal squamous epithelium lining; the tumour cells show pleomorphism, multinuclei, bizarre nuclei, prominent nucleoli, and mitosis (inset). **b** Some tumour cells contain fine melanin granules. **c** Immunostaining with anti-Melan A antibody; most tumour cells shows strong cytoplasmic staining with anti-Melan A antibody. **d** Immunostaining with programmed death-ligand 1 (PD-L1) antibody (28–8) shows membranous and cytoplasmic staining in 20% of tumour cells

Table 1 ^{18}F-fluoro-deoxyglucose positron emission tomography–computed tomography findings

Location		Day 6 SUVmax	Day 30 SUVmax
Vagina		28	23
Lymph nodes (short axis)	Left pelvis	22.7	11.7
	Left inguinal	19.8	14.6
Right foot		9	7.4

The SUVmax levels of the primary vaginal tumour and metastatic tumours on day 6 and day 30 were compared. After treatment, SUVmax decreased in all the four areas. SUVmax, maximum standard uptake value

inguinal nodes got enlarged and her left lower abdomen became oedematous, causing mild pain.

Initiation of nivolumab (3 mg/kg, repeated every 2 weeks) gradually improved her symptoms; however, the primary vaginal tumour increased in size after 10 weeks. After 12 weeks of nivolumab administration, the patient developed grade 3 immune-related hepatitis with elevated alanine transaminase (ALT; 194 U/l) but no bilirubin elevation. We discontinued nivolumab, and immediately administered 1 mg/kg of methylprednisolone. After 6 days, her liver enzyme levels promptly decreased to a grade 1 level. She developed left inguinal and left pelvic pain, which was treated with diclofenac rectal suppositories. FDG/PET–CT showed growth of the primary vaginal tumour and increased 18-FDG uptake in both the metastatic lymph nodes and the primary vaginal tumour. The patient was administered itraconazole, and her pain diminished the next day. During the 6-week steroid tapering, her ALT increased to 408 U/l, and 2 mg/kg of methylprednisolone was re-administered. After 3 months of itraconazole re-challenge, the primary vaginal tumour size gradually increased. However, a CT scan revealed that metastatic lymph nodes were stabilized, and no new metastatic lesions were found. Head MRI revealed remission of brain metastases. She has not reported any pain in the 4 months after the itraconazole re-challenge.

Table 2 The results of tissue cDNA microarray analysis

	Pre-treatment	Day 13		Day 30	
Gene symbol	Signal	Signal	Log2 ratio	Signal	Log2 ratio
STATH	14,257	50	−8.16	275	−5.7
EEF1A2	3329	16	−7.7	3847	0.21
TTR	164,093	790	−7.7	75,548	−1.12
CDH2	3933	37	−6.72	1716	−1.2
FGF9	5098	50	−6.66	792	−2.69
WNT11	1493	18	−6.38	77	−4.29
MCHR1	1183	17	−6.14	3568	1.59
GNAO1	1759	26	−6.07	1325	−0.41
HS6ST2	1019	15	−6.06	2396	1.23
CA8	1510	37	−5.34	4297	1.51
PNPLA3	11,614	344	−5.08	16,371	0.5
AQP7	5427	165	−5.04	20	−8.12

mRNA was isolated from tissues biopsied before treatment, and 13 and 30 days after the initiation of itraconazole. Genes down-regulated to < 1/32 of baseline levels after 13 days of itraconazole administration are listed in the table

Discussion and conclusions

This case report provides two new insights. Firstly, to our knowledge, this is the first study to report a clinical response achieved by itraconazole treatment of primary malignant melanoma, and to evaluate the response using PET–CT. Secondly, the biopsied specimens were analysed by cDNA microarray, providing information on changes in gene expression in response to itraconazole treatment.

Itraconazole treatment resulted in an early clinical response. The patient's left inguinal lymph nodes were a useful target for visual and palpatory evaluation. Within a week of the first itraconazole challenge, her inguinal nodes were smaller on palpation, and her pain was relieved. She discontinued itraconazole after a window period designed in the clinical trial, and subsequently experienced left inguinal pain and oedema with tumour growth. The rapid progression after cessation of itraconazole appeared to be a 'flare-up' phenomenon, which has been reported after discontinuation of tyrosine kinase inhibitors [15, 16]. To monitor the response to itraconazole, we used FDG/PET–CT. Imaging of the aggregated inguinal lymph nodes showed a 24% reduction in tumour size and a 26% reduction in the SUVmax. Because itraconazole was found to be effective in this patient in a window-of-opportunity trial, it was used for treatment, at a lower cost and with minimal side effects, after discontinuation of nivolumab because of progressive disease and immune-related adverse events.

Malignant melanoma is highly vascular, and expression of vascular endothelial growth factor and its receptors are associated with poor prognosis [17, 18]. A recent prospective study investigating the repurposing of propranolol as an anti-angiogenesis agent showed improved disease-free survival among propranolol-treated patients with melanoma [19]. Itraconazole also possesses anti-angiogenetic activity. In 2007, Gupta et al. screened US Food and Drug Administration (FDA)-approved drugs, and identified itraconazole as a promising anti-angiogenic agent [20]. Therefore, itraconazole might inhibit the growth of melanoma cells by inhibiting angiogenesis, as well as melanoma-associated fibroblasts [21, 22].

A previous study investigated the anti-melanoma effect of itraconazole in vitro and in vivo in mice [12], and the results indicated that itraconazole suppresses the Hedgehog, Wnt, and PI3K/mTOR signalling pathways in melanoma cells. These signalling alterations were also demonstrated in cervical cancer CaSki cells treated with

itraconazole [23]. In the present study, cDNA microarray analysis did not reveal any effects of itraconazole on Hedgehog or WNT/β-catenin pathways. Itraconazole interferes with multiple pathways in both cancer cells and the surrounding stromal cells, and a variety of effects on different types of cancer cells have been previously reported [4]. Itraconazole inhibited angiogenesis by inhibiting AKT (protein kinase B)/mTOR signalling in human umbilical vein endothelial cells, which was a result of both aberrant mitochondrial metabolism and blockage of cholesterol trafficking [24]. Using cell lines derived from vaginal melanoma and other malignancies, we found that itraconazole inhibited cholesterol transport in cancer cells (unpublished data).

In the present case, tissue cDNA microarray analysis revealed intriguing results. Transcriptions of four genes (*STATH, EEF1A2, TTR*, and *CDH2*) were down-regulated 100-fold. The *STATH* gene encodes statherin, *EEF1A2* encodes eukaryotic translation elongation factor 1 alpha 2, *TTR* encodes transthyretin, and *CDH2* encodes N-cadherin. These results differ markedly from those of Liang et al. in melanoma cells [12], and the downregulation of these four genes by itraconazole treatment has not been reported in previous preclinical and clinical reports [4]. This might be because our study was conducted in a human patient, and because the molecular interactions in vaginal melanoma might be different from those in cutaneous melanoma [13]. We found that itraconazole inhibited growth of vaginal melanoma cells in vitro using a cell line, but inhibition of Hedgehog, Wnt, or PI3K/mTOR pathways was not observed (unpublished data). Although the precise mechanism underlying the anti-tumour activity of itraconazole in this patient is unknown, the tissue cDNA microarray did reveal transcript alterations in melanoma cells as well as the surrounding microenvironment, providing clues to the mechanisms of itraconazole in human tissue.

Statherin has been reported to play a role in oral health, including putative protective activity against carcinogenesis [25]. Expression of statherin RNA or protein has not reported in any other organ in healthy humans [26], and, to the best of our knowledge, there have been no reports of statherin expression in any the other malignancy except oral cancer. Plitidepsin, which targets EFF1A2, has been studied in two clinical trials conducted in patients with melanoma [27, 28]. Duanmin et al. reported that EEF1A2 is highly expressed in pancreatic ductal adenocarcinoma, and is associated with lymph node metastasis [29]. It is not clear from the present case if these previous findings are applicable to vaginal malignant melanoma; further studies are needed to investigate this. A previous study on the use of serum biomarkers for the early detection of malignancy showed that transthyretin levels were elevated in melanoma as well as ovarian, endometrial, and lung cancer; however, the underlying mechanisms through which transthyretin is involved in these cancers remain unknown [30–33]. The transition from E-cadherin to N-cadherin is an essential step in the migration and invasion of cancer cells, including melanoma cells [34]. In vitro transfection of melanoma cells with small interfering RNA targeting the N-cadherin gene resulted in a decrease in matrix metalloproteinase-2 and -9 activity, and inhibited invasion [35].

In conclusion, itraconazole was effective in a patient with primary malignant melanoma of the vagina. The early response to itraconazole treatment was evaluated with FDG/PET–CT, and lymph node metastases were well-controlled. cDNA microarray analysis of the biopsied tissue of the vaginal tumour revealed down-regulation of genes that might be the targets of itraconazole. The interaction between itraconazole and these genes, and the effects of these interactions warrant further investigation in future studies.

Abbreviations

AKT: Protein kinase B; ALT: Alanine transaminase; CT: Computed tomography; FDG/PET: 18F-fluoro-deoxyglucose positron emission tomography; MRI: Magnetic resonance imaging; mRNA: Messenger ribonucleic acid; mTOR: Mammalian target of rapamycin; PD-L1: Programmed death-ligand 1; PI3K: Phosphatidylinositol 3-kinase; SUVmax: Maximum standard uptake value

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Availability of data and materials

All data presented in the manuscript are available from the corresponding author upon reasonable request.

Authors' contributions

KI, HT, and KS wrote the manuscript and participated in the analysis and interpretation of the data. RIN participated in the collection and interpretation of the case data. NN participated in the interpretation of the data. All authors participated in drafting and revising the paper, and approved the final manuscript.

Ethics approval and consent to participate

This study was a part of a clinical trial titled 'Prospective evaluation of the molecular effects of itraconazole as an anti-cancer agent: a window of opportunity study', which was approved by the institutional review board of Hyogo College of Medicine (No. 282). Authors obtained written informed consent and publication consent from the patient.

Consent for publication

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the editor of this journal.

Competing interests

The authors declare that they have no competing interests.

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Author details
[1]Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan. [2]Department of Medical Oncology, Meiwa Hospital, Nishinomiya, Hyogo 663-8186, Japan. [3]Department of Surgical Pathology, Hyogo College of Medicine, 1-1 Mukogawa, Nishinomiya, Hyogo 663-8501, Japan.

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Case report

Bevacizumab with metronomic chemotherapy of low-dose oral cyclophosphamide in recurrent cervical cancer: Four cases

Rose Isono-Nakata, Hiroshi Tsubamoto*, Tomoko Ueda, Kayo Inoue, Hiroaki Shibahara

Department of Obstetrics and Gynecology, Hyogo College of Medicine, Japan

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ABSTRACT

Standard chemotherapy for women with advanced or recurrent cervical cancer involves a combination of paclitaxel, platinum, and bevacizumab. However, for patients who experience anaphylaxis in response to paclitaxel or platinum, have permanent peripheral neuropathy, or develop early recurrence or progressive disease during first-line chemotherapy, the development of a non-taxane non-platinum regimen is mandatory. Clinical trials using anti-angiogenic treatment demonstrated favorable outcomes in cases of highly vascularized cervical cancer. Metronomic chemotherapy has been considered an anti-angiogenic treatment, although its use in combination with bevacizumab has not been studied in cervical cancer. We treated four patients with recurrent cervical cancer with 50 mg of oral cyclophosphamide daily and 15 mg/kg of intravenous bevacizumab every 3 weeks (CFA-BEV). One patient experienced disease progression after 4 months, whereas the other three patients continued the regimen until their last follow-up at 13, 14, and 15 months, respectively. One patient suffered from grade 3 neutropenia; however, no grade 2 or higher non-hematological toxicities were observed. These cases demonstrate the use of CFA-BEV with minimal toxicity and expected anti-cancer activity and indicate that this regimen should be considered for second-line chemotherapy in advanced recurrent cervical cancer.

1. Introduction

Platinum-based chemotherapy has been a standard treatment for advanced or recurrent cervical cancer, and a combination regimen of paclitaxel, platinum, and bevacizumab (TP-Bev) is widely recognized as the current standard treatment (Tewari et al., 2017). However, some patients suffer from anaphylaxis after taxane- or platinum-based therapy. There is no standard treatment for progressive disease that develops during or shortly after platinum-based chemotherapy (Takekuma et al., 2017). Development of a non-taxane, non-platinum regimen for recurrent cervical cancer is therefore warranted.

Clinical trials using anti-angiogenic drugs, including bevacizumab and cediranib, for treatment of highly vascularized cervical cancer have demonstrated high efficacy (Symonds et al., 2015). Metronomic chemotherapy—which is described as frequent administration of low-dose chemotherapeutic drugs despite the maximum tolerated dose (MTD) therapy—has also demonstrated high clinical efficacy and only mild toxicities in patients with various cancers, which is likely due to inhibition of angiogenesis (Mpekris et al., 2017). The combination of metronomic chemotherapy with bevacizumab has been reported in several cancer types. For ovarian cancer, there have been six studies that have reported the results of a regimen including 50 mg of oral cyclophosphamide (CFA) daily and 10 mg/kg of intravenous bevacizumab (BEV) repeated every 2 weeks. Alagkiozidis et al. reported 2 cases with endometrial cancer that were treated with 50 mg of oral CFA daily and 15 mg/kg of BEV repeated every 3 weeks. The two patients had a progression-free survival (PFS) of greater than 16 months, with prolonged quality of life (Alagkiozidis et al., 2015).

We treated four patients with recurrent cervical cancer with 50 mg of oral CFA daily and 15 mg/kg of BEV repeated every 3 weeks (CFA-BEV). Among three patients (cases 1, 2, and 4) who received salvage chemotherapy, case 2 had a PFS of 4.2 months and cases 1 and 4 continued treatment for more than 13 months without disease progression. One patient (case 3) continued the regimen as maintenance chemotherapy for more than 13 months after radiotherapy for metastases in the pubic region and surrounding soft tissues (Table 1). Written informed consent was obtained from all patients to publish their data in this case report.

* Corresponding author at: Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1–1 Mukogawa –cho, Hyogo, Nishinomiya-shi 663-8501, Japan.
E-mail address: tsuba@hyo-med.ac.jp (H. Tsubamoto).

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Table 1
Description of each case.

Case	Histology	Primary treatment	Treatment after recurrence	Baseline of CFA-BEV				Response of CFA-BEV	AEs of CFA-BEV CTCAE ver 4.0		Current status
				Age (years)	PS	Moore criteria		PFS (months)	Non-hematologic	Hematologic	
						Score	Risk		≥ grade 2	≥ grade 3	
1	SCC	RH + CCRT	RT, anaphylaxis to platinum, UFT, TAE, RFA	73	0	1	low	15.2*	No	Grade 3 neutropenia	AWD
2	Adeno-SCC	CCRT	Surgery, RT	47	0	1	low	4.2	No	No	AWD
3	SCC	RH	RT, surgery, anaphylaxis to platinum	75	1	NA		12.8*	No	No	NED
4	SCC	RH + CCRT	Progression during platinum, RT, UFT	47	0	2	intermediate	13.8*	No	No	NED

Moore criteria included black race, performance status 1, pelvic disease, prior cisplatin, and a progression-free interval < 365 days. CFA-BEV, oral cyclophosphamide and bevacizumab; AEs, adverse events; CTCAE, Common Terminology Criteria for Adverse Events; PS, performance status; PFS, progression free survival; SCC, squamous cell carcinoma; RH, radical hysterectomy; CCRT, concurrent chemoradiotherapy; RT, radiotherapy; UFT, tegafur/uracil; TAE, trans-arterial embolization; RFA, radiofrequency ablation; NA, not available; AWD, alive with disease; NED, no evidence of disease.
* Denotes patients who continued to receive CFA-BEV until their last follow-up.

2. Case reports

2.1. Case 1

A 66-year-old woman was diagnosed with stage IB1 squamous cell carcinoma and underwent radical hysterectomy. The surgical specimen revealed pelvic lymph node metastases. Adjuvant concurrent chemoradiotherapy with weekly administration of 40 mg/m^2 cisplatin for 5 cycles was administered. Five years after first-line treatment, the patient was diagnosed with metastases in the left axillary lymph nodes. Following surgical resection, she received adjuvant radiotherapy to the field and received triweekly administration of 80 mg/m^2 nedaplatin for 2 cycles. She discontinued nedaplatin because of anaphylaxis and was administered tegafur/uracil for 7 months. At the age of 73 years, she was diagnosed with a solitary liver metastasis and multiple lung metastases. After transcatheter arterial chemoembolization and radiofrequency ablation for local control of liver metastasis, she continued to receive CFA-BEV. Computed tomography (CT) was conducted 5.2, 9.2, 11.3, and 14.8 months after treatment and revealed that her lung disease had stabilized (Fig. 1). During CFA-BEV treatment, she developed grade 3 neutropenia (950 cells/mm^3) and grade 1 fatigue, and refrained from chemotherapy for one month after 10 months of treatment.

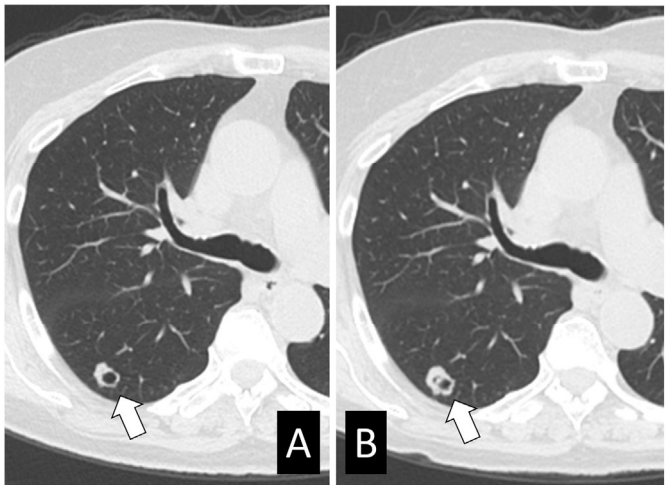


Fig. 1. Stable disease of lung metastases during CFA-BEV treatment in case 1. Images from computed tomography performed before (A) and 14.8 months after (B) the initiation of CFA-BEV treatment. The arrow shows the maximum size of the lung metastases (diameter, 10 mm).

2.2. Case 2

A 40-year-old woman was diagnosed with stage IIIB adenosquamous cell carcinoma. A computed tomography (CT) scan revealed pelvic and paraaortic lymph node metastases. She received extended-field radiotherapy with 80 mg/m^2 of nedaplatin every 3 weeks for 5 cycles. After 30 months, she underwent a thoracoscopic pulmonary upper lobectomy for right solitary lung metastasis. After 14 months, she received radiotherapy for Virchow metastasis. After 29 months, at the age of 47 years, she was diagnosed with multiple lymph node and lung metastases on 18F-fluoro-deoxyglucose (18-FDG) positron emission tomography (PET)-CT. She displayed no symptoms and rejected taxane-based chemotherapy owing to its side effect of alopecia. She was treated with CFA-BEV. After 2 months, PET-CT showed stable disease. She discontinued treatment because of onset of cough caused by progressive lung metastasis after 4.2 months of treatment. Following this, she agreed to receive a regimen of paclitaxel, carboplatin and bevacizumab, which resulted in partial response of lung metastases.

2.3. Case 3

A 66-year-old woman was diagnosed with stage IB1 squamous cell carcinoma. She received radical hysterectomy. Two years later, she received high-dose-rate intracavitary radiotherapy because of sustained evidence of the presence of malignant cells (class V) on vaginal wall cytology. After 6 months, she was diagnosed with left inguinal lymph node metastases; these were resected. Six months later, a recurrence in the anterior vaginal orifice and left inguinal lymph node metastases were identified. Resection of the urethra and vaginal wall, suprapubic cystostomy and left inguinal lymph node resection were conducted. Four years later, she experienced pelvic recurrence, therefore pelvic exenteration with urostomy, and colonostomy was performed. Eight months later, metastases in the pubic symphysis and the surrounding soft tissues were identified, for which concurrent chemoradiotherapy with nedaplatin was administered. She discontinued nedaplatin after 3 cycles because of anaphylaxis, by which point she was 75 years old. The patient continued to receive CFA-BEV. No evidence of recurrence was detected on CT after 11.9 months of treatment.

2.4. Case 4

A 45-year-old woman was transferred to our hospital because of massive vaginal bleeding. A cervical tumor 7 cm in diameter was identified, and stage IB2 squamous cell carcinoma was diagnosed.

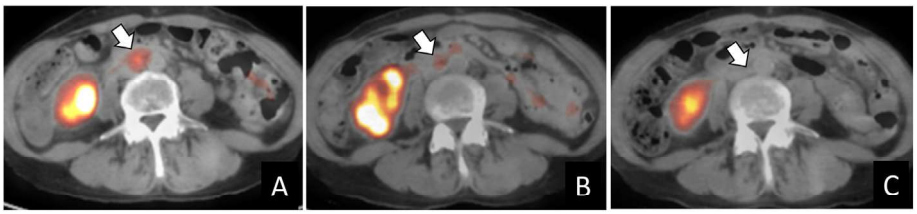


Fig. 2. Decreased 18F-fluoro-deoxyglucose uptake of paraaortic lymph node during CFA-BEV treatment in case 4. The short axis was 8.7 mm in diameter, and the maximum standard uptake value (SUVmax) was 2.7 (A) before CFA-BEV initiation. After 5.1 months, the SUVmax decreased to 2.4 (B); no abnormal uptake was detected 9.9 months (C) and 13.8 months after treatment. The arrow indicates the paraaortic lymph node.

Following transarterial chemoembolization with 50 mg of cisplatin, she underwent a radical hysterectomy which resulted in incomplete lymph node resection because of dense adhesion to the vessels. She received pelvic radiotherapy with nedaplatin for 6 cycles. One month after the last cycle of nedaplatin, PET-CT scan revealed paraaortic and mediastinal lymph node metastases, for which additional irradiation was performed. After 12 months, at the age of 47 years, a PET-CT scan revealed recurrence of the paraaortic lymph node metastases. She continued to receive CFA-BEV. PET-CT scanning was conducted at 5.1, 9.9, and 13.8 months after treatment and revealed complete remission (Fig. 2).

3. Discussion

The mainstay of treatment for recurrent cervical cancer is to maintain the quality of life, and prolonged survival is expected by administration of bevacizumab, especially in high-risk women. The four patients presented in this case report had no cancer-associated symptoms before the initiation of CFA-BEV therapy. Therefore, metronomic chemotherapy with bevacizumab was considered because it has the advantage of prolonged tumor stabilization with mild toxicities, rather than the rapid and short-term tumor regression responses caused by standard MTD therapy.

Metronomic chemotherapy has been most extensively studied in breast cancer (Liu et al., 2017), where, in combination with bevacizumab, it has demonstrated potent anti-tumor activity with only mild toxicities. Metronomic weekly administration of paclitaxel has been shown to have anti-angiogenic activity. In a randomized clinical trial in which a combination of chemotherapy and bevacizumab was used to treat platinum-resistant ovarian cancer, weekly administration of paclitaxel had the highest efficacy compared with other drugs, presumably because of the synergistic effect of weekly dosing of paclitaxel and bevacizumab on angiogenesis (Pujade-Lauraine et al., 2014). The combination of low dose oral cyclophosphamide and bevacizumab presumably had a synergic anti-angiogenic effect on recurrent cervical cancer in this study, as it did on ovarian cancer in previous studies.

Although metronomic chemotherapy was initially considered to target angiogenesis, recent studies have shown other anti-tumor mechanisms targeting stem-like cells (CSCs) and the immune system (Kareva, 2017). Metronomic chemotherapy in combination with an anti-angiogenic drug showed a reduction in CSCs in rat glioma (Folkins et al., 2007). Metronomic cyclophosphamide therapy reduced CD4 + CD25+ regulatory T cells and activated cytotoxic NK and CD8+ T cells (Ghiringhelli et al., 2007), although immunotherapy alone using ipilimumab or pembrolizumab showed limited activity against cervical cancer (Frenel et al., 2017; Lheureux et al., 2017).

A non-platinum-based regimen for recurrent cervical cancer could comprise topotecan, paclitaxel, and bevacizumab (TP-BEV); however, the toxicities would be greater than those experienced with CFA-BEV. In the GOG-0240 trial, a regimen of topotecan-paclitaxel with or without bevacizumab resulted in grade 3–4 neutropenia in 64% of cases and grade 2–3 sensory neuropathy in 18% (Tewari et al., 2017). Garcia et al. treated 70 patients with recurrent ovarian cancer with CFA-BEV, and only one (1.4%) patient developed grade 3 neutropenia (Garcia et al., 2008). Patients with low and intermediate risk, according to the Moore criteria (Moore et al., 2010), and those administered bevacizumab-containing chemotherapy doublets in the GOG-0240 trial had a PFS of 10.9 and 7.9 months, respectively (Tewari et al., 2015). In our report, cases 1 and 4 had similar risks and had a PFS of over 13 months, confirmed by CT and PET-CT, respectively. CFA-BEV could be a promising potential treatment for patients who experience recurrent disease shortly after platinum treatment, anaphylaxis in response to paclitaxel or platinum, or peripheral neuropathy. In addition, the application of this therapy regimen to asymptomatic patients with minor or dormant recurrent disease should be further investigated.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Clinical Image

Removal of a Retroperitoneal Foreign Body by Laparoscopic Surgery

Saeki Shinichiro, Yu Wakimoto*, Hidetake Kamei, Atsushi Fukui, Hiroaki Shibahara
Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Hyogo, Japan

Abstract

Migration of intrauterine devices (IUDs) into the abdominal cavity is rare. In this report, we describe a patient in whom a levonorgestrel intrauterine system (LNG-IUS) device was initially misplaced outside of the uterus, likely due to stenosis of the cervix following a conization procedure for carcinoma *in situ*. The patient presented with persistent abdominal pain and vaginal bleeding. The LNG-IUS was not visible on physical examination and ultrasound imaging, requiring intraoperative abdominal radiography and postoperative computed tomography for localization. Once localized, we proceeded with the removal of the foreign body in the retroperitoneal space by laparoscopy. Misplacement of an IUD such as LNG-IUS outside of the uterus after a conization procedure should be suspected in women with persisting symptoms, and this possibility should be diligently assessed.

Keywords: Contraceptive device, intrauterine device, intrauterine system, levonorgestrel, perforation

Introduction

In Japan, the levonorgestrel intrauterine system (LNG-IUS) was included in the public insurance plan for the treatment of hypermenorrhea and dysmenorrhea in 2014.[1] Migration of an intrauterine device (IUD) into the peritoneal cavity is rare, with an incidence rate of 0%–0.28%.[2] In this report, we describe a case in which the LNG-IUS was misplaced outside of the uterine cavity, which required removal from the retroperitoneal cavity using a laparoscopic approach.

Clinical Image

A 29-year-old female, gravida 3, para 1, had previously undergone cervical conization, for the treatment of carcinoma *in situ* of the uterine cervix, at the age of 22 years. An LNG-IUS was inserted into the uterus for the treatment of dysmenorrhea and to prevent cervical stenosis at the local clinic at the age of 24 years.

The patient visited our hospital after surgery with persisting abdominal pain and was admitted for investigation. On speculum examination, the LNG-IUS thread was visible; however, due to the previous conization procedure, the location of the uterine cervical canal was difficult to confirm. The foreign body could not be located during vaginal examination. For evacuation of the hematoma, we proceeded the dilation using a 14-Fr catheter, and we attempted to locate and remove the misplaced LNG-IUS [Figure 1]. As we were unable to locate the LNG-IUS, intraoperative radiography was performed, with the device visible outside of the uterine cavity [Figure 2].

Two months after the transcervical procedure, we performed a laparoscopic procedure to remove the foreign body. We inserted a 12-mm trocar through the umbilicus and two 5-mm trocars at both lateral regions and the lower abdomen. After confirmation of the position of the device in the left side retroperitoneal space near ureter [Figure 3], we confirmed

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Address for correspondence: Dr. Yu Wakimoto, Department of Obstetrics and Gynecology, Hyogo College of Medicine, 1-1 Mukogawa-Cho, Nishinomiya, Hyogo 663-8501, Japan.
E-mail: yuuwakimoto@gmail.com

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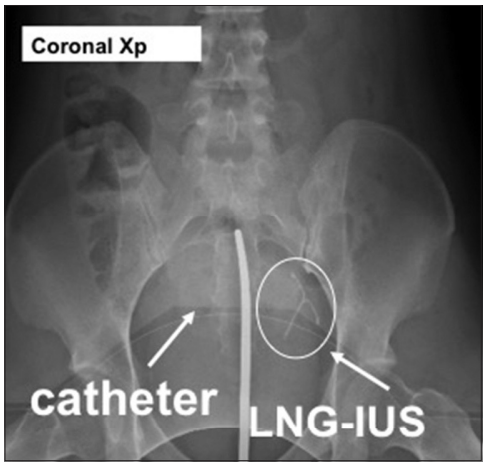


Figure 1: Intraoperative abdominal radiograph at dilation and curettage point showing the location of the levonorgestrel intrauterine system outside of the uterine cavity

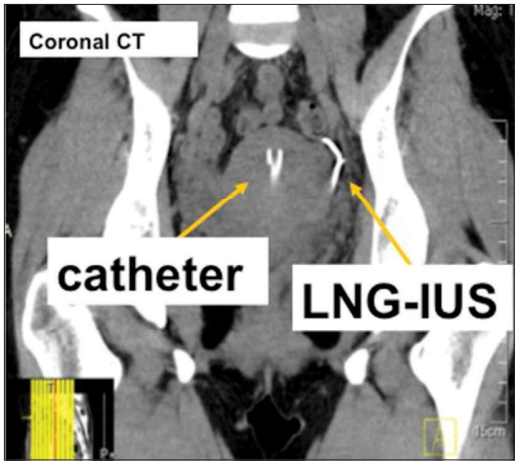


Figure 2: Postoperative at dilation and curettage point computed tomography confirming the location of the levonorgestrel intrauterine system in the retroperitoneal space. The catheter was implanted during initial dilation and curettage to prevent restenosis

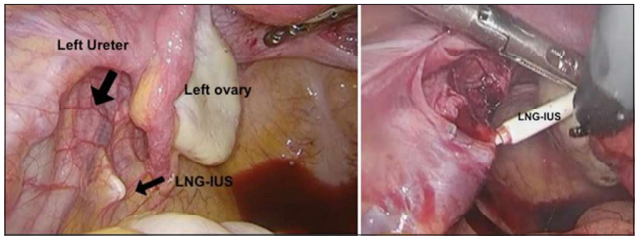


Figure 3: Localization of the levonorgestrel intrauterine system under the left ovary in the pelvic peritoneum

the absence of perforation or adhesions to adjacent organs by laparoscopic observation.

The ureters were noted to have bilateral peristalsis transperitoneally. A 1.5-cm linear incision was made in the long axis of the device using monopolar electrical scalpel. The device was coherent at the retroperitoneal space, and it was removed carefully. The chromotubation was successfully performed to confirm the tubal patency. The retroperitoneal wound was carefully cover the with adhesion barrier (Seprafilm, Kaken Pharmaceutical Co., Ltd. Tokyo, Japan).

The operation time was 1 h 21 min, with minimal blood loss. Abdominal pain resolved completely after surgery.

Discussion

In our case, the LNG-IUS was incorrectly placed in the retroperitoneum at the time of initial insertion. A previous review of women with a misplaced LNG-IUS indicated that 50% were asymptomatic, 28.7% became pregnant, 17.8% reported abdominal pain, and 4.7% presented with irregular vaginal bleeding.[3] The patient in our case was admitted due to persisting lower abdominal pain and vaginal bleeding, both of which were initially considered as resulting from an intrauterine hematoma.

Certainly, there is no need for the removal of an intra-abdominal LNG-IUS in asymptomatic cases,[4] except for the purpose of planning a pregnancy, which would require removal to lower the plasma levels of LNG to permit ovulation.[5]

In our case, we suspected that the LNG-IUS was incorrectly placed into the retroperitoneum, through the posterior vaginal vault, and did not migrate from the uterus into the peritoneal cavity due to uterine perforation.[6] Certainly, IUD migration can cause serious complications, including bowel and bladder perforation, ileus, abdominal abscess, and fistula formation.[7] Kno *et al*. reported that an abnormal uterine orientation, such as retroversion or acute reflection, increases the risk of perforation, with 42% of uterine perforation occurring in patients with a retroverted uterus, with the incidence rate of uterine retroversion estimated at 20%–25%.[8] Although 43% of women in Kno's case series (16 of 37 cases) did not experience symptoms associated with a perforation of the uterus, the authors did still advocate for the prompt localization and removal of the IUD in these cases to prevent complications and limit the need for a more extensive procedure later. Based on our experience, we propose that it is important to be cognizant of the possibility of IUD migration and therefore to obtain at a minimum upper and lower abdominal radiographs. It would also be advisable to clearly explain the possible complications to patients and stress the importance of annual examinations and regular removal of the IUD.

Conclusion

We describe a case of IUD misplacement in the retroperitoneal space that required removal using a laparoscopic technique. Misplacement of an IUD outside of the uterus after a conization procedure should be suspected in women with persisting symptoms, and this possibility diligently assessed.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient has given her consent for her images and other clinical information to be reported in the journal. The patient understands that her name and initial will not be published and due efforts will be made to conceal her identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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Recurrent uterine serous carcinoma with a germline pathogenic *BRCA2* variant treated using olaparib: A case report

Kayo Inoue[a,*], Hiroshi Tsubamoto[a], Tomoko Ueda[a], Chihiro Tajima[a], Nami Nakagomi[b]

[a] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Japan
[b] Department of Surgical Pathology, Hyogo College of Medicine, Japan

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ABSTRACT

A germline pathogenic variant in *BRCA2* was secondarily found through genomic sequencing of uterine serous carcinoma. Clinical response to olaparib was observed in recurrent uterine serous carcinoma with a germline *BRCA2* mutation. Here, we report, for the first time, a long-term clinical response to olaparib in a patient with uterine serous carcinoma and a germline pathogenic *BRCA2* variant.

1. Introduction

Case report

A 57-year-old, Japanese woman (gravida 2) presented to our hospital with postmenopausal vaginal bleeding. She had no past medical or family history, and had a body mass index of 25.7 kg/m^2. Endometrial biopsy revealed the presence of a serous carcinoma. Pelvic magnetic resonance imaging (MRI) revealed a tumor invading the cervix and lower uterine segment (Fig. 1-A). ^{18}F-fluoro-deoxyglucose positron emission tomography (FDG-PET) / computed tomography (CT) findings suggested a tumor in the lower uterine segment and multiple metastases to the pelvic lymph nodes. Complete cytoreduction was achieved by total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and retroperitoneal lymphadenectomy. The final pathologic diagnosis was uterine serous carcinoma stage IIIC1, according to the International Federation of Gynecology and Obstetrics (FIGO) staging for uterine cancer (2008). The tumor was 5 cm in diameter located in the lower uterine segment. There was no lymphovascular invasion, and less than half the myometrium appeared to have been invaded (Figs. 2 and 3). Metastases were found in the right ovary, pelvic lymph nodes, and the peritoneum of the Douglas pouch. The tumor in the right ovary was microscopic, and the tumor in the uterus and right ovary showed positivity for immunohistochemical staining of WT-1 (Fig. 2). There were no findings indicative of malignancy in the right fallopian tube and left adnexa.

The patient underwent six cycles of adjuvant chemotherapy with paclitaxel, carboplatin, and bevacizumab. She remained disease-free for 10 months after the completion of chemotherapy, at which point she complained of upper abdominal pain. FDG-PET/CT findings suggested tumor recurrence in the peritoneum under the right diaphragm and the greater omentum (Fig. 1-B). A multi-gene panel examination of the tumor obtained from the primary surgery was performed via next-generation sequencing. The results revealed a *BRCA2* loss-of-function mutation (p.I1859fs, c.5576_5579delTTAA). After the patient and family members underwent genetic counseling, germline testing was conducted, and the same mutation was detected in the patient's germline cells.

While waiting for the above-mentioned genetic test results, the patient underwent second-line chemotherapy with doxorubicin and cisplatin. After two cycles of chemotherapy, the patient's serum CA125 level decreased from 397 to 201 IU/mL. FDG-PET/CT revealed a significant reduction in peritoneal dissemination and decreased maximum standardized uptake (SUVmax from 10.1 to 3.4) in the recurrent tumors (Fig. 1-C). The recurrent tumors were found to be platinum sensitive, but chemotherapy was halted due to ileus that occurred each time after cisplatin administration and for which hospitalization was required.

Due to limited therapeutic options and the somatic and germline *BRCA2* mutations, olaparib was considered as a candidate for treatment. A multidisciplinary team conference was held, and institutional review board approval was granted. Informed consent was obtained from the patient and her family, and off-label use of olaparib was chosen as the treatment plan, though the treatment was not covered by medical insurance. Olaparib was administered orally (300 mg twice daily, a total daily dose of 600 mg). The patient was relieved of the abdominal pain after being treated with olaparib for 3 months, and her serum CA125 level decreased to 26.6 IU/mL. However, she also experienced anemia, which is an adverse effect of olaparib; thus, intermittent cessations and several rounds of blood transfusion were

* Corresponding author.
E-mail address: inouekayomana51713@yahoo.co.jp (K. Inoue).

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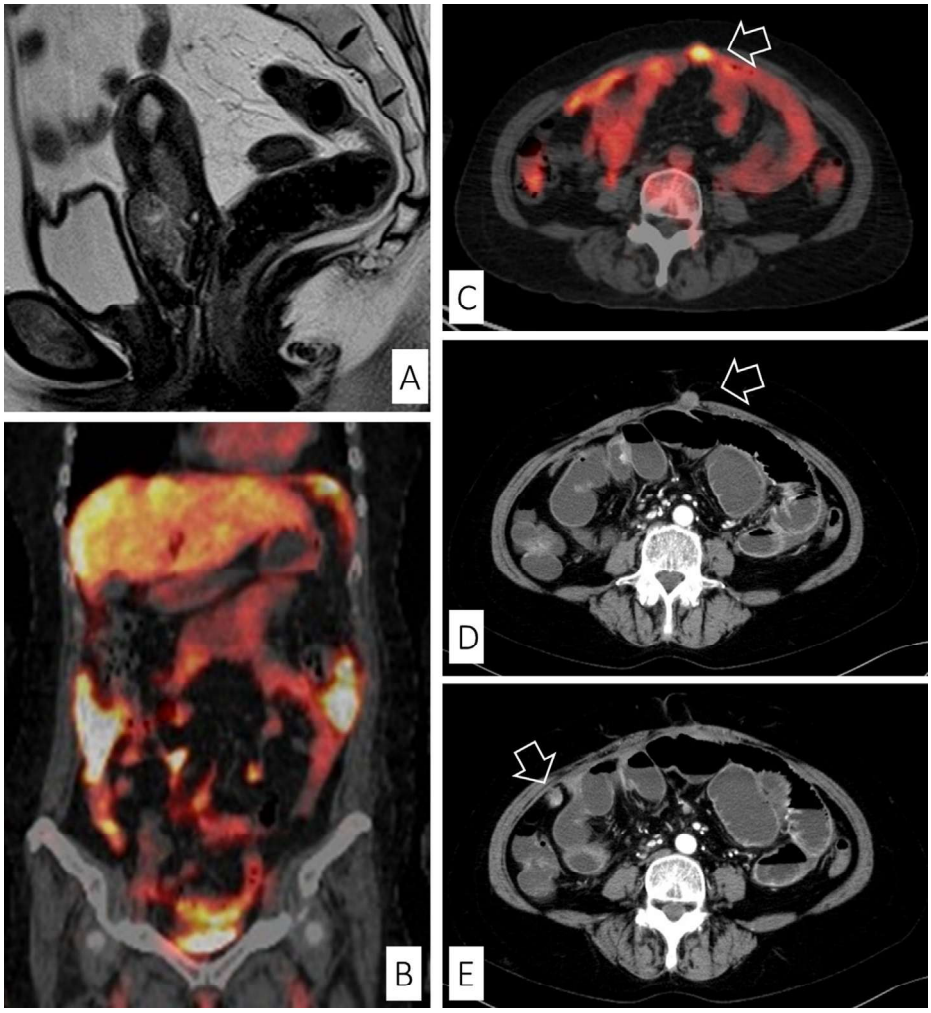


Fig. 1. A: Magnetic resonance image (T2 image) acquired before the primary surgery. The tumor was located in the lower uterine segment and cervix. B: ^{18}F-fluoro-deoxyglucose positron emission tomography (FDG-PET)/ computed tomography (CT) performed after first-line chemotherapy for the diagnosis of recurrence. Peritoneal dissemination in multiple sites, and strong accumulation of FDG in the lesions under the right diaphragm and in the greater omentum and parietal peritoneum can be observed. C: FDG-PET/CT after two cycles of second-line chemotherapy showing a significant reduction in peritoneal dissemination and decreased accumulation of FDG. D and E: Enhanced CT performed 11 months after olaparib initiation showing peritoneal dissemination in the abdominal wall and small bowel obstruction. The arrow in panel D indicates the lesion seen in panel C; this lesion became slightly larger after 11-month treatment with olaparib. The arrow in panel E shows one of the new peritoneal dissemination lesions.

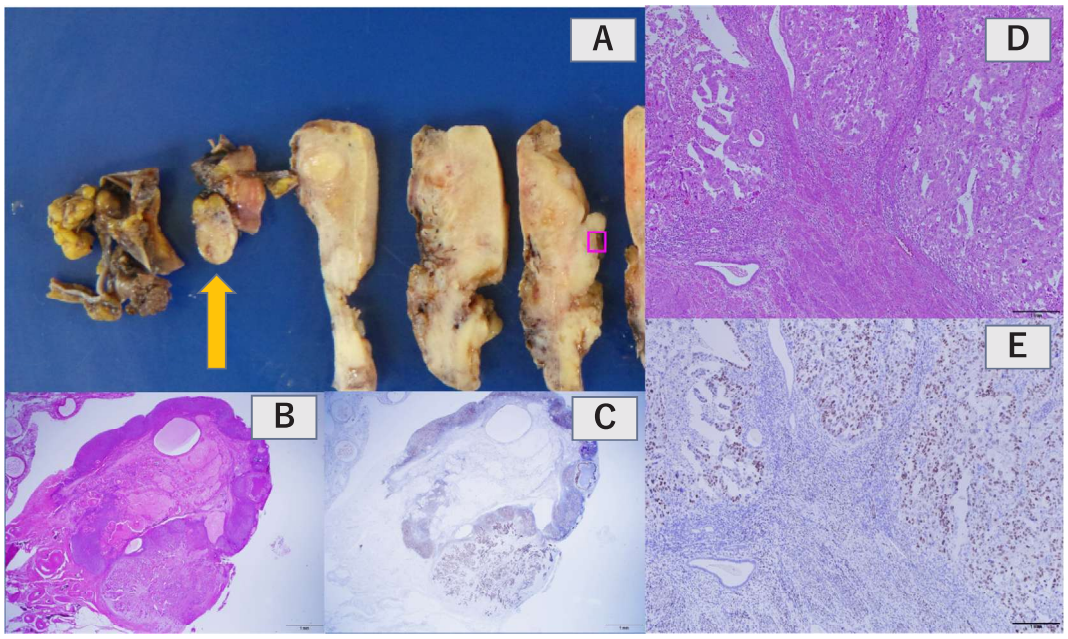


Fig. 2. A: Macroscopic findings of the uterus and adnexa obtained from the primary surgery. The uterus and cervix were cut through the midline and a parallel axis. The tumor was 5 cm in diameter and was located in the lower uterine segment. The arrows show the normal-sized and macroscopically normal ovaries. B and C: Loupe view of the right ovary shows 4 × 5 mm and 1 × 1 tumors. B: Hematoxylin and eosin (H&E) staining. C: Immunohistochemical staining of WT-1. The tumors show positivity for WT-1. D and E: Tumor in the uterus. D: H&E staining. E: Tumor cells are positive for WT-1.

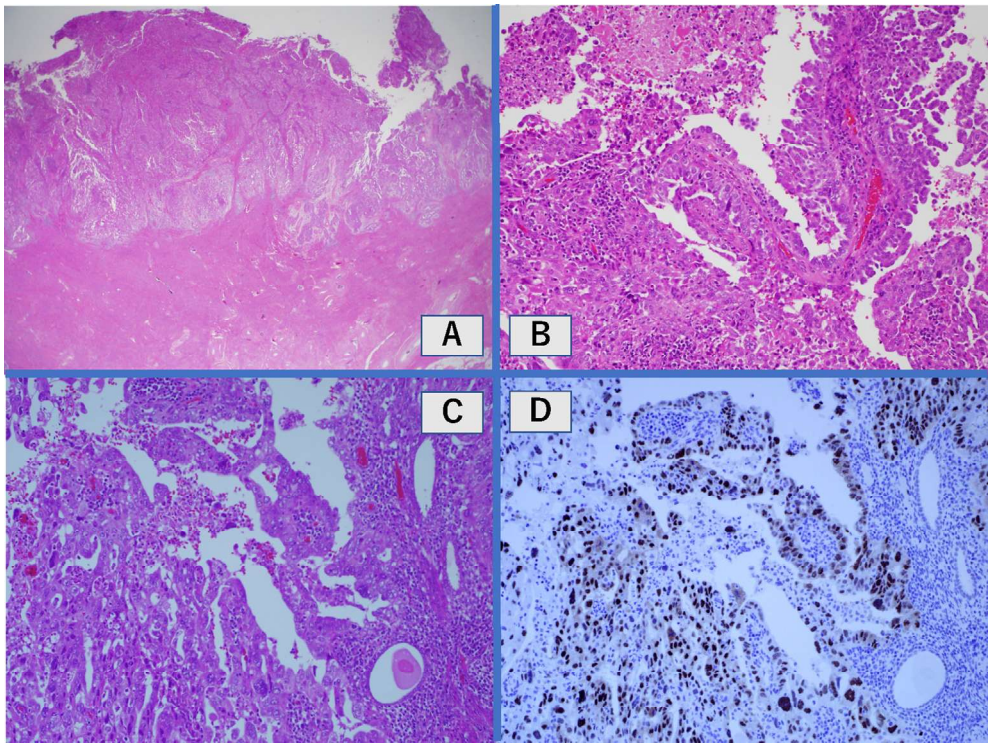


Fig. 3. Histological findings of the uterus: hematoxylin and eosin staining (A, B, and C) and immunohistochemical staining for p53 (D). A: Tumor cells were observed mainly in the surface of the endometrium. B: Papillary growth of tumor cells with high-grade cellular and nuclear atypia. C: Coexistence of tumor cells on the left and residual non-neoplastic atrophic glands on the right; these findings suggest that tumors developed from the non-neoplastic atrophic endometrial glands and grew replacing the non-neoplastic glands. D: Serous carcinoma with strong and diffuse positivity for p53, and non-neoplastic endometrial glands negative for p53. These findings are consistent with high-grade serous carcinoma of the uterus origin.

required. The dosage of olaparib was reduced to two daily doses of 200 mg (for a total daily dose of 400 mg). Subsequently, the patient's appetite recovered, and she became free of pain. Olaparib was administered for another 8 months, during which the patient remained asymptomatic. The patient developed bowel obstruction during taking olaparib, thus olaparib was discontinued. The placement of an ileus tube was ineffective in treating bowel obstruction. Enhanced CT showed pelvic peritoneal dissemination without masses or ascites (Fig. 1-D and E). The patient decided to discontinue olaparib and other anticancer treatments. The patient has since been receiving total parenteral nutrition and supportive care at home; at the time of writing this report, the patient has been alive at home for two months with disease.

Written informed consent to report the case was obtained from the patient.

2. Discussion

Here, we report two new insights from a case of uterine serous carcinoma. First, a *BRCA2* pathogenic variant was secondarily identified via genomic sequencing of uterine serous carcinoma. Second, we report the long-term clinical response of a patient treated with olaparib for 11 months. Considering that there are limited standard chemotherapeutic options for recurrent endometrial cancer, and hormonal therapy is not effective for uterine serous carcinoma, our patient underwent tumor DNA sequencing to identify possible targeted treatments. The results of the somatic tumor panel led to a secondary finding of a germline *BRCA2* mutation. The patient had no family or medical history of cancer, and the *BRCA2* mutation was unexpected.

It remains unclear whether mutations in *BRCA1* and *BRCA2* are associated with increased risks for endometrial cancer, and especially uterine serous carcinoma. A case-controlled study found no differences in risks of endometrial cancer between individuals with and without *BRCA1/2* mutations (Segev et al., 2015). In contrast, studies show that patients with *BRCA1* mutations undergoing risk-reducing salpingo-oophorectomy have increased risks of uterine serous carcinoma (Shu et al., 2016, Saule et al., 2018).

Conversely, the frequency of *BRCA1/2* mutations has been reported to be higher among patients with uterine serous carcinoma than among those with all histologic types of endometrial cancer. A different study reported that among unselected 381 endometrial cancer patients who underwent cancer panel testing, 26 had serous carcinoma, and among them *BRCA2* mutations were found in one patient with uterine serous carcinoma. A *BRCA1* mutation was found in one of 289 patients with endometrioid carcinoma (Ring et al., 2016). A *meta*-analysis demonstrated three out of 207 Caucasian women with uterine serous carcinoma had germline *BRCA1/2* mutations (Jonge et al., 2017). Accordingly, it is estimated that 1–3% of uterine serous carcinomas are associated with *BRCA1/2* mutations.

Olaparib, the first poly (ADP-Ribose) polymerase (PARP) inhibitor, was approved by the Food and Drug Administration for the treatment of recurrent platinum-sensitive ovarian cancer with germline or somatic pathogenic *BRCA1/2* mutations and also for patients with germline *BRCA*-positive, HER2-negatvie metastatic breast cancer. The efficacy of olaparib for the treatment of patients with metastatic pancreatic cancer with *BRCA1/2* mutations was demonstrated in a randomized-controlled trial (Golan et al., 2019). Olaparib also showed positive phase III results in metastatic prostate cancer treatment.

The efficacy of olaparib in endometrial cancer patients with germline *BRCA1/2* mutations remains unknown. To our knowledge, there has only been one case report showing a durable response (more than 15 months) to olaparib treatment in a patient with recurrent grade 1 endometrioid carcinoma with a germline *BRCA2* mutation and a different somatic *BRCA2* mutation (Gockley et al., 2018). This case report was the first to show a 11-month clinical response to olaparib in a patient with uterine serous carcinoma with a germline pathogenic *BRCA2* variant.

It also remains unclear whether uterine serous carcinoma is a manifestation of homologous recombination deficiency (HRD). Previous work has suggested that HRD and sensitivity to PARP inhibitors were tied to *BRCA*-associated cancer types in patients with *BRCA1/2* mutations (Jonsson et al., 2019). Jonge et al. showed HRD occurs in high-grade, non-endometrioid endometrial cancers (serous carcinoma and carcinosarcoma) and is related with pathogenic *BRCA1*

variants or high somatic copy-number losses of HR genes. In their study, 24% (n = 6) of endometrial cancers were HR deficient, with either serous carcinoma or carcinosarcoma with a serous component, and two of them had pathogenic *BRCA1* variants (Jonge et al., 2019a). In addition, Jonge et al. also reported that, among 40 endometrial cancer patients with germline *BRCA1/2* mutations, there were six with uterine serous carcinoma (3 *BRCA1* and 3 *BRCA2* mutations), five of whom were positive for loss of heterozygosity of the *BRCA1/2* wild-type allele (3 *BRCA1* and 2 *BRCA2* mutations). They thus suggested that endometrial carcinoma might be a form of germline *BRCA*-associated hereditary breast and ovarian cancer (Jonge et al., 2019b).

Although the frequency of germline *BRCA1/2* mutations in patients with uterine serous carcinoma may not be high, olaparib could be an efficient treatment strategy for uterine serous carcinoma in cases involving HRD or germline *BRCA1/2* mutations. Finally, screening for germline *BRCA1/2* mutations may be worthwhile.

In summary, olaparib provided 11-month progression free survival for the patient in our case report. Our report suggests that uterine serous carcinoma may be a *BRCA*-associated cancer. Germline or tumor DNA testing for pathogenic *BRCA1/2* mutations in patients with uterine serous carcinoma might be beneficial, and olaparib could be a potential treatment option in patients with a germline pathogenic *BRCA2* variant.

3. Conclusion

Olaparib could be a promising candidate for the treatment of uterine serous carcinoma associated with germline *BRCA2* mutations.

Author contributions

Kayo Inoue drafted the article, Hisroshi Tsubamoto supervised, Tomoko Ueda and Chihiro Tajima collected information, and Nami Nakagomi drafted the pathological information and selected the figures. All authors read and approved the final article.

Statement of informed consent

Written informed consent was obtained from the patient for publication of this case report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal on request.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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NIPTの現状

NIPTで予期しない結果となる原因は何か？

澤井 英明

- NIPTでは陽性・陰性以外に結果の報告ができない「判定保留」がある．日本人では判定保留は初回採血の約0.3%に起こり，2度目の採血でも判定保留となったものは約0.1%ある．
- NIPTでは陰性と報告されていても当該染色体異常の罹患児が出生する「偽陰性」が，日本人では約0.01%ある．
- NIPTでは陽性や判定保留となった場合に，目的とした染色体異常以外の染色体異常やがんなど，本来の検査目的ではない疾患が偶然に発見される「偶発的所見」がある．

はじめに

NIPTは日本では日本産科婦人科学会の指針により，胎児のトリソミー21と18，13が検査対象となっている．結果は，胎児がこうした特定の染色体の異数性（数的異常）である可能性が高い場合を陽性，低い場合を陰性として報告される．NIPTは非確定診断検査であるため，その検査結果については，NIPT陽性の場合は絨毛検査や羊水検査などの確定診断検査で確認する必要がある．NIPTコンソーシアムの報告によると，日本人ではNIPTの陽性的中率（NIPT陽性となった場合に本当に罹患している確率）はトリソミー21で96.5%，18で82.8%，13で63.6%となり，全体では89.4%となる[1]．これは偽陽性が全体の10.6%にみられることを意味するが，偽陽性はNIPT陽性となった直後に確定診断検査で結果が明らかになるので，いわば想定の範囲内である．

しかし，陽性・陰性以外にも，結果の報告ができない「判定保留」という報告がある．NIPTコンソーシアムの報告によると，日本人では判定保留は初回採血の約0.3%に起こり，2度目の採血でも判定保留となったものは約0.1%ある[1]．判定保留は検査前の遺伝カウンセリングでは説明されていたとしても，実際に生じる頻度は少なく，妊婦にとって

さわい ひであき：兵庫医科大学病院遺伝子医療部（〒663-8501　兵庫県西宮市武庫川町1-1）

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予期しない結果と認識される．NIPT の実施データの蓄積により，どのような場合に判定保留が生じやすいのか，また判定保留となった場合の対応をどうすべきかなどが徐々に明らかになってきた．

また NIPT は陰性的中率（NIPT 陰性となった場合に本当に罹患していない確率）は 99.99%以上とされている．しかし 100%ではないので，NIPT では陰性と報告されたにもかかわらず，検査対象であった染色体異常の罹患児が出生する「偽陰性」が，NIPT コンソーシアムの報告ではこれまでに 13,481 例中 2 例（約 0.01%）ある[1]．偽陰性は判定保留よりもはるかに頻度は少なく，NIPT 実施から数か月経過した出生時に判明することになり，その頻度からみても妊婦にとって予期しない結果の 1 つである．これも NIPT の実施データの蓄積により，偽陰性は偶然生じるものなのか，何か偽陰性が生じやすい原因があるのかが徐々に明らかになってきた．

NIPT が開発されて，臨床応用され始めた時点で，頻度は少ないものの，こうした判定保留や偽陰性といった事象が生じること自体は認識されていた．頻度が少ないゆえに妊婦にとっては予期しない結果であるが，医療者にとっては生じうることはもともと想定されていたものである．しかし，NIPT で陽性や判定保留となった場合に，目的とした染色体異常以外の染色体異常やがんなど，本来の検査目的ではない疾患が偶然に発見される「偶発的所見」があることがわかってきた．これらは当初の検査目的ではなく，医療者にとっても予期しない結果といえる．

判定保留

判定保留は海外の報告では，初回採血で約 1.9%，再採血しても約 1.3%と報告されていた[2]．また，初回採血での判定保留が 5.4%という報告もある[3]．報告により異なるが，おおむね 0.32～5.30%の範囲と思われる[4]．NIPT コンソーシアムの報告では，判定保留は初回採血の約 0.3%，再採血では判定保留となったのは約 0.1%であった[1]．海外に比して，日本では著明に判定保留が少ないことが示されている．胎児 DNA 濃度には採血可能な妊娠 10 週から 21 週までの間の妊娠週数や妊婦の年齢，検査までにかかる時間は関連せず，一方で母体体重や BMI の増加により胎児 DNA 濃度は低下すると報告されている[5]．母体の体重の違いが，スリムな体型の多い日本人で判定保留が少ない原因と考えられる．なお，胎児がトリソミー 13 または 18 の場合は胎児 DNA 濃度が低下するとされるが，トリソミー 21 の場合は上昇するという報告と正常核型と変わらないという報告があり，明確ではない[6]．

判定保留となった場合には，胎児 DNA 濃度が低値の場合には後日に再採血を実施することで検査結果が得られる可能性があるが，侵襲的検査を実施したほうがよい場合もある．また判定保留の原因として，不育症の原因となる抗リン脂質抗体症候群を含めた自己免疫疾患合併が報告されている．一方で，不育症の治療として，ヘパリンなどの抗凝固薬を投与されているケースも判定保留が増加することが知られており，自己免疫疾患合併自体の影響と，抗リン脂質抗体症候群や凝固線溶系の異常に対して治療薬として投与されて

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いるヘパリンの影響の2つが考えられる．

NIPTコンソーシアムの検査実施37,506人のうち，初回の採血で判定保留となったのは110人（0.29%）である[1]．このうち79人が再採血を受けており，そのうち24人が再度の判定保留となっている．これら判定保留のうち，詳細の判明している初回の採血の判定保留62人では何らかの理由によりヘパリンを投与されていたのは10人（16%）で，自己免疫疾患と判明しているのは4人（6.5%）（うち重複1人）となっている．判定保留群におけるこれらの割合は，一般頻度に比べると高いと推定される．判定保留となったヘパリン投与10人のうち再採血により再び判定保留となったのは5人で，再採血による判定保留率は，全体では30%（24/79）に対してヘパリン投与患者では50%（5/10）と高率になっている．判定保留となった自己免疫疾患合併妊婦4人のうち再採血により再び判定保留となったのは3例で，75%（3/4）と高率になっている．特にSLE合併妊婦3人（1人はヘパリン投与も実施）は全員が判定保留となっており，関連性が示唆される．

自己免疫疾患合併自体の影響については，症例報告がいくつかあり，自己免疫性血小板減少性紫斑病（IPT）を合併した妊婦で反復して判定保留になった症例が複数報告されている[7, 8]．自己免疫疾患合併妊婦の判定保留の原因は，胎児由来細胞フリーDNAの割合が低い（low cell-free fetal fraction）とされ，これは自己免疫により母体の炎症反応が惹起され，母体血中の母体由来の細胞フリーDNAが増加することで，相対的に胎児由来の細胞フリーDNAの割合が低下するのではないかと考察されている．またSLE患者ではT細胞においてDNAのメチル化異常が観察され，そのT細胞のアポトーシスに伴い，長さが極端に短く低メチル化を受けた細胞フリーDNAが血中で増加することが知られている．そのことで次世代シーケンサーを用いたNIPTの解析において健常な受診者と異なる解析パターンを示すと考察されている[9]．

ヘパリン投与がNIPTの判定保留と関連するという報告もある．抗リン脂質抗体症候群などで流産を防止するために使用するヘパリンが，NIPTの解析に影響を与えることが判定保留に関連するという[10]．その理由は，ヘパリン投与中の患者では健常者に比べて胎児由来細胞フリーDNA断片のサイズがやや短く，GC含有量の高い胎児由来細胞フリーDNA断片が血中で増加し，NIPTの解析において次世代シーケンサーのカウント数に影響を及ぼすとされているためである．ヘパリンが母体血中の白血球と結合してアポトーシスを誘導し，母体由来の細胞フリーDNA断片が血中で増加することが一因と考えられている．ヘパリンは半減期が短いので，血中濃度が低いときに採血を行うことでヘパリンの影響を最小限に抑えられるとされている．

妊娠時には双胎であったが一児が早期に流産するvanishing twinも判定保留や偽陽性に関連する可能性が報告されている[11, 12]．流産の原因は胎児の染色体異常が半数程度を占めるとされており，二卵性双胎では，一児が正常核型，もう一児が染色体異常で早期に流産して，見かけ上は単胎妊娠という場合がありうる．この場合に染色体異常を有する流産胎児の胎盤が完全に吸収されずに残っており，そのDNAが影響して，判定保留や偽陽性になる可能性がある．

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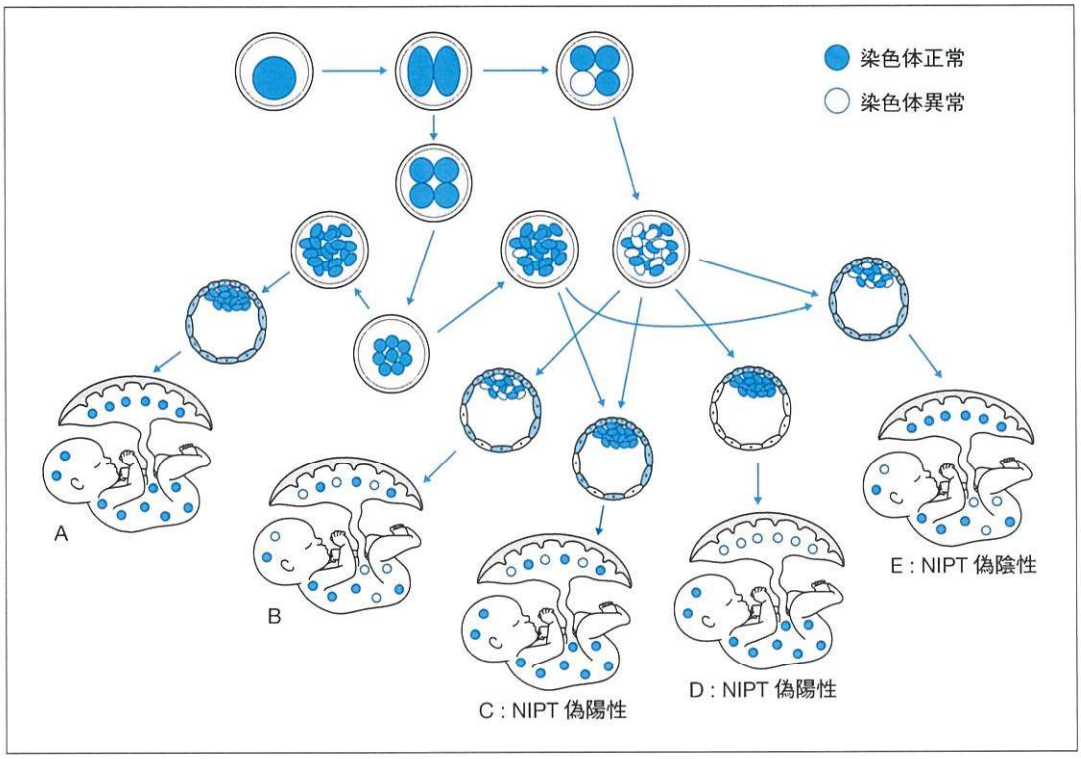


図1　NIPT の偽陽性と偽陰性のメカニズム

受精時には染色体が正常の受精卵も，細胞分裂を繰り返す過程で，染色体異常が生じる可能性がある．すべての細胞が正常であればAとなる．二分割や四分割程度の段階から桑実胚の段階までの間で染色体異常を生じた場合には，Bのように胎児も胎盤もモザイクである標準的なモザイクとなる．Cのように桑実胚までは正常で，胚盤胞になってから栄養外胚葉の細胞のみに異常が生じたり，桑実胚の段階で異常が生じていても，染色体異常の細胞が胎盤に配分されることで，胎児は正常で胎盤だけがモザイクという絨毛検査でみられるパターンの胎盤限局性モザイク（NIPT の偽陽性も）となる．Dのように桑実胚の段階で異常が生じていても染色体異常の細胞がすべて胎盤に配分されると，胎盤がすべて染色体異常で胎児はすべて正常ということもある（NIPT の偽陽性）．そして，Eのように胚盤胞の段階で内細胞塊がモザイクで栄養外胚葉が正常になっていると，胎児のみにモザイク型の染色体異常が生じて NIPT では偽陰性となる．

判定保留になった場合には，その原因が胎児 DNA 濃度不足であれば，後日に再採血することで報告できる可能性がある．また初回採血時にヘパリンを使用している場合には，もし一時的にでも中止できるのであれば，再採血時にはヘパリンを中止して採血する．それ以外の理由で判定保留になっている場合には，原則的には再採血は行わずに，次は確定診断検査を行うことが望ましい．その場合に，超音波検査で異常の可能性が示されているのであれば，染色体検査はG分染法だけでなく，マイクロアレイ法を併用することでより診断精度が向上する．

偽陰性

胎児に染色体異常があるのに検査で正常核型と診断される偽陰性の原因としては，絨毛限局性モザイク（confined placental mosaicism：CPM）が知られている[11, 12]．従来

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からCPMは絨毛穿刺の際にみられる所見として知られており，絨毛穿刺で正常核型と染色体異常がモザイクの形で検出され，羊水検査をするとすべて正常核型であることを指していた．これはNIPTであれば，胎児は正常核型であるのにNIPTで陽性となる偽陽性の原因となる．一方でNIPTコンソーシアムのデータでも，0.1%程度と非常に稀ではあるが，偽陰性が報告されている．その原因として胎児は染色体異常であるが，絨毛組織にのみ正常核型の染色体を持つ細胞が存在し，正常核型とのモザイクになるというケースが文献でもNITPコンソーシアムでも報告されている[13]．NIPTの結果は絨毛内の染色体異常と正常核型の細胞の割合により判定されるため，絨毛内のモザイクで正常核型を持つ細胞の割合が高いと，陰性と判定される．胎児は染色体異常であるがNIPT陰性のため，確定診断検査を受けることなく出生してその時点で初めて染色体異常と判明する．

偽陰性の発生するメカニズムを**図1**に示す．受精卵から分割胚が形成される過程で，染色体異常が生じると，モザイク型の染色体異常となる．その時期や偶然を含めたさまざまな要因により，モザイクのパターンは分かれる．このような偽陰性は，NIPTが陰性と診断されていたにもかかわらず罹患児が出生することで，妊婦や家族に相当の精神的ストレスを与えると考える．場合によってはNIPTの検体の取り違えの可能性などを指摘される場合もある．児の染色体検査ですべての細胞が染色体異常であっても，胎盤のモザイクの可能性があるので，胎盤のマイクロアレイ法などで確認することが望ましい．また胎児DNA濃度が低値の場合にも起こりうるので，NIPTの結果の見直しも重要である．

偶発的所見

悪性腫瘍

悪性腫瘍は染色体異常を有する可能性があるので，妊婦が悪性腫瘍に罹患している場合に，その影響でNIPTが偽陽性や判定保留になることがある．妊婦の悪性腫瘍合併は比較的珍しいが，それでも1,000人に1人程度はあるとされる[14]．NIPT偽陽性例の15%程度を占めるともいわれる[12]．悪性腫瘍が疑われるNIPTの結果として，複数の染色体の異数性について陽性となっている妊婦が報告されており，そうした39例のうち7例でがんがみつかっている[15]．ただし，頻度としてはNIPT実施の10,000例に1例程度であり，稀なケースには違いないが，このような複数の染色体にわたるNIPT陽性の妊婦で羊水検査を実施して胎児の染色体異常がないという結果になった場合には，悪性腫瘍の合併に注意する必要がある．悪性腫瘍の種類としては，白血病やリンパ腫などの血液系のがんが比較的多いが，大腸がんなど他のものでも報告されている．これは血液系のがんではがん細胞が血液中にあるため，その壊死が直接的に母体血中に流入することからNIPTに影響を及ぼしやすいものと推定される．なお，悪性腫瘍ではなくて良性腫瘍の子宮筋腫が原因で偽陽性になった例もNIPTコンソーシアムで報告されている．

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検査目的対象外の胎児染色体異常

日本ではNIPTは21と18，13の異数性を調べる目的で実施される．これ以外の染色体異常については検査対象となっていないが，これらの検査対象外の染色体異常が染色体21と18，13の定量値に影響して，偶発的にみつかる可能性がある．NIPTで最も普及しているMPS法（massively parallel sequencing法）は各染色体由来のゲノムの量を定量して，標準範囲からどの程度解離しているかで胎児の染色体異常を予測する．胎児がトリソミー21の場合は全体のゲノム量に占める21番由来の染色体の割合が増加することで陽性の判定を行う．必ずしも検査対象となる染色体の異数性でなくても，いずれかの染色体由来のゲノム量が増加すると，他の染色体のゲノム量は相対的に，すべて低下することになる．

おわりに

NIPTはDNA解析装置の高性能化に伴い，海外ではすべての染色体の異数性や7Mb以上の微小欠失，突然変異や常染色体優性遺伝による単一遺伝子疾患，一部の常染色体劣性遺伝病の診断にも広がっている．適応が拡大することで，従来は想定されていない，または頻度は少ないが妊娠の予後に大きな影響を及ぼす病態がみつかる可能性も拡大することになる．NIPTを提供するうえで留意しておく必要がある．

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特集　不妊症・不育症の標準的治療と先端技術の課題

生殖医療と遺伝カウンセリング

澤井英明

キーワード：遺伝カウンセリング，生殖医療，不妊治療，着床前診断

はじめに

生殖医療は受精という遺伝の本質に迫る領域を扱う医療であり，その点から生殖医療は本来，遺伝医療と密接な関連がある．しかし，以前の“不妊治療”の時代は長らく卵管通過性の確保や排卵誘発，そして人工授精までがその守備範囲であり，生殖補助医療（ART）が導入されるまでは，不妊治療で直接受精を扱うことはなかった．そのため生殖医療と遺伝医療はそれぞれ別々に発展してきたが，医療技術の発展に伴い相互に接近することとなり，体外受精・胚移植（IVF-ET），顕微授精（ICSI），そして着床前診断（preimplantation genetic testing：PGT）に至る過程で生殖医療は遺伝子医療や出生前診断と密接な関連を持つようになった（図1）．

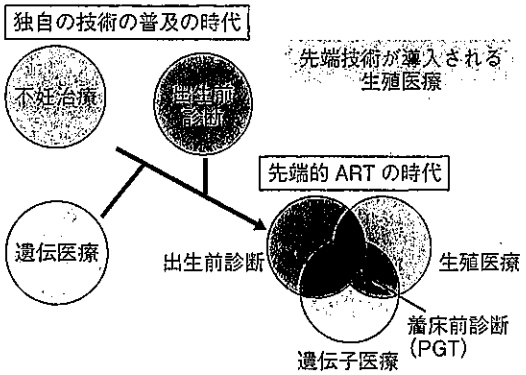


図1　生殖医療と遺伝医療
不妊治療，出生前診断，遺伝医療がそれぞれ発展する過程で，相互に接近融合してきた．
（柴原浩章他編：図説よくわかる臨床不妊症学 一般不妊治療編，第3版，中外医学社，東京，2016；104より改変）

Ⅰ 遺伝カウンセリング

遺伝カウンセリングとは，本人または家族（以下，クライアント）が遺伝や遺伝性疾患に関して気になる，知りたいと希望する場合に具体的な内容を聴取し，それに対する情報提供を行い，クライアントが課題を解決したり，完全に解決できなくても，自主的に適切な選択が行えるように支援することである．本人が疾患に罹患しているか否か，症状の有無にかかわらず実施されるという点で，患者を対象とする一般診療とは全く異なる．

通常は遺伝カウンセリングの結果，提示された選択肢がいくつかあるとして，どれを選んでも間違いではないが，その選択の結果が必ずしもクライアントにとって望ましい結果になるとは限らない．また，遺伝性疾患の要因は家系内で共有されている可能性があり，1人の患者の病気だけではなく，潜在的に家系内でその疾患に関連している人が複数いることもある．遺伝カウンセリングの結果によっては，家系内の遺伝的な背景が意図せず明らかになる場合もある．

Ⅱ 生殖医療における遺伝カウンセリング

染色体異常などの遺伝学的要因が妊娠に及ぼす影響を考えると，不妊症・反復流産（不育症）・先天異常児の出生は同じ原因の影響の程

さわい・ひであき：兵庫医科大学産科婦人科学 教授，兵庫医科大学病院遺伝子医療部 部長

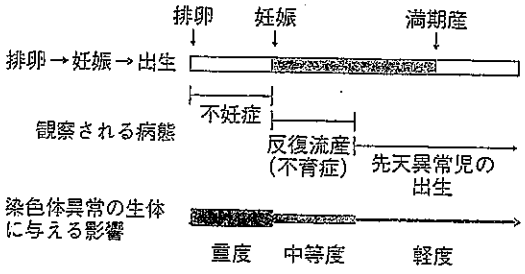


図2　不妊症・反復流産（不育症）・先天異常児の出生と染色体異常の関係

遺伝学的要因が原因の場合は不妊症，反復流産（不育症），先天異常児の出生は，染色体異常の及ぼす影響の程度の差にすぎないこともある．

（柴原浩章他編：図説よくわかる臨床不妊症学 一般不妊治療編．第3版，中外医学社，東京，2016；101より改変）

度の差にすぎないことが分かる．たとえば，染色体異常の及ぼす影響が大きければそもそも受精ができず不妊症となり，中等度であれば妊娠はするが途中で流産し反復流産（不育症）となり，軽度であれば妊娠は継続し先天異常を有する児が出生する（図2）．

ARTは標準的な不妊治療として広く臨床応用され，その成績を大きく向上させており，高い治療効果は明らかである．しかし，いくつかの遺伝学的な課題があることも確かであり，ARTによる治療を開始する前にあらかじめ遺伝カウンセリングとして対応しておくことが適切である．

III 生殖医療を実践するうえでの遺伝学的な課題

1. 不妊症や反復流産の原因としてカップルのどちらかが染色体異常保因者である可能性

不妊症のカップルが染色体検査を行う時期について明確なコンセンサスはない．一般人口の染色体異常の頻度は，新生児の調査で0.84～0.92%程度とされている[1]．不妊症で治療対象となるのは，多くの場合は外見が正常で知的障害もない女性や男性であり，その頻度は染色体の均衡型構造異常（0.52%）と性染色体異常（男性0.115%，女性0.075%）の合計に近いと考えられ[2]，男性では0.64%，女性では0.6%程度となる．したがって，不妊を主訴に受診したカップル全員に染色体検査を行うのは効率的とは言えない．

しかし，IVF-ETに進んだカップルでは女性の1.5%，男性の1.1%に染色体異常を認めたという報告があり[3]，ICSIの治療を受けているカップルでは女性の1.2～2.1%，男性の2.2～6.1%に染色体異常を認めたという報告がある[3,4]．これらの報告を考慮すると，ART（IVF-ETやICSI）治療を行っても，良好胚を得られないようなケース（特に原因不明の場合）では，カップルの染色体検査を考慮してよいと思われる．

染色体検査を行う場合は，できるだけカップルで同時に実施するほうがその後の方針を明確にするうえで望ましい．ただし，染色体構造異常が見つかった場合に，どちらかが染色体構造異常の保因者であるということは知りたいが，それがどちらであるかまでは知りたくないというカップルもいるので，検査を実施する際と結果に異常があって説明する際には，必ずカップルにその意向を確認してから告知する．

遺伝学的検査を受ける場合の原則は，染色体検査を受けるか受けないか，また検査結果の開示を求める（知ること）か求めない（知らないこと）かは，被検者の意思によるということである．この知る権利と知らないでいる権利についてはしばしば誤解されているが，これはあくまで被検者が選択肢を提示されたうえで，それを受けるか受けないか（検査結果を知るか知らないでおくか）を決める権利を意味するもので，選択可能なオプションがあるのに，知らないでいる権利があるから被検者には知らせないでおくという意味ではない．不妊症のカップルは治療を受けて，妊娠し分娩に至ることを望んで受診しているのだから，標準的な検査方法として提示できるものは提示する必要がある．

2. ARTによる妊娠で出生した児の先天異常のリスク

新生児の明らかな外見的形態異常の一般頻度は約2%とされており，これに精神発達遅滞と遺伝性疾患を加えると約3〜4%に先天異常を認めることになる[5]．各種の大規模調査では，ARTによる妊娠と先天異常の関連について，ARTによる妊娠で先天異常児の出生率が上昇するというものと上昇しないというものがある[6~9]．

ARTによる治療自体が新生児異常を増加させるかは明確ではないが，結果的にARTによる妊娠では新生児異常率が上昇すると思われる．これは自然妊娠と比較した場合，ARTによる不妊女性特有の不利な条件がいくつかあるためである．たとえば，一般に不妊女性は自然妊娠女性より高年齢であること，不妊原因としてカップルのどちらかが染色体構造異常の保因者であること，重度の男性不妊症では精子に染色体異常を生じる可能性が上昇することなどである．ARTが直接の原因ではないが，結果的にARTを受けたカップルから出生する児の先天異常率は上昇する．

3. ゲノム刷り込み現象（インプリンティング）の異常による疾患発症

ゲノム刷り込み現象（インプリンティング）とは，父親由来の遺伝子と母親由来の遺伝子で発現が異なる現象を言う．通常の遺伝子は父親または母親由来の染色体のどちらでも発現の制御は等しく受けるのが基本であるが，一部の遺伝子では父親または母親由来のどちらかのみが発現する．この場合に発現しないほうの遺伝子は刷り込みを受けていると表現され，遺伝子のメチル化による不活性化がその本態とされている．

本来発現するべき父親または母親由来の遺伝子が，何らかの原因（遺伝子変異や欠失）により正常に発現しなかった場合や，本来は父親と母親から1本ずつ由来すべき染色体が，2本ともいずれか一方に由来し，かつそれが不活性化遺伝子を持つ場合（片親性ダイソミー）には，遺伝子が不活性化されてしまい，これが疾患につながることがある．このゲノム刷り込み現象が関連する疾患として，Prader-Willi症候群やSilver-Russell症候群，網膜芽細胞腫などが知られている[10]．Angelman症候群，Beckwith-Wiedemann症候群は，ARTによる妊娠でゲノム刷り込み現象の関連する疾患の頻度が上昇する可能性が指摘されている．ただ，Prader-Willi症候群やSilver-Russell症候群への関連性は否定的である[11]．

4. ICSIと染色体異常妊娠の関連性

ICSIによる妊娠は1992年に初めて報告されて以来，胎児異常，特に染色体構造異常妊娠との関連について情報が蓄積されてきた．当初の適応であった男性不妊，すなわち重度乏精子症患者の精子を用いることの安全性について，重度乏精子症の男性不妊症にICSIを行った場合に一部の染色体異常妊娠が増加するとされている．

信頼のある多数ICSI妊娠症例の解析報告の1つに，2002年にBonduelleらが出生前診断による染色体異常の頻度を報告したものがある[1]．それによると，1,586例のICSI妊娠での出生前診断による染色体異常は47例（2.96%）で，このうち親由来と考えられる転座型などの染色体構造異常が22例（1.39%），それを除外した純粋な突然変異（*de novo*）の染色体異常が25例（1.58%）となっている．この25例のうち性染色体異常は10例（0.63%），常染色体数的異常が8例（0.5%），転座型などの構造異常が7例（0.44%）となっている．

これらの染色体異常率はいずれも報告による一般頻度の3〜5倍程度に増加している．このうち常染色体数的異常（21トリソミーなど）はICSI妊娠女性の年齢が比較的高齢であることから，年齢を考慮すると異常率の増加は説明できる．しかし，性染色体異常と染色体構造異常の頻度は年齢の影響はほとんどないとされてお

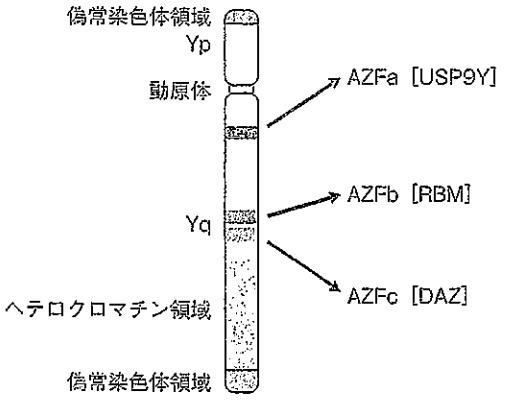
り，高年齢妊娠では説明できず，ICSI妊娠で性染色体異常と染色体構造異常は増加していると考えざるをえない．

ただし，これらの染色体異常が見られた症例はICSIの適応が男性因子であり，重度乏精子症の男性では染色体は正常でも精子が染色体異常を有している比率が一般頻度より高いと報告されていることから，精子での染色体異常の増加によってICSIで性染色体異常と染色体構造異常が増加すると考えられる．ICSIの操作自体が胚に障害を与えて染色体異常が増加するわけではないとしても，結果的にはICSI妊娠での染色体異常は一般頻度に比べて増加することになるので，ICSI実施の際にはこうした点を事前に説明しておくことが必要と考える．

5. 重度乏精子症におけるY染色体の微小欠失と次世代への伝播の問題

重度乏精子症や非閉塞性無精子症の男性の約3～15%にY染色体長腕に位置するazoospermia factor（AZF）遺伝子の微小欠失が検出される（図3）．重度乏精子症の不妊カップルでは，自然妊娠では妊娠不可能であったとしても，精巣生検などでわずかでも精子が回収できればICSIで妊娠が可能である．しかし，微小欠失の部位や範囲が関連するとされている[12,13]．

また，精子が回収でき妊娠が成立した場合に児が男児であれば，Y染色体を受け継ぐので，微小欠失も受け継ぐことになり，その男児が成人になった際に乏精子症となる可能性が高い．この微小欠失は世代を経る際に新たに生じたり，拡大したりすることもあり，Y染色体の欠失を持たないまたは微小欠失が非常に軽度の乏精子症の父親から受け継いだY染色体が，新たに微小欠失を生じさせたり，欠失の範囲を拡大させたりして，その男児が重度乏精子症になったりする可能性もある[14]．これらはまだ十分な実証には至っていないものの可能性については留意しておく必要がある．造精機能にかかわる遺伝子は常染色体上にも存在するため，その機能の全体像が解明されたわけではないが，ICSIによる造精機能障害の次世代への伝播の可能性についてはARTを行う前に遺伝カウンセリングで説明する必要がある．



USP9Y：ubiquitin specific peptidase 9, Y-linked
RBM：RNA binding motif
DAZ：deleted in azoospermia

図3　Y染色体長腕に位置するAZF遺伝子の微小欠失

微小欠失の部位や範囲が関連するとされ，AZFa完全欠失やAZFb完全欠失，AZFb+c完全欠失などでは精巣内精子採取術（testicular sperm extraction：TESE）を行っても精子回収の可能性はきわめて低いとされている．一方，AZFcのみの完全欠失であればTESEによる精子回収の可能性は高い（70%程度）．

（柴原浩章他編：図説よくわかる臨床不妊症学 一般不妊治療編．第3版，中外医学社，東京，2016；99より改変）

おわりに

生殖医療における遺伝カウンセリングでは，不妊治療を受けるカップルにとってARTの遺伝的リスクや未解明な点を理解するということが重要である．しかし，ARTを提供する医療者側にとっても，一定の確率で生じる先天異常や染色体異常のリスクを患者にあらかじめ理解してもらうことは，トラブルを未然に防ぐという意味でも大きな役割を持つ．これまで遺伝カウンセリングは臨床遺伝専門医や生殖医療専門医などの医師が行うことが多かったが，近年は遺伝カウンセリングの専門職である認定遺伝カウンセラーがART施設でも活躍を始めており，充実した遺伝カウンセリングがなされつつある．

[COI 開示] 本論文に関して発表者に開示すべき COI 状態はない

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特集　ビッグデータ時代のゲノム医学

Ⅵ. 胎児期・周産期疾患のゲノム医学

胎児・周産期疾患のゲノム医学

澤井　英明

Key words：胎児，周産期，ゲノム医学，NIPT，遺伝学的検査

ここ数年の胎児の出生前診断の大きな変化は，“超音波検査”と“母体血中細胞フリー胎児DNAを用いた新しい無侵襲的出生前遺伝学的検査(non-invasive prenatal testing；NIPT)”によりもたらされた。妊娠10-13週ごろに実施される超音波検査では，胎児の頸部浮腫(nuchal translucency；NT)，静脈管血流量，三尖弁逆流，鼻骨低形成などを調べることで，胎児が21トリソミーや他の染色体異数性に罹患しているリスクを調べることができる。また，同様の目的では，超音波検査と母体血清マーカー検査(inhibin-Aとpregnancy associated plasma protein；PAPP)を組み合わせた複合検査(combined test)も実施されている。超音波検査は特定の妊娠週数に限らず実施されていることから，偶発的に見つかる先天性の胎児異常には，単一遺伝子疾患のこともあるが，超音波検査のみでは確定診断は困難である。そのような場合には遺伝学的検査が必要になる。

Ⅰ. 遺伝カウンセリング

出生前診断の範囲は様々であるが，図1に示すように遺伝カウンセリングとインフォームド・コンセントの区別と重複，そして超音波検査とNIPTなど遺伝学的検査の区別と重複は非常に複雑化している。臨床遺伝専門医と超音波専門医を両方取得している医師は極めて少なく，それぞれの領域が現在以上に高度化すると，1人ですべての検査を担当できる医師はほとんどいなくなる状況が想定される。出生前診断を専門にする特定の施設への集中が進む可能性がある一方で，こうした幅広い出生前診断の普及は，妊婦健診に通院している施設で実施することへのニーズが高まるとも思われる。

Ⅱ. 確定診断

偶発的な先天性の胎児異常は妊娠の2-3%にみられ，単一遺伝子疾患であれば遺伝学的検査により確定診断が可能なこともある。確定診断できれば予後の予測が可能となり，妊娠21週以前であれば妊娠継続の可否を判断できる。妊娠継続の場合には妊娠経過の予測や分娩方式の決定が可能になり，出生後の新生児については早期の治療が可能になる。原則的には，こうした胎児の疾患の確定診断のための遺伝学的検査は絨毛検査や羊水検査など侵襲的検査が必要である。

羊水検査は妊娠16週ごろから実施され，流産リスクは海外では約1%(わが国では0.3%)とされてきたが，近年は技術や器械の技術・精度の向上から，このリスクは実際には0.1%程度とされている[1]。絨毛検査は妊娠10週以降14週までに実施され，わが国では流産率が1%程度とされている。羊水検査より流産率は高いが，これは羊水検査より実施週数が早いからで，検査自体に伴う流産リスクは絨毛検査と同様とされている[1]。

Genomic medicine of fetus and pregnancy
Sawai Hideaki：兵庫医科大学病院遺伝子医療部(〒663-8501　兵庫県西宮市武庫川町1-1)

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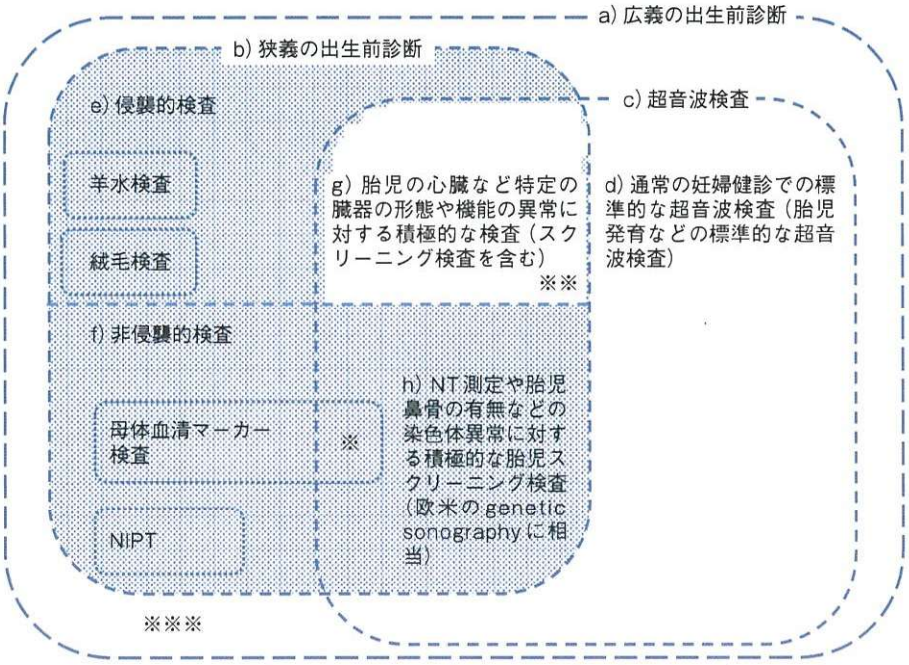


図1　出生前診断における遺伝学的検査の位置づけ

広義の出生前診断a）は，出生前に胎児の状態を調べるすべての検査・診断を含んでいる。そして，超音波検査のうち，通常の妊婦健診で実施される標準的な超音波検査d）を除くものが狭義の出生前診断b）に位置づけられる。超音波検査c）は通常の妊婦健診で実施される標準的な超音波検査d）と積極的なスクリーニング検査g）h）に分類される。この図で遺伝学的検査とは網掛けの部分e）f）h）を示し，この部分は遺伝カウンセリングを必要とする。狭義の出生前診断にはインフォームド・コンセント（IC）を必要とするが，そのうちこの網掛けの部分は遺伝学的検査に相当するので，ICだけではなく，遺伝カウンセリングが必要である。

通常の妊婦健診で実施される標準的な超音波検査d）は狭義の出生前診断b）には含まれず，実施にあたってICは必ずしも必要とはしない。しかし，超音波検査であっても積極的なスクリーニング検査g）h）については，遺伝学的検査であってもh）なくてもg），狭義の出生前診断b）に位置づけられ，ICが必要である。また，母体血清マーカー検査や母体血を用いた新しい出生前遺伝学的検査（NIPT）は狭義の遺伝学的検査b）である。

日本産科婦人科学会の産科診療ガイドライン2018でd）は通常超音波検査，h）は胎児超音波検査と呼んでいるものに相当する。

※は妊娠初期に母体血清マーカーと超音波検査を組み合わせたcombined testによるfirst trimester screening；FTS（妊娠10-13週）であり，わが国では普及していないが海外では標準的なスクリーニング検査とされている。また，この図は超音波検査についてしか記載していないが，超音波検査以外の胎児の画像診断検査（MRIやCT）などは位置的には※※の部分に該当する。また，NSTなど胎児心拍数図やE_3など胎盤機能検査はa）の内側でb）c）の外側に位置する※※※。

かつてはG分染法による染色体検査か，サンガー法による特定の疾患遺伝子変異の同定しか方法がなかったが，染色体マイクロアレイ法（chromosomal microarray analysis；CMA）や次世代シーケンシング（next generation sequencing；NGS）により詳細な分析が可能になり，従来の細胞遺伝学的出生前検査と比較して解像度が向上し，診断の範囲と精度が拡大した。すなわちCMAにより，異数性と大きな染色体構造異常のみならず，微小欠失および重複が検出されるようになった。具体的にCMAが胎児染色体異常の検出率を改善するかどうかの研究では，通常は検査の適応とはされない低リスクグループで，従来の核型分析と全ゲノムCMAの両方を解析した報告がある[2]。CMAを併用すると，超音波検査で異常があった場合にはこれらの遺伝学的検査の実施で27.4％に原因が見つかるとされた。更に，G分染法やCMAに加えて，出生前診断にNGSを取り入れることで，疾患の原因を同定することができるようになる。具体的な方法としては，疾患特異的遺伝子パネル検査や全エクソーム解析（whole exome sequencing；WES），全ゲノム解析（whole

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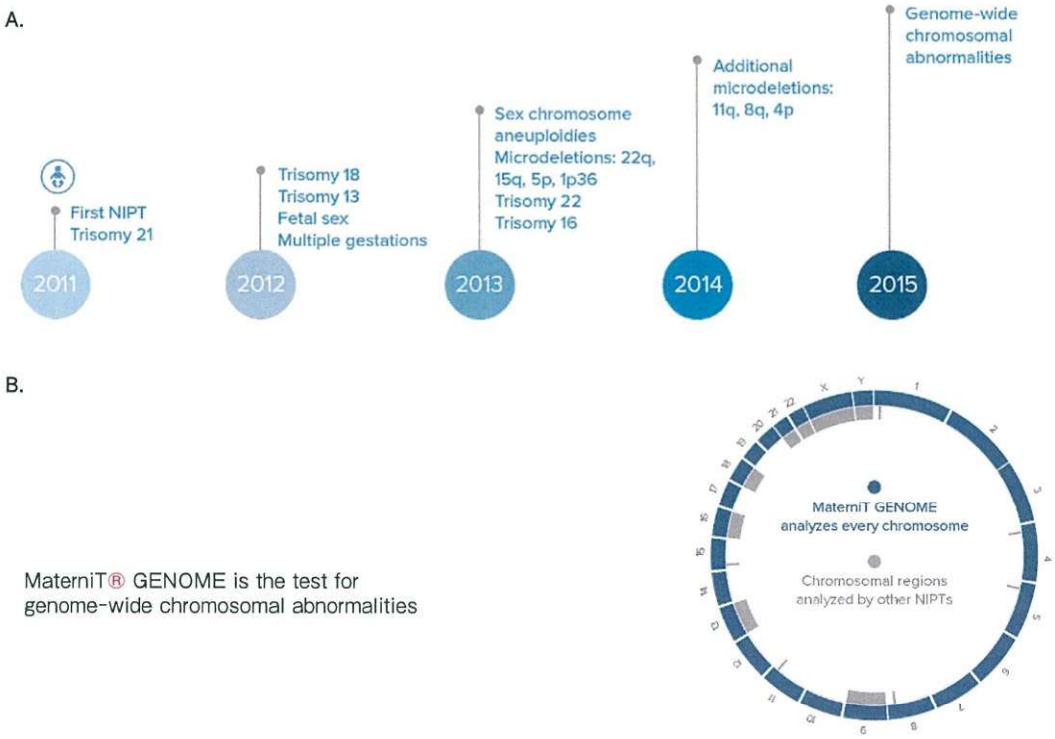


図 2　NIPT の検査対象疾患の拡大

わが国では NIPT の対象疾患は 13，18，21 トリソミーに限定されているが，米国では毎年のように対象疾患が拡大し続けている。(周産期医学 2016 年 46 巻増刊号周産期医学必修知識より引用)

genome sequencing；WGS)が検討されつつある。WES のコホートでは，胎児形態異常がある場合に WES を実施し，全体的な診断率は 8.5％と 10.3％と報告された[3,4]。

Ⅲ．スクリーニング検査としての NIPT

NIPT は，妊婦の血液の中に含まれている細胞フリーの胎児 DNA (cell-free DNA；cfDNA) を NGS を用いて定量し，それがいずれの染色体に該当するかの割合を算出して，コントロールと比較し，胎児が染色体異常に罹患しているかどうかを検出する。検査の結果が陽性と診断された場合は罹患の可能性が高く，羊水検査や絨毛検査などの侵襲的検査によって確定診断をする必要がある。母体血中の胎児由来 cfDNA は胎盤栄養膜に由来する。染色体の異数性 (13，18，21 番) を対象とするものが広く普及している。cfDNA の感度と特異性に関する様々な研究のメタ分析では，21 トリソミーの検出率は 99.7％であり，偽陽性率は 0.04％，18 トリソミーの検出率は 98.2％であり，偽陽性率 0.05％，13 トリソミーの検出率は 99％，偽陽性率 0.04％，モノソミー X の検出率は 95.8％で，偽陽性率 0.38％とされている[5]。いずれも陰性と診断された場合には，胎児がこれらの染色体の異常を持つ可能性は極めて低く (偽陰性率は 0.01％以下)，流産リスクのある侵襲的検査を避けることができる。

米国では Sequenom 社は 2015 年 9 月から MaterniT GENOME という最新の検査方法を提供している (**図 2**)。これによると，常染色体と性染色体のすべての染色体の数的異常や 7 Mb 以上の部分欠失・重複の同定が可能であり，また 22q11 など比較的頻度の高い部分欠失 7 種類が検査可能である。しかし，NIPT で胎児由来とされる DNA は厳密には絨毛組織由来であり，このことが NIPT の偽陽性や偽陰性の可能性を精度向上だけでは解決し得ず，確定診断検査にはならないという限界がある。また，胎児 DNA 濃度が低い

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表　出生前診断・検査の比較

	確定診断検査		非確定診断検査	
	羊水検査	絨毛検査	母体血清マーカー検査（＋超音波検査）	NIPT
検体	羊水（胎児細胞）	絨毛（絨毛細胞）	母体血（母体血中胎児・胎盤由来タンパク質）	母体血（母体血中絨毛由来DNA）
安全性の限界	流産リスク0.3% 出血・破水等あり	流産リスク1% 出血・破水等あり	流産リスク等なし	流産リスク等なし
検出可能疾患	染色体の異数性と構造異常 遺伝子変異	染色体の異数性と構造異常 遺伝子変異	21，18トリソミーと神経管欠損症	全染色体の異数性と構造異常，特定染色体の微小欠失，全染色体の7 Mb以上の欠失（わが国では21, 18, 13トリソミーのみ）
位置づけ	侵襲的・確定的検査	侵襲的・確定的検査	非侵襲的・スクリーニング検査	非侵襲的・スクリーニング検査
検査自体の限界		胎盤限局性モザイクが1%程度あり	低陽性的中率	モザイク・低胎児DNA濃度・母体合併症による判定保留

場合には検査ができないことや，母体の疾患によっては母体血中のDNAの検出パターンに影響を及ぼすことがあり，例えば母体の悪性腫瘍や自己免疫疾患などの場合に，判定不能などの結果になり得る。従来の侵襲的検査に迫るNIPTであるが，実施にあたっては他の検査方法との比較，限界などに留意する必要がある（表）。

染色体の異数性については，全染色体に対して実施可能となっており，突然変異を中心に多数の単一遺伝子疾患の検査としても実施可能となってきた[6]。しかし，結果がでないという“判定保留”が報告される場合がある。胎児DNAの割合が過少，母体の肥満，胎盤モザイク，母体の腫瘍などがその原因とされている。

Ⅳ．全ゲノム解析のもたらす危惧

NIPTでも侵襲的検査でも，遺伝学的検査は技術的には全ゲノム解析を実施することが可能になっており，こうした高度化は意義不明の変異（variant of unknown significance；VUS）の検出という重大な危惧を含んでいる。例えば，従来から報告のある明確に疾患意義のある欠失や重複が検出されて，しかも超音波検査などで疾患の症状が同定される場合は遺伝子検査としての診断的意義は確実と思われるが，一方で高年妊娠というだけで妊娠経過に異常なく経過している場合に，こうした診断意義不明の変異が検出されると，それをどう解釈してよいかが判断しにくい。すなわち，ヒト遺伝子の塩基配列は全く正常であっても全く同じではなく個人差があり，個体間の塩基配列の違いが存在する。一般集団中で100人に1人（1%）以上の頻度で認められる病的意義のない遺伝子変異は遺伝子多型（genetic polymorphism）とされる。しかし，一部には既知の遺伝性疾患の原因遺伝子に認められる比較的低リスクの，原因遺伝子変異となる場合がある。こうした低頻度の変異がVUSとして報告される。このような変異が胎児に検出されると，それが突然変異か親由来かの検討に進むが，突然変異であった場合には病的意義が否定できない。しかし，その場合も重症度や表現型の予測は極めて困難で，出生前診断をしなければ健常児を得たのに，はっきりしない結果で不安になり妊娠を諦めるといったことが起こり得る。そのため，WESを実施する際にはより確実さを担保するために，胎児と両親のいわゆるトリオ解析を行うことで，かなりの部分は正確に判断可能となるが，それでも胎児の表現型に異常がない場合は解釈が困難であったり，父性が異なる可能性も否定はできないなど，確実ではない。

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おわりに

ゲノム医学の進歩が，総体的には人類の福祉に貢献してきたことは疑いがない。しかし，個々の点では進歩したことで生じる矛盾や後退があり，これは出生前診断についても同じである。検査の限界や課題を認識しつつ進めていくことが重要である。

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ARTICLE

Maternal age-specific risk for trisomy 21 based on the clinical performance of NIPT and empirically derived NIPT age-specific positive and negative predictive values in Japan

Takahiro Yamada[1,2,11] · Akihiko Sekizawa[1,3] · Yosuke Fujii[4] · Tatsuko Hirose[1,3] · Osamu Samura[1,5] · Nobuhiro Suzumori[1,6] · Kiyonori Miura[1,7] · Hideaki Sawai[1,8] · Fumiki Hirahara[1,9] · Haruhiko Sago[1,10]

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Abstract

The data collected by nation-wide study of noninvasive prenatal genetic testing (NIPT) for trisomy 21 from 21,610 pregnant women with advanced maternal age in Japan were reported. Among 188 NIPT-positive cases, 180 cases were true positive. The incidence of aneuploidy according to maternal age was estimated using a state-space model. Although, the frequency of trisomy increased exponentially with maternal age as previously reported, the maternal age-specific risk for trisomy 21 that was based on the clinical performance of NIPT was lower than the predicted risk in previous Western cohorts based on the data from invasive prenatal testing (Bayesian two-sided tail-area probability $P = 0.0156$). The empirical positive predictive value (PPV) of NIPT is likely to turn out higher than that of the theoretical PPV calculated from the sensitivity/specificity of the test and the incidence of trisomy 21 from this study.

Introduction

Information regarding the live birth incidence of trisomy is indispensable in prenatal genetic counseling. The incidence at different stages of pregnancy is also useful for identifying necessary prenatal genetic tests [1]. The sensitivity and specificity are used to assess the efficacy of each screening test and the positive predictive value (PPV) and negative predictive value (NPV) are of great concern in clinical testing because they depend on prior probability, or maternal age-specific and gestational age-specific risks of trisomy. The maternal age and gestational age-specific risks have been previously reported [1–3], and are calculated using the data from chorionic villus sampling (CVS) (10–14 weeks), amniocentesis (15–20 weeks), and live birth (term) cases.

NIPT is a noninvasive and highly reliable screening test. Unlike invasive procedures, NIPT carries no risk of miscarriage or harm to the unborn child, and the characteristics of NIPT clients at advanced maternal age are similar to those of the natural population.

✉ Takahiro Yamada
taka0197@kuhp.kyoto-u.ac.jp

1 Japan NIPT consortium, Tokyo, Japan

2 Department of Obstetrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan

3 Department of Obstetrics and Gynecology, The Showa University School of Medicine, Tokyo, Japan

4 Kyoto University Graduate school of Medicine, Kyoto, Japan

5 Department of Obstetrics and Gynecology, The Jikei University School of Medicine, Tokyo, Japan

6 Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

7 Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

8 Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan

9 Department of Obstetrics and Gynecology, Yokohama City University Graduate School of Medicine, Yokohama Japan

10 Center of Maternal-Fetal, Neonatal and Reproductive Medicine, National Center for Child Health and Development, Tokyo, Japan

11 Present address: Clinical Genetics Unit, Kyoto University Hospital, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

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In Japan, NIPT is not a standard medical test, is permitted only as part of clinical research, and all the collected data are always secured and controlled.

The objective of the present study is to empirically estimate the maternal age-specific risk for trisomy 21 using the data collected by nation-wide NIPT research and maternal age-specific NIPT-PPV and NIPT-NPV, which is based on clinical NIPT data in Japan.

Method

This was a multicenter prospective cohort study conducted in Japan with the approval of the institutional review board of ethics at each respective institution. The data were collected from a total of 21,610 pregnant women undergoing NIPT (massively parallel sequencing: MaterniT21 Plus®□ and GeneTech NIPT) with advanced maternal age (age: 38.48 ± 0.02, gestational weeks: 13.36 ± 0.01) between April 2013 and December 2015 at 38 medical institutions in Japan. To avoid any biased feedback, the data from the patients who had undergone ultrasound screening before NIPT were excluded.

The estimated risk of trisomy 21 for our data set was compared with that of previously reported data from Western countries [1, 2, 4–7].

The incidence of aneuploidy according to maternal age was estimated using a state-space model [8-10]. The incidence of aneuploidy and observed aneuploidy were modeled according to the following two formulae (where Eq. 1 denotes state process and Eq. 2 denotes observed process):

$$p_t - p_{t-1} \sim p_{t-1} - p_{t-2} + \epsilon_t \tag{1}$$

$$w_t \sim \text{binomial}(p_t), \tag{2}$$

where t was maternal age, $p_t \in (0, 1)$ was incidence to be estimated, $w_t \in \{0, 1, 2\ldots\}$ was the observation count of aneuploidy, and ϵ_t was Gaussian noise. The incidence of aneuploidy generally monotonically increases according to maternal age [1, 2, 4, 6]. Monotonic increase was assumed to occur according to the formula:

$$p_i \leq p_j, (i<j) \tag{3}$$

Besides estimating the incidence of aneuploidy in our data set, we estimated the incidence in other data sets from Australia [2] and Japan [3] as a reference. Estimation was performed using R 3.4.0 (https://cran.r-project.org/) and rstan 2.15.1 (http://mc-stan.org).

PPV and NPV were calculated exclusively based on the data from the patients whose birth outcomes were confirmed.

In this study, "theoretical PPV" (PPV_t) and "empirical PPV" (PPV_e) were defined as following:

$$\text{PPV}_t = \frac{pS_n}{pS_n + (1-p)(1-S_p)}, \tag{4}$$

$$\text{PPV}_e = 100\frac{w_{\text{true}}}{w_{\text{NIPT}}}, \tag{5}$$

where p was incidence of trisomy, S_n was sensitivity, S_p was specificity, w_{true} was the number of the true trisomy confirmed by diagnostic test, and w_{NIPT} was the number of positive cases in NIPT data set.

The theoretical PPV and NPV for trisomy 21 were calculated based on a sensitivity of 99.1% and specificity of 99.9% that were reported by Palomaki et al. in the validation study of MaterniT21 Plus® [11].

Results

The number of NIPT-positive cases for trisomy 21 was 188, of which 180 cases were confirmed by invasive testing or karyotyping of POC (PPV 95.7%) (Table 1, Fig. 1). The negative predictive value was 100% because there were no false-negatives identified in this cohort (Fig. 1). Among 21,381 NIPT-negative cases, the cases of which outcomes were confirmed were 16,585. There was no false-negative case in 16,585 cases. In another paper of our study group [12], there were two false-negative cases (one trisomy 21

Table 1 Incidence and positive predictive values of trisomy 21

Maternal age	N	NIPT positive	True positive	Risk	PPV (%)
34	721	2	2	0.28	100
35	2126	5	5	0.24	100
36	2496	9	7	0.28	77.8
37	2785	10	9	0.32	90.0
38	2967	13	12	0.40	92.3
39	3091	30	29	0.94	96.7
40	2803	29	29	1.03	100
41	2021	26	24	1.19	92.3
42	1325	33	32	2.42	97.0
43	733	19	19	2.59	100
44	355	7	7	1.98	100
45	127	4	4	3.15	100
46	38	0	0	0	ND
47	16	1	1	6.25	100
48	3	0	0	0	ND
49	2	0	0	0	ND
50	1	0	0	0	ND
Total	21,610	188	180	0.83	95.7

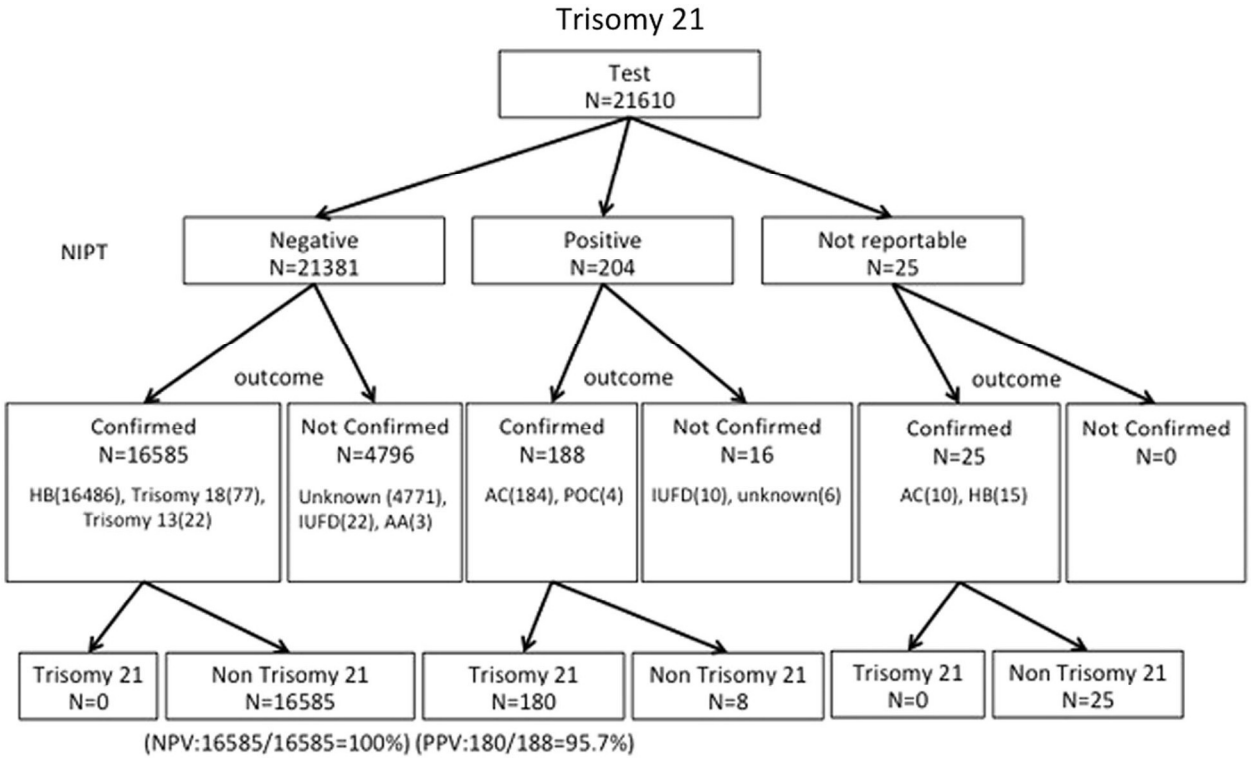


Fig. 1 NIPT results and outcomes for detection of trisomy 21 in 21,610 women. 'Confirmed outcomes' represents clinical confirmation of a healthy baby, or actual karyotyping. IUFD intra uterine fetal death without karyotyping, AA artificial abortion without karyotyping, HB healthy baby, AC karyotyping with amniocentesis, POC karyotyping with product of conception after IUFD

and one trisomy 18) in 13,491 cases. These cases were not included in this study because the indication of these cases was abnormal findings of ultrasound. The frequency of trisomy increased exponentially with maternal age, as previously reported [1-7] (Table 1, Fig. 2a). However, the maternal age-specific risk for trisomy 21 during the CVS period (10–14 weeks) that was based on the clinical performance of NIPT was lower than the predicted risk [1] in Western cohorts (Bayesian two-sided tail-area probability $p = 0.0156$) (Fig. 2a), while the risk for trisomy 18 and 13 were approximately the same (data not shown).

We then calculated the maternal age-specific PPV curve from the maternal age-specific risk for trisomy 21, the detection rates, and the test specificities (Fig. 3). The risk according to the empirical data was higher than that according to the theoretical data, especially among younger age groups.

Discussion

The first aim of the present study was to empirically estimate the maternal age-specific risk for trisomy 21 using NIPT data in Japan. For evaluating the obtained risk for trisomy 21, we compared our data with previous studies. The maternal age and gestational age-specific incidence of trisomy as reported by Snijders et al. [1] is often cited in established textbooks [13]. However, our data of maternal age-specific risk for trisomy 21 was much lower than that of Snijders et al [1]. We re-evaluated the available data by comparing other independent studies. Although the maternal age-specific risk of trisomy reported by Kratzer [5] was similar to that reported by Snijders et al. [1], Halliday [2] confirmed the results of the present study. In comparing studies based on amniocentesis period (Fig. 2b), and term (Fig. 2c) with other studies, large variation was observed among studies, especially in mothers older than 41 years, while the maternal age-specific risks reported by Snijders was much higher. The risk of trisomy 21 might be lower in Japanese people, as the Japanese risk seems to be the lowest based on CVS (our NIPT data) and amniocentesis periods [3]. Other studies[2–7], have also indicated that the incidence of trisomy 21 at each gestational period is lower than that reported by Snijders et al [1]. Our data might therefore represent a more accurate depiction of the trisomy risk than that of Snijders [1]. The results of the present study could be used to evaluate the significance of NIPT data as well as that of other screening tests (maternal serum markers, combined testing, etc.). In Japan, the artificial reproductive technology is prevalent, especially among advanced maternal age. Limitations of this study include that the age of oocyte retrieval was not considered in the analysis.

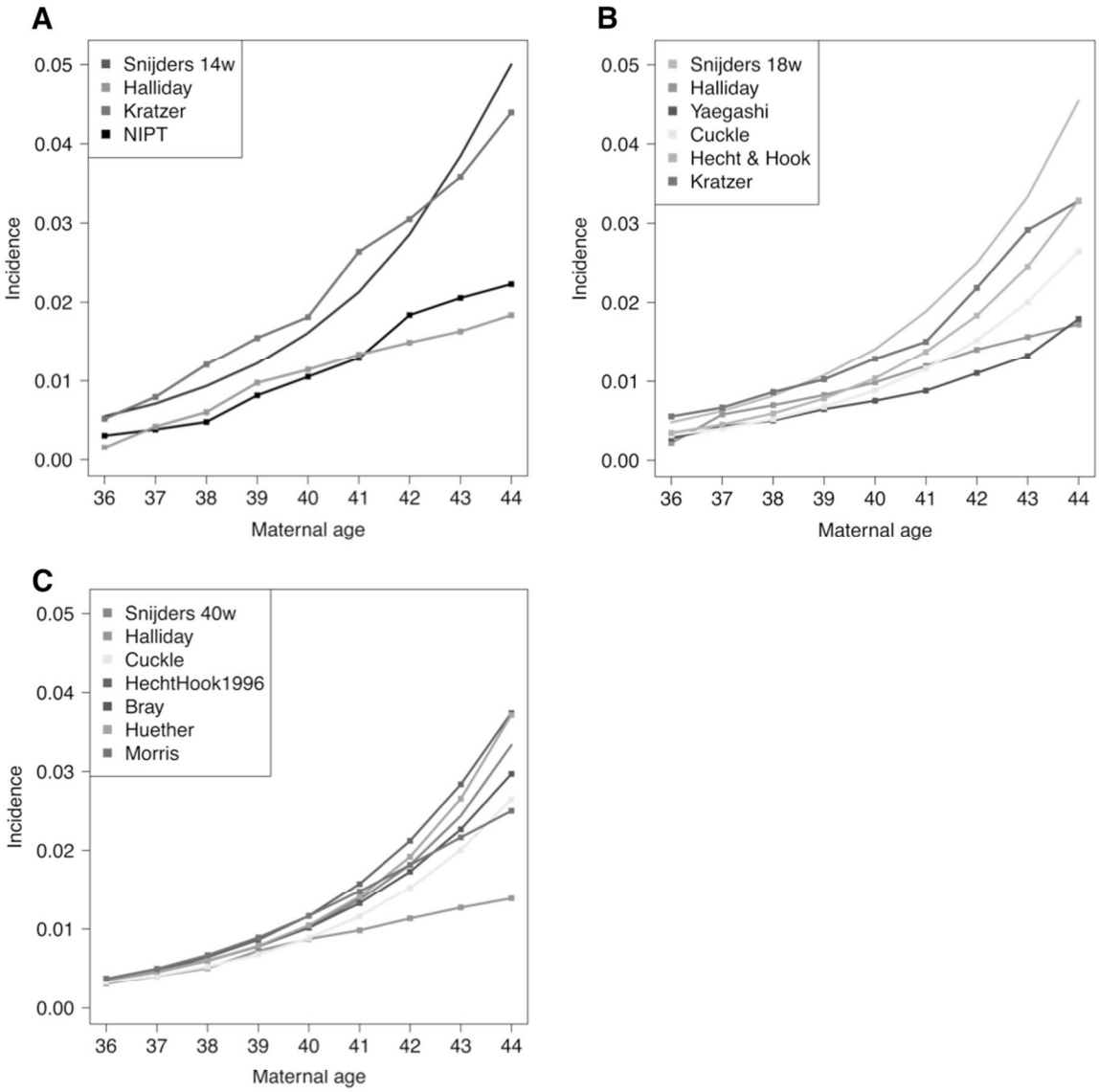


Fig. 2 The maternal age-specific risk for trisomy 21 based on the clinical performance of NIPT in the chorionic villus sampling period (10–14 weeks) (**a**), amniocentesis period (15–20 weeks) (**b**) and live birth period (term) (**c**)

Empirical NIPT/PPV is likely to turn out higher than the theoretical PPV extracted from the lower trisomy 21 incidence, which our study showed, and this is especially true for younger generation. Although the etiology remains unknown, we can postulate a possible explanation. For the calculation of theoretical PPV, we used the sensitivity (99.1%) and specificity (99.9%) reported by Palomaki et al [11]. However, the actual specificity might be much higher now than it was 5 years ago. When we assume the specificity is 99.98%, the theoretical PPV curve rises and is similar to the curve based on the empirical data (Fig. 3). At the least, this is an area that can use further research; A larger sample size in NIPT positive will allow empirical PPVs to be more accurate, but it is safe to say that what is found in this study is rather significant.

In Japan, NIPT is not a standard medical test, but a clinical research performed for patients in higher risk groups. NIPT is permitted only for high-risk pregnant women, including those with advanced maternal age, because the utility of NIPT for low-risk pregnant women had not been established when the Japanese guidelines on new prenatal genetic testing methods using maternal blood were released [12]. As a result, young women may not receive NIPT even if they request it. Recently, in some non-approved clinics, some low-risk pregnant women have received NIPT without proper genetic counseling, resulting in serious problems for patients who received positive results. These patients do not receive the appropriate genetic counseling, and suffer from persistent anxiety, often visiting our hospitals and demanding proper genetic counseling and

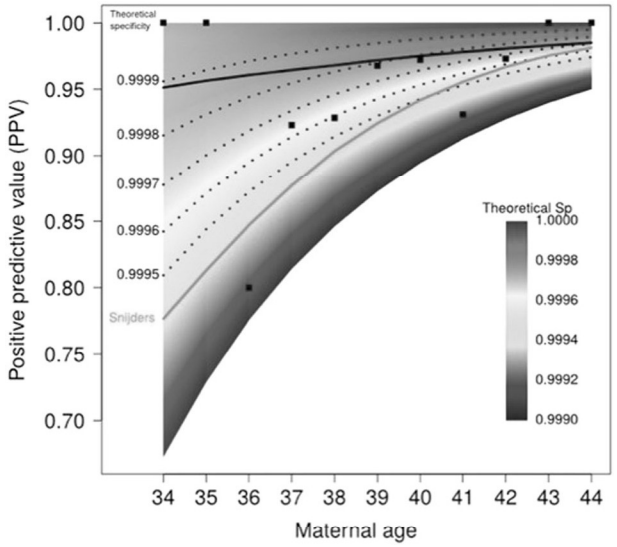


Fig. 3 Maternal age-specific positive predictive value (PPV) of trisomy 21 at 14 weeks of gestation. The color gradation represents the variation in NIPT specificity. The dark blue line located in the bottom of the graph is the PPV curve, which was calculated from the specificity reported by Palomaki et al. (99.90%) [11]. The pink line is the PPV curve, which was calculated based on Snijders et al [1]. The black line is the regression curve of the outlier model from the empirical data obtained in the present study. The empirical PPV was much higher than the theoretical PPV, especially among younger age groups. The theoretical PPV increased with increasing specificity

care. Although some evidence of the efficiency of NIPT in low-to-middle-risk women [14, 15] has been reported, since the release of the guidelines, the guidelines have not been updated. The present study provides further evidence to support the utility of NIPT for low-risk pregnant women, and prompt the revision of the guidelines.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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CORRECTION

Correction: Maternal age-specific risk for trisomy 21 based on the clinical performance of NIPT and empirically derived NIPT age-specific positive and negative predictive values in Japan

Takahiro Yamada[1,2,13] · Akihiko Sekizawa[1,3] · Yosuke Fujii[4] · Tatsuko Hirose[1,3] · Osamu Samura[1,5] · Nobuhiro Suzumori[1,6] · Kiyonori Miura[1,7] · Hideaki Sawai[1,8] · Fumiki Hirahara[1,9] · Jun Murotsuki[1,10] · Yoshimasa Kamei[1,11] · Haruhiko Sago[1,12] · Japan NIPT consortium*[1]

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Correction to: [illegible] advance online publication 30 May 2018: https://www.nature.com/articles/s10038-018-0453-8

Since the advance online publication of this article, the authors of the above paper have noticed errors in the list of authors and affiliations. The article with correct author information now appears in this issue.

The online html and pdf versions have also been rectified.

The authors would like to apologize for the error.

The correct authors and affiliations are listed as follows:

Takahiro Yamada[1,2,13], Akihiko Sekizawa[1,3], Yosuke Fujii[4], Tatsuko Hirose[1,3], Osamu Samura[1,5], Nobuhiro Suzumori[1,6], Kiyonori Miura[1,7], Hideaki Sawai[1,8], Fumiki Hirahara[1,9], Jun Murotsuki[1,10], Yoshimasa Kamei[1,11], Haruhiko Sago[1,12], and Japan NIPT Consortium*[1]

1) Japan NIPT Consortium, Tokyo, Japan
2) Department of Obstetrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan
3) Department of Obstetrics and Gynecology, The Showa University School of Medicine, Tokyo, Japan
4) Kyoto University Graduate School of Medicine, Kyoto, Japan
5) Department of Obstetrics and Gynecology, The Jikei University School of Medicine, Tokyo, Japan
6) Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan
7) Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan
8) Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan
9) Department of Obstetrics and Gynecology, Yokohama City University Graduate School of Medicine, Yokohama, Japan
10) Department of Maternal and Fetal Medicine, Tohoku University Graduate School of Medicine, Miyagi-Children's Hospital, Sendai, Japan
11) Department of Obstetrics and Gynecology, Saitama Medical University, Saitama, Japan
12) Center of Maternal-Fetal, Neonatal and Reproductive Medicine, National Center for Child Health and Development, Tokyo, Japan
13) Present address: Clinical Genetics Unit, Kyoto University Hospital, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

Study collaborators of Japan NIPT Consortium:

Takashi Kaji (The University of Tokushima Faculty of Medicine), Masanobu Ogawa (National Hospital Organization Kyushu Medical Center), Keiichi Matsubara (Ehime University School of Medicine), Haruka Hamanoue (Yokohama City University Graduate School of Medicine), Akimune Fukushima (Iwate Medical University School of Medicine), Masayuki Endo (Osaka University), Kazufumi Haino (Niigata University Medical and Dental Hospital), Hideaki Masuzaki (Nagasaki University Graduate School of

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*Study Collaborators of Japan NIPT Consortium are described separately

✉ Takahiro Yamada
taka0197@kuhp.kyoto-u.ac.jp

Extended author information available on the last page of the article.

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Biomedical Sciences), Masaki Ogawa (Tokyo Women's Medical University Hospital), Shinya Tairaku (Kobe University Graduate School of Medicine), Masato Mizuuchi (Sapporo Medical University School of Medicine), Yoko Okamoto (Osaka Medical Center and Research Institute for Maternal and Child Health), Yukie Kawano (Oita University), Hisashi Masuyama (Okayama University Graduate School of Medicine), Hisao Osada (Chiba University Graduate School of Medicine), Taihei Tsunemi (Nara Medical University), Kazuhisa Maeda (Shikoku Medical Center for Children and Adults), Yasuyo Kasai (Japanese Red Cross Medical Center), Reiko Neki (National Cerebral and Cardiovascular Center), Yukiko Katagiri (Toho University Omori Medical Center), Shunichiro Izumi (Tokai University School of Medicine), Setsuko Nakayama (Aiiku Hospital), Yuko Yokohama (Asahikawa Medical University), Masaya Hirose (Hyogo Prefectural Amagasaki General Medical Center), Kousuke Kawakami (National Hospital Organization Kokura Medical Center), Kiyotake Ichizuka (Showa University Northern Yokohama Hospital), Masakatsu Sase (Yamaguchi Grand Medical Center), Satoru Sakatsume (Dokkyo Medical University Koshigaya Hospital), Tomohiko Tsuruta (Kansai Rosai Hospital)

Affiliations

Takahiro Yamada[1,2,13] · Akihiko Sekizawa[1,3] · Yosuke Fujii[4] · Tatsuko Hirose[1,3] · Osamu Samura[1,5] · Nobuhiro Suzumori[1,6] · Kiyonori Miura[1,7] · Hideaki Sawai[1,8] · Fumiki Hirahara[1,9] · Jun Murotsuki[1,10] · Yoshimasa Kamei[1,11] · Haruhiko Sago[1,12]Japan NIPT consortium*[1]

1 Japan NIPT Consortium, Tokyo, Japan

2 Department of Obstetrics, Hokkaido University Graduate School of Medicine, Sapporo, Japan

3 Department of Obstetrics and Gynecology, The Showa University School of Medicine, Tokyo, Japan

4 Kyoto University Graduate School of Medicine, Kyoto, Japan

5 Department of Obstetrics and Gynecology, The Jikei University School of Medicine, Tokyo, Japan

6 Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

7 Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

8 Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan

9 Department of Obstetrics and Gynecology, Yokohama City University Graduate School of Medicine, Yokohama, Japan

10 Department of Maternal and Fetal Medicine, Tohoku University Graduate School of Medicine, Miyagi-Children's Hospital, Sendai, Japan

11 Department of Obstetrics and Gynecology, Saitama Medical University, Saitama, Japan

12 Center of Maternal-Fetal, Neonatal and Reproductive Medicine, National Center for Child Health and Development, Tokyo, Japan

13 Present address: Clinical Genetics Unit, Kyoto University Hospital, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan

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ORIGINAL ARTICLE

WILEY PRENATAL DIAGNOSIS

Classification of factors involved in nonreportable results of noninvasive prenatal testing (NIPT) and prediction of success rate of second NIPT

Nobuhiro Suzumori[1,2] | Akihiko Sekizawa[3] | Eri Takeda[1,2] | Osamu Samura[4] | Aiko Sasaki[5] | Rina Akaishi[5] | Seiji Wada[5] | Haruka Hamanoue[6] | Fumiki Hirahara[6] | Hiroko Kuriki[6] | Hideaki Sawai[7] | Hiroaki Nakamura[8] | Takahiro Yamada[9] | Kiyonori Miura[10] | Hideaki Masuzaki[10] | Takahiro Yamashita[11] | Yoshimasa Kamei[12] | Akira Namba[12] | Jun Murotsuki[13] | Tomohiro Tanemoto[4] | Akimune Fukushima[14] | Kazufumi Haino[15] | Shinya Tairaku[16] | Keiichi Matsubara[17] | Kazutoshi Maeda[18] | Takashi Kaji[19] | Masanobu Ogawa[20] | Hisao Osada[21] | Haruki Nishizawa[22] | Yoko Okamoto[23] | Takeshi Kanagawa[23] | Aiko Kakigano[24] | Masayuki Endo[24] | Michihiro Kitagawa[25] | Masaki Ogawa[26] | Shunichiro Izumi[27] | Yukiko Katagiri[28] | Naoki Takeshita[28] | Yasuyo Kasai[29] | Katsuhiko Naruse[30] | Reiko Neki[31] | Hisashi Masuyama[32] | Maki Hyodo[33] | Yukie Kawano[34] | Takashi Ohba[35] | Kiyotake Ichizuka[36] | Takeshi Nagamatsu[37] | Atsushi Watanabe[38] | Naomi Nishikawa[39] | Naoki Hamajima[39] | Nahoko Shirato[3] | Junko Yotsumoto[40] | Miyuki Nishiyama[5] | Keiko Koide[3] | Tatsuko Hirose[3] | Haruhiko Sago[5]

[1] Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

[2] Division of Clinical and Molecular Genetics, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan

[3] Department of Obstetrics and Gynecology, Showa University School of Medicine, Tokyo, Japan

[4] Department of Obstetrics and Gynecology, Jikei University School of Medicine, Tokyo, Japan

[5] Division of Fetal Medicine, Center for Maternal-Fetal, Neonatal and Reproductive Medicine, National Center for Child Health and Development, Tokyo, Japan

[6] Department of Clinical Genetics, Yokohama City University Hospital, Yokohama, Japan

[7] Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan

[8] Department of Obstetrics, Osaka City General Hospital, Osaka, Japan

[9] Clinical Genetics Unit, Kyoto University Hospital, Kyoto, Japan

[10] Department of Obstetrics and Gynecology, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

[11] Department of Obstetrics and Gynecology, Maternal and Child Health Center, Aiiku Hospital, Tokyo, Japan

[12] Departments of Obstetrics and Gynecology, Saitama Medical University School of Medicine, Saitama, Japan

[13] Department of Maternal and Fetal Medicine, Tohoku University Graduate School of Medicine, Miyagi Children's Hospital, Sendai, Japan

[14] Department of Clinical Genetics, School of Medicine, Iwate Medical University, Morioka, Japan

[15] Department of Obstetrics and Gynecology, Niigata University Medical and Dental Hospital, Niigata, Japan

[16] Department of Obstetrics and Gynecology, Kobe University Graduate School of Medicine, Kobe, Japan

[17] Department of Obstetrics and Gynecology, Ehime University School of Medicine, Matsuyama, Japan

[18] Department of Obstetrics and Gynecology, Shikoku Medical Center for Children and Adults, Kagawa, Japan

[19] Department of Obstetrics and Gynecology, The University of Tokushima Faculty of Medicine, Tokushima, Japan

[20] Department of Obstetrics and Gynecology, Clinical Research Institute, National Hospital Organization Kyushu Medical Center, Fukuoka, Japan

[21] Department of Gynecology, Chiba University Graduate School of Medicine, Chiba, Japan

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[22] Department of Obstetrics and Gynecology, Fujita Health University, Aichi, Japan
[23] Department of Obstetrics, Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan
[24] Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan
[25] Department of Obstetrics and Gynecology, Sanno Hospital, Tokyo, Japan
[26] Division of Obstetrics, Perinatal Medical Center, Tokyo Women's Medical University Hospital, Tokyo, Japan
[27] Department of Obstetrics and Gynecology, Tokai University School of Medicine, Kanagawa, Japan
[28] Department of Obstetrics and Gynecology, School of Medicine, Faculty of Medicine, Toho University, Tokyo, Japan
[29] Department of Obstetrics and Gynecology, Japanese Red Cross Medical Center, Tokyo, Japan
[30] Department of Obstetrics and Gynecology, Nara Medical University, Nara, Japan
[31] Department of Perinatology and Gynecology, National Cerebral and Cardiovascular Center, Osaka, Japan
[32] Department of Obstetrics and Gynecology, Okayama University Graduate School of Medicine, Okayama, Japan
[33] Department of Obstetrics and Gynecology, Hiroshima University Graduate School of Medicine, Hiroshima, Japan
[34] Department of Molecular Pathology, Faculty of Medicine, Oita University, Oita, Japan
[35] Department of Obstetrics and Gynecology, Kumamoto University, Kumamoto, Japan
[36] Department of Obstetrics and Gynecology, Showa University Northern Yokohama Hospital, Yokohama, Japan
[37] Departments of Obstetrics and Gynecology, Tokyo University Hospital, Tokyo, Japan
[38] Division of Clinical Genetics, Nippon Medical School Hospital, Tokyo, Japan
[39] Division of Clinical Genetics, Nagoya City West Medical Center, Nagoya, Japan
[40] Departments of Genetic Counseling, Ochanomizu University, Tokyo, Japan

Correspondence
Nobuhiro Suzumori, Department of Obstetrics and Gynecology, Nagoya City University Graduate School of Medical Sciences, One Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya 467-8601, Japan.
Email: og.n.suz@med.nagoya-cu.ac.jp

Abstract

Objective: To evaluate the reasons for nonreportable cell-free DNA (cfDNA) results in noninvasive prenatal testing (NIPT), we retrospectively studied maternal characteristics and other details associated with the results.

Methods: A multicenter retrospective cohort study in pregnant women undergoing NIPT by massively parallel sequencing (MPS) with failed cfDNA tests was performed between April 2013 and March 2017. The women's data and MPS results were analyzed in terms of maternal characteristics, test performance, fetal fraction (FF), *z* scores, anticoagulation therapy, and other details of the nonreportable cases.

Results: Overall, 110 (0.32%) of 34 626 pregnant women had nonreportable cfDNA test results after an initial blood sampling; 22 (20.0%) cases had a low FF (<4%), and 18 (16.4%) cases including those with a maternal malignancy, were found to have altered genomic profile. Approximately half of the cases with nonreportable results had borderline *z* score. Among the women with nonreportable results because of altered genomic profile, the success rate of retesting using a second blood sampling was relatively low (25.0%-33.3%). Thirteen (11.8%) of the women with nonreportable results had required hypodermic heparin injection.

Conclusions: The classification of nonreportable results using cfDNA analysis is important to provide women with precise information and to reduce anxiety during pregnancy.

1 | INTRODUCTION

Noninvasive prenatal testing (NIPT) using massively parallel sequencing (MPS) has been performed worldwide for the detection of common chromosomal aneuploidies.[1-3] It is currently being used in over 60 countries in Europe, Asia, Africa, and North and South America.[4] In Japan, a nationwide NIPT trial for common trisomies 21, 18, and 13 has been performed by the Japan NIPT consortium since April 2013.[5] From April 2013 to March 2016, the Japan NIPT consortium performed 30 613 tests using MPS at 55 medical institutions certified by the Japanese Association of Medical Sciences as part of this multicenter clinical trial.[6]

The analysis of cell-free DNA (cfDNA) in maternal blood for the purpose of NIPT has been shown to be highly accurate for the detection of the main fetal trisomies 21, 18, and 13.[1,2,7-9] However, a small percentage of women have nonreportable results after undergoing cfDNA screening for fetal trisomies. The reasons for these result failures include a low fetal fraction (FF) of fetal DNA (defined as the presence of a fetal cfDNA fraction <4%), insufficient cfDNA, extensive

changes in the maternal genome, an abnormal or borderline *z* score for trisomies, and so on.

The placenta discharges detectable cell-free FF into the maternal blood,[2,3] however, the FF is affected by maternal weight, the maternal body mass index (BMI), gestational age, and fetal aneuploidy.[2,8] Except for maternal reasons, the most common reasons for false-positive and false-negative results are vanishing twin, placental or fetal mosaicism, and fetal chromosomal rearrangement.[10] In addition, other reasons for result failure include the presence of maternal neoplasms such as lymphoma, leukemia, breast cancer, colorectal cancer, multiple myeloma, and uterine fibroids.[10,11]

A recent report suggests that anticoagulation therapy and obesity are associated with an increased incidence of failed cfDNA test results because of a low FF.[12] In contrast, Gromminger et al reported that low molecular weight heparin therapy reduced the plasma cfDNA level during pregnancy.[13] The objective of the present study was to determine whether maternal characteristics, test performance, and anticoagulation therapy influence the results and the details of nonreportable reasons in pregnant women undergoing NIPT. We not only determined which factors negatively influence the success rate of NIPT but more importantly whether these factors influence the success rate of a repeated NIPT.

What is already known about this topic?

- The main reasons why NIPT results are nonreportable are a low FF of cfDNA, a high maternal weight, multiple gestation, vanishing twin, placental or fetal mosaicism, cancer, uterine fibroids, or maternal medical treatment.

What does this study add?

- Success rate of retest by second blood sampling was low in women with altered genomic profile.
- Positive predictive value of retest by second blood sampling was low, and approximately 10% women of initial nonreportable results had received heparin administration.
- The classification of nonreportable results using cfDNA analysis could provide useful information for retest.

2 | MATERIALS AND METHODS

The present study is a retrospective study analyzing data from NIPT using MPS obtained during a clinical study of cfDNA testing for fetal trisomies 13, 18, and 21 performed between 10 and 20 weeks' gestation. After sequencing analysis by MPS, segmental and whole chromosomal duplication and deletion were analyzed with all the chromosomes, and only trisomies 13, 18, and 21 were reported. Most of the indications for our study were an advanced maternal age (≥35 y), but other indications included abnormal ultrasound findings for the fetus, abnormal serum screening results, a personal history of a child with aneuploidy, or a parent carrying a balanced Robertsonian translocation. The study's design was approved by all the hospitals' Institutional Review Boards, and all the women provided written informed consent prior to their participation. Details of the Japan NIPT consortium's practices are provided on their website (http://www.nipt.jp/).

Blood samples (20 mL) were collected between April 2013 and March 2017 from pregnant women at each hospital and were sent to Sequenom Laboratories (San Diego, California, United States) or the Kazusa DNA institute (Chiba, Japan) for MateriT21 Plus testing within 3 days of their collection. Cases with missing information regarding the maternal characteristics or with fetal demise before NIPT could be performed were excluded from this study.

Maternal plasma cfDNA extracted from each sample was used for library preparation and MPS using an Illumina HiSeq 2000 (San Diego, United States), as described previously.[1,5,7,8] The SeqFF method is a multivariate regression model that verifies the fetal DNA fraction.[8] The sequencing data were used to calculate the *z* scores for trisomies 21, 18, and 13, which represent robust assessments of normalized chromosomal representation compared with the euploid genome. A low FF of less than 4% for singleton pregnancies and less than 8% for twin pregnancies was reported as a result failure. We classified these nonreportable cases using cfDNA analysis after the initial blood sampling as insufficient cfDNA, altered genomic profile, borderline *z* score, or uncertain.

Cases with positive NIPT results for trisomies 21, 18, or 13 were subsequently undergone chorionic villus sampling or amniocentesis to determine the fetal genotype. Pregnant women with nonreportable results after the initial blood sampling were followed-up using a retest of a second blood sampling and/or cytogenetic analysis by chorionic villus sampling or amniocentesis. The clinical data, test results, and pregnancy outcomes were collected and aggregated every 6 months at the data center. We excluded cases with missing clinical or follow-up data. Information on maternal outcome, such as neoplasia, was collected pre-NIPT and post-NIPT. This study was part of a clinical trial registered with the University Medical Information Network clinical trials registry (UMIN000009338).

Statistical analyses using the Kolmogorov–Smirnov test or the Shapiro–Wilk test were used to confirm the normality of the distributions. Differences between mean values were also examined using *t* tests (SPSS version 23.0, Chicago, Illinois, United States). A *P* value of less than or equal to 0.05 was considered statistically significant.

3 | RESULTS

The characteristics of the 34 626 women who underwent NIPT using MPS during the study period are shown in Table 1. The median maternal age at the time of examination was 38.0 years, and the median gestational age was 13.3 weeks. Over 94% of the women were 35 years or older at the time of the initial blood sampling. Over 98%

TABLE 1 Characteristics of women undergoing noninvasive prenatal testing (NIPT)

		Overall (n = 34 626)	Nonreportable Results (n = 110)
Maternal age (y)	Mean ± SD	38.4 ± 2.7	38.0 ± 3.4
	Median	38.0	38.0
	IQR	4.0	4.0
	Range	20.0-58.0	25.0-47.0
Gestational age (weeks)	Mean ± SD	13.3 ± 1.5	13.3 ± 1.6
	Median	13.1	13.1
	IQR	2.1	2.2
	Range	8.4-37.0	9.7-17.6
Maternal weight (kg)	Mean ± SD	53.2 ± 7.8*	55.5 ± 11.7 *
	Median	52.0	53.0
	IQR	9.0	11.0
	Range	34.0-148	37.9-114
FF (%)	Mean ± SD	13.0 ± 4.2†	10.6 ± 4.8†
	Median	12.5	11.0
	IQR	4.7	7.0
	Range	2.2-57.5	0.9-24.8
Twins (n)		324 (0.9%)	8 (7.2%)
	Percentage of nonreportable results among twin pregnancies: 2.5%		

Abbreviations: SD: standard deviation; IQR: interquartile range; FF: fetal fraction

Significant differences in maternal weight

*(P = 0.04) and fetal fraction (FF).

†(P = 0.0001) were found.

women were Japanese, and approximately 1% women were Asian but not Japanese. Significant differences in maternal weight (P = 0.04) and FF (P = 0.0001) were found between the women with and those without reportable results (Table 1), with higher maternal weights and lower FFs tending to be associated with nonreportable results.

Of the 34 626 high-risk pregnant women who were tested in this study, the numbers of positive, negative, and nonreportable results were 604, 33 912, and 110, respectively. Three false negative (0.009%) cases were found in the present study. No significant differences in maternal age or gestational age at the time of NIPT were found between the cases with reportable and those with nonreportable results, although the women with nonreportable results had a significantly lower FF, compared with the other participants (P = 0.0001).

Among the women who underwent NIPT, the numbers of singleton and twin pregnancies were 34 302 and 324, respectively. Among the women with twin pregnancies, 2.5% (eight of 324) had nonreportable results; this rate was higher than that among women with singleton pregnancies (0.30%, 102 of 34 302). In addition, six of the 22 women (27.2%) with nonreportable results and a low FF were pregnant with twins.

The details of the nonreportable NIPT results are shown in Figure 1. Of the 110 initially nonreportable cases, 94 cases underwent retesting using a second blood sampling; 49 (52.1%) of these cases tested

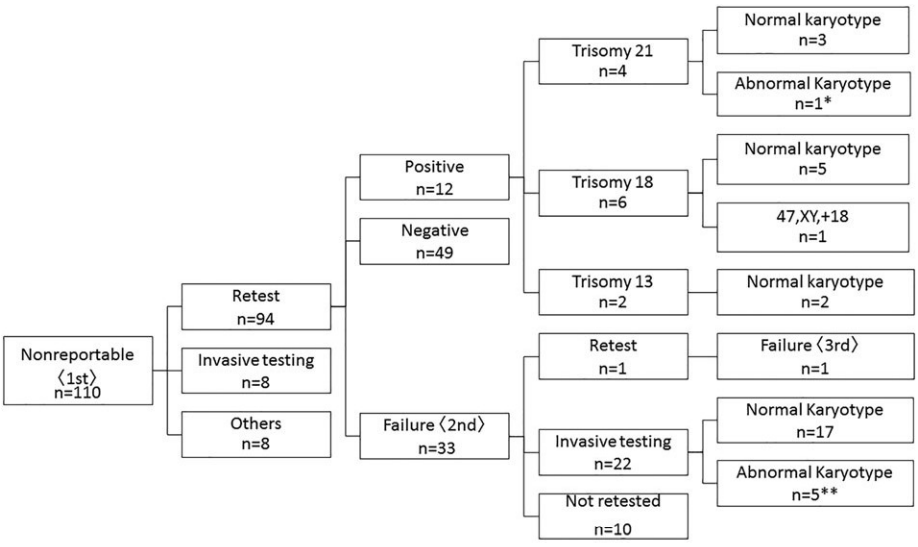


FIGURE 1 Outcomes of noninvasive prenatal testing (NIPT) nonreportable results after initial blood sampling.*The trisomy 21 positive case by retest was twin pregnancy, and it was confirmed that one was 47,XN,+21(4)/46,XN(12), and the other was 46,XN by amniocentesis. **Five abnormal karyotypes (46,XN,dup[8][p23.2], 46,XN,del[18][q21.3], 46,XN,del[18][10], 45,XN,-15,der[18]t[15;18][q11.2;p11.2], 47,XN,+21[10]/46,XN[4]) were detected with invasive testing after the second failure

negative, and 12 (12.8%) tested positive. Among 49 negative cases, 46 had no evidence of any trisomies, one was intrauterine fetal demise (IUFD) at 18 weeks of gestation, and two were unclear. Approximately one third of the initially nonreportable cases (33/94) also had failed results after the retest. Among the 12 women with positive NIPT retest results, abnormal fetal karyotype was identified in two (16.7%) cases. Among the initial nonreportable cases, eight women opted for (received) invasive prenatal testing, and all were found to have normal fetal karyotypes. In contrast, eight women did not opt for retesting or an invasive prenatal procedure (six were no evidence of any trisomies after birth, one case was miscarriage at 10 wk of gestation, and one had selective abortion for curative surgery of ovarian tumor).

Twenty-two of the 33 women with nonreportable results after retesting were subsequently undergone amniocentesis (Figure 1). Among these 22 women, 17 were found to have normal fetal karyotypes, and five were found to have abnormal fetal karyotypes (46, XN,dup[8][p23.2] with genome wide change, 46,XN,del[18][q21.3]; 46,XN,del[18][p10]; 45,XN,-15,der[18]t[15;18][q11.2;p11.2] with specific genome changes, and 47,XN,+21[10]/46,XN[4] with uncertain). Nonreportable reasons of two cases confirmed trisomies (47,XN, +21[4]/46,XN[12] and 47,XY,+18) were borderline *z* scores.

The details of the cases with NIPT nonreportable results after the initial blood sampling (n = 110) are shown in Table 2. Approximately half of the cases with nonreportable results had a borderline *z* score (50.9%). "Uncertain" in Table 2 included cases with "cause unknown," "uncertain cfDNA results," or "laboratory error." Of the 110 cases with initially nonreportable results shown in this table, 22 had a low FF (<4%), and 18 (16.4%) cases had altered genomic profile (either genome wide change or specific genome change). Six of these women had maternal neoplasia (uterine fibroids: n = 4; Ewing sarcoma: n = 1; colorectal cancer: n = 1). There were four women who were already known with neoplasia before the initial blood sampling, and two women were identified with NIPT.

The retest data for the second NIPT blood samples from the women with initially nonreportable results are shown in Table 3. Among the nonreportable women with an altered genomic profile (abnormal *z* score), the success rate of the retest by blood sampling was lower than that for women with insufficient cfDNA or a borderline *z* score. Most of the "uncertain" cases (11/12, 91.7%) in Table 3 after the initial sampling were successfully retested resulting in negative using a second blood sampling.

Overall, 93 pregnant women underwent hypodermic heparin injection as an anticoagulation therapy (Table 4). Among the 110 cases with initially nonreportable NIPT results, 13 cases (11.8%) had received heparin administration because of maternal antiphospholipid syndrome or to prevent thrombosis. The rate of nonreportable NIPT results was significantly higher among women who received heparin administration during pregnancy, compared with those who did not (11.8% and 0.23%, respectively; $P < 0.0001$).

TABLE 2 Details of initial nonreportable noninvasive prenatal testing (NIPT) results (n = 110)

Nonreportable Reasons	n	%	Explanation
Insufficient cfDNA	24	21.8	
Insufficient cfDNA	2	1.8	
Low FF	22	20.0	n = 6: twin pregnanciesn = 1: heparinn = 6: BMI ≥ 25n = 2: maternal neoplasia (uterine fibroids)
Altered genomic profile	18	16.4	
Genome wide change	10*	9.0	n = 5: maternal neoplasia (uterine fibroids: n = 4*; Ewing sarcoma: n = 1) n = 1: heparinn = 1: BMI ≥ 25
Specific genome change	9*†	8.2	n = 2: maternal neoplasia (uterine fibroids: n = 1*; colorectal cancer: n = 1†)n = 1: CPMn = 4: suspected microdeletionn = 2: maternal genomic condictionn = 1: BMI ≥ 25
Borderline *z* score	56	50.9	
Borderline *z*13 score	9†	8.2	n = 5: vanishing twinn = 3: maternal neoplasia (colorectal cancer: n = 1†, uterine fibroid: n = 2)n = 10: heparinn = 12: others (CPM: n = 3, autoimmune disease: n = 5, suspected microduplication: n = 3, 8q23.2duplication [derived paternally]: n = 1)
Borderline *z*18 score	26	23.6	
Borderline *z*21 score	20	18.2	
Borderline *z*13.18.21 score		1.8	
Uncertain	12	10.9	n = 1: heparin, n = 2: others (CPM: n = 1, trisomy 21 mosaic: n = 1)
Total	110	100	

Abbreviations: DD twins: dichorionic diamniotic twins; MD twins: monochorionic diamniotic twins; BMI: body mass index; CPM: confined placental mosaicism

*†Note: Duplication (two cases have two nonreportable reasons)

4 | DISCUSSION

In this retrospective study, a total of 34 626 women were analyzed using MPS and the SeqFF method, and only 0.30% of singleton pregnancies had nonreportable results. A recent review of NIPT failure results reported that MPS had the lowest failure rate (1.58%), compared with SNP-based analysis (6.39%) and chromosome specific sequencing (3.56%).[14] Our present data suggest that NIPT is appropriate for pregnant women of advanced maternal age in Japan because of the relatively low failure rates for initial and second blood draws, compared with the rates in the above-mentioned review of studies performed in other countries.

A recent report suggests that the rate of informative redraw is strongly dependent on the maternal weight and the FF at the time of the initial blood sampling.[15] Overall, 22 of the 110 (19.6%) cases with initially nonreportable results had a low FF (<4%). Among the 94 cases who were retested with a second blood sampling, 61 (64.9%) had either positive or negative results; this rate is comparable with recently reported findings (62.9%-70.6%).[15] Our present data for the initial blood sampling results also support the findings of this previous report, since a relatively high maternal weight or a low FF was significantly associated with a nonreportable result. In contrast, our findings present classification of factors involved in nonreportable results and prediction of success rate of second NIPT.

TABLE 3 Retest data for second noninvasive prenatal testing (NIPT) blood sampling (n = 94)

Nonreportable Reasons	Number Undergoing Retest	Retest Results	Success Rate of Retest (%)
Insufficient cfDNA	22		
Insufficient cfDNA	2	Negative: n = 2	100
Low FF	20	Negative: n = 14 (BMI ≥ 25: n = 3, twin pregnancies: n = 5, heparin: n = 1, maternal neoplasia: n = 1) failures: n = 6 (BMI ≥ 25: n = 2, BMI ≥ 25 + twin pregnancy: n = 1, maternal neoplasia: n = 1)	70.0
Altered genomic profile	10		
Genome wide change	4	Negative: n = 1 (maternal neoplasia: n = 1)failures: n = 3 (maternal neoplasia: n = 3)	25.0
Specific genome change	6†	Negative: n = 2 (others: n = 1) failures: n = 4 (maternal neoplasia: n = 1†, others: n = 4)	33.3
Borderline *z* score	51		
Borderline *z*13 score Borderline *z*18 score Borderline *z*21 score Borderline *z*13.18.21 score	6† 25 18 2	Negative: n = 18 (heparin: n = 3, vanishing twin: n = 1, others: n = 1)21 positive: n = 4 (vanishing twin: n = 1, others: n = 1)18 positive: n = 6 (vanishing twin: n = 1, others: n = 2)13 positive: n = 2 (others: n = 1)failures: n = 20† (heparin: n = 6, vanishing twin: n = 1, maternal neoplasia: n = 2†, others: n = 7)	60.8
Uncertain	12	Negative: n = 11failures: n = 1 (others)	91.7
Total	94		

Abbreviations: cfDNA: cell-free DNA; BMI: body mass index

†Duplication (one case has two nonreportable reasons). Obesity is defined by body mass index (BMI) ≥ 25 in Japan. The average BMI was 21.7 (range: 17.1-40.4).

Vanishing twin cases can be identified by ultrasound findings during the first trimester, and these cases sometimes result in discordant NIPT data.[16] Almost 70% of spontaneous miscarriages are caused by fetal chromosomal abnormalities,[17] so pregnant women with a vanishing twin should be counseled before undergoing NIPT about the possibility of a redraw being required after the initial blood sampling.

Regarding NIPT for twin pregnancies, Tan et al reported that the rate of MPS result failure for women treated with assisted reproductive techniques was only 0.9% (5/565).[18] Among the twin pregnancies in the presently reported series, 2.5% (8/324) had nonreportable results from the initial blood sampling, which was higher than that of the above-mentioned report.[18] The most likely reason for this discrepancy is that an FF of less than 8% in women with twin pregnancies was regarded as a nonreportable result in this study. We recently reported obtaining nonreportable results for 60 women with twin pregnancies, excluding those with vanishing twin.[19] No false-negative cases were found among the twin pregnancies in either our previous study or the present study.

Dharajiya et al recently reported that 38 out of 55 nonreportable NIPT cases had altered genomic profiles, with 18 cases having malignancies and 20 having benign uterine fibroids.[11] A recent review suggests that neuroendocrine cancer, angiosarcoma, and small-cell carcinoma can also cause false-positive results.[10] In our 10 cases with an abnormal *z* score reflecting genome wide change, one patient had a malignant sarcoma, and four had benign uterine fibroids. In contrast, one woman with colorectal cancer and one with uterine fibroids had nonreportable results because of a minus *z* score reflecting a specific genome change. These findings suggest that the screening and evaluation of cfDNA by blood sampling could be a potential predictor of cancer. Detection of the copy number in plasma samples using an NIPT platform has reportedly been used as a prognostic biomarker in patients with gynecological cancer.[20] Our data suggest that invasive prenatal procedures should be recommended without retesting in women with altered genomic profiles, since the likelihood of obtaining reliable results is relatively low for plasma samples exhibiting multiple copy number variations that can influence fetal mortality.

Gromminger et al reported that low molecular weight heparin injections increase the proportion of smaller plasma DNA with a higher GC content.[13] In the 110 cases with initially nonreportable

TABLE 4 Number of women who received heparin prior to undergoing noninvasive prenatal testing (NIPT)

NIPT Results			n	%
Positive (n = 604)	Trisomy 21		0	0
	Trisomy 18		1	1
	Trisomy 13		0	0
Subtotal			1	1
Negative(n = 33 912)			79	85
Nonreportable (n = 110)	Retest	Positive	0	0
		Negative	6	6.5
		Failures	6	6.5
	Invasive testing		0	0
	Others		1	1
Subtotal			13	14
Total			93	100

results, a relatively high percentage of women (14%) had received heparin because of antiphospholipid syndrome or to prevent thrombosis. However, we were unable to determine whether other medications taken during pregnancy might have similarly influenced the accuracy of NIPT.

In summary, our present data suggest that women with twin pregnancies, vanishing twin, maternal neoplasia, or who have received a heparin injection during pregnancy might receive a nonreportable NIPT result and should be informed of this possibility and undergo genetic counseling prior to NIPT. For women with nonreportable results because of an altered genomic profile, invasive prenatal procedures should be recommended without retesting. We need to recommend invasive testing for the women themselves with altered genomic profile. The classification of nonreportable results using cfDNA analysis is also important for providing women with accurate information and to reduce anxiety during pregnancy.

As of the end of August 2018, NIPT has been performed at over 90 hospitals in Japan, of which 84 were participants in the Japan NIPT consortium. Additional studies are needed to define the significance and accuracy of NIPT including false-positive and false-negative cases. Because pregnant women undergoing NIPT in Japan have a low nonreportable result rate after initial blood sampling, further studies by the Japan NIPT consortium, including genome-wide cfDNA analyses, should be initiated in the near future.

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CONFLICTS OF INTEREST

None declared.

ORCID

Nobuhiro Suzumori https://orcid.org/0000-0001-9003-1289
Takahiro Yamada https://orcid.org/0000-0002-5370-7873
Haruhiko Sago https://orcid.org/0000-0001-9387-1849

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Research Article

Challenges in Managing Patients with Hereditary Cancer at Gynecological Services

Mako Ueda,[1,2] Hiroshi Tsubamoto,[1] Mina Kashima-Morii,[2] Yoshitaka Torii,[3] Mariko Kamihigashi,[1] Yu Wakimoto,[1] Nami Nakagomi,[3] Tomoko Hashimoto-Tamaoki,[2] Hideaki Sawai,[1,2] and Hiroaki Shibahara[1]

[1]*Department of Obstetrics and Gynecology, Hyogo College of Medicine, Nishinomiya, Japan*
[2]*Department of Clinical Genetics, Hyogo College of Medicine, Nishinomiya, Japan*
[3]*Department of Surgical Pathology, Hyogo College of Medicine, Nishinomiya, Japan*

Correspondence should be addressed to Hiroshi Tsubamoto; tsuba@hyo-med.ac.jp

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Aim. To reveal current problems and challenges faced by our gynecologic services department in managing patients with hereditary cancers. *Methods*. We collected clinical data of patients with hereditary cancers, identified via genetic testing (or clinically diagnosed in cases of Cowden syndrome or Peutz–Jeghers syndrome), and treated in our gynecological department from 2012 to 2018. *Results*. Fifteen patients had hereditary breast and ovarian cancer (HBOC), 6 had Lynch syndrome, 2 had Cowden syndrome, and 2 had Peutz–Jeghers syndrome. Five patients diagnosed with HBOC were younger than 40 years at diagnosis. Risk-reducing salpingo-oophorectomy (RRSO) was performed on 1 patient with a *BRCA1* mutation at age 38 years. Seven patients overall underwent RRSO, and none had malignancies on pathological examinations. Peritoneal washing cytology (PWC) was suspicious for malignancy in one patient; however, subsequent PWC at 6 months after RRSO was negative. A patient with endometrial cancer and Lynch syndrome and a patient with atypical endometrial hyperplasia (AEH) and Cowden syndrome strongly desired fertility preservation. They achieved remission after medroxyprogesterone acetate treatment and multiple dilations and curettages, respectively. One patient with Lynch syndrome developed AEH after 11 years of surveillance. Laparotomy revealed adjacent low-grade and high-grade serous ovarian cancer with positive ascites cytology. She had no recurrence during 7-year follow-up after laparotomy. *Conclusion*. Managing patients with hereditary cancer, positive or false-positive ascites cytology discovered during RRSO, and desired preservation of fertility is highly challenging.

1. Introduction

Hereditary cancer comprises a group of diseases caused by gene mutations that are associated with risks of synchronous, metachronous, and multiorgan cancer. Affected patients need to be identified, managed by a multidisciplinary team, and placed under periodic surveillance for cancer treatment and prevention. The Familial Tumor Center was established at our hospital to conduct family surveys, registrations, and gene analyses from 2001 to 2009. A multidepartment coordination system was established to accommodate the needs of patients and families after the assignment of certified genetic counselors began in 2011 [1]. Familial Tumor Data Sharing in our hospital, risk-reducing salpingo-oophorectomy (RRSO) for patients with *BRCA* gene mutations, and regional coordination of hereditary breast and ovarian cancer (HBOC) management were instituted upon approval of the ethical review board (no. 187, no. 256, and no. 274, respectively). More patients with hereditary cancers identified via genetic testing are now being treated in the gynecological services department after the establishment of the coordination system. As such, we have taken on some difficult cases in daily practice.

In Japan, the Basic Plan to Promote Cancer Control Programs was revised in March 2018, which promoted genomic cancer medicine programs. Increasing numbers of

patients have requested sequencing tumor genomes including germline mutations. The use of poly ADR-ribose polymerase (PARP) inhibitor, olaparib, approved for recurrent breast cancer in July 2018, requires a concurrent companion diagnostic test for pathogenic *BRCA* mutations (variants). The frequency of treating patients with hereditary cancer at gynecological services is thus expected to increase rapidly. We report the current practice at our clinic and the challenges we have faced in managing these patients.

2. Methods

We collected the clinical data of patients with hereditary cancer who were identified via genetic testing or were clinically diagnosed with Cowden syndrome or Peutz–Jeghers syndrome and who were treated in the gynecological services department from 2012 to 2018. The patients were also registered in the Familial Tumor Data Sharing system. We also listed the more difficult cases individually. Informed consent was obtained from each of the patients for inclusion in this research (the ethical review board of no. 187).

3. Results

Fifteen patients (13 families) had HBOC, 6 had Lynch syndrome, 2 had Cowden syndrome, and 2 had Peutz–Jeghers syndrome. None of the patients had Li–Fraumeni syndrome (Table 1). Of the 15 patients with HBOC, 8 were tested at our clinic and 7 were tested at other clinics. Six patients were *BRCA1* positive, and 9 patients were *BRCA2* positive. Seven of the 15 patients with HBOC (2 *BRCA1* positive and 5 *BRCA2* positive) underwent RRSO. One of the patients is scheduled for bilateral salpingo-oophorectomy (BSO) for an ovarian cyst. The mean age of the patients at the time of RRSO was 53 years. No occult cancers or serous tubal intraepithelial carcinomas (STIC) were found during RRSO. One patient had positive ascitic cytology suspicious for malignancy. Seven patients who have not undergone RRSO are currently under surveillance, and their mean age was 39 years.

Lynch syndrome was diagnosed based on genetic testing in 6 patients. Three of them were tested at our clinic. Three patients had mutL homolog 1 (*MLH1*), 1 had mutS homolog 2 (*MSH2*), 1 had mutS homolog 6 (*MSH6*), and 1 patient's result was not disclosed. The mean age at the time of diagnosis was 38 years. One patient (no. 20) was diagnosed with Lynch syndrome after resection of sigmoid colon cancer in her 30s. Atypical endometrial hyperplasia (AEH) was found after 11 years of surveillance. Two tumors adjacent to the fallopian tube and adherent to the ovarian surface were identified during laparotomy, necessitating hysterectomy and bilateral salpingo-oophorectomy (BSO). These were pathologically diagnosed as low-grade and high-grade serous cancers with no apparent stromal invasion [2]. The patient has been followed without treatment, despite having positive ascitic cytologies, and has had no evidence of recurrence at 7 years after hysterectomy and BSO. Two patients were diagnosed with Cowden syndrome, one was diagnosed based on genetic testing, and another was diagnosed clinically. Peutz–Jeghers syndrome was diagnosed in 1 patient in our clinic based on the presence of gastric hamartomatous polyposis and mucocutaneous pigmented spots.

3.1. Difficult Cases

3.1.1. Positive Ascites Cytology at the Time of RRSO (Patient No. 6). This patient was referred to our clinic by two of her physicians, an oncologist and a gynecologic oncologist, after her mother (Patient no. 16) underwent RRSO. There were no abnormalities on her preoperative examination. There were no findings of malignancy or endometriosis upon surveillance of the pelvis during laparoscopy. A detailed pathological examination of the excised ovary and fallopian tube was negative for malignancy; however, the peritoneal washing cytology (PWC) indicated suspected adenocarcinoma (Figure 1). Further examination of a paraffin-embedded sample was also negative for malignancy. After an assessment by an outside cytopathologist and consensus with the referring oncologist and gynecologic oncologist, a positron emission tomography (PET) scan was performed, followed by a second-look laparoscopy 6 months later. The PET scan found no abnormality. No macroscopic abnormalities were observed during second-look laparoscopy. Biopsies were taken from 3 different parts of the peritoneum in the pouch of Douglas, and peritoneal washing ascites was collected. No abnormalities were identified in any of the samples.

3.1.2. Fertility Preservation in a Patient with Endometrial Cancer Associated with Lynch Syndrome (Patient No. 19). An unmarried nulligravida in her 30s with endometrial cancer was referred to our clinic in consideration of fertility preservation because of an extensive family history suggestive of Lynch syndrome. The patient did not wish to undergo genetic testing. After being thoroughly informed of the possibility of Lynch syndrome based on relevant study reports, she consented to medroxyprogesterone acetate (MPA) therapy at 600 mg/day for 6 months. She ultimately achieved remission. A right triple negative breast cancer (TNBC) developed 2 years later. At that time, she requested genetic testing, which was required for developing a treatment protocol to manage the breast cancer and promote fertility preservation. Lynch syndrome was diagnosed based on the pathogenic *MLH1* mutation. Both ovaries and oocytes were cryopreserved prior to neoadjuvant chemotherapy. She has had no endometrial cancer recurrence at 3 years after MPA therapy.

3.1.3. Fertility Preservation in a Patient with AEH Associated with Cowden Syndrome (Patient No. 22). An unmarried nulligravida in her 30s had a left breast cancer in her 20s. Breast-conserving surgery was performed followed by tamoxifen therapy. The patient underwent genetic testing because her father had been diagnosed with Cowden syndrome. A pathogenic mutation of the phosphatase and

Table 1: Demographics of patients with hereditary cancer treated in our gynecological services department between 2012 and 2018.

	Case no.	Age at diagnosis	Genetic diagnosis				Cancer	Family history	Gynecological management
			Pathogenic mutation	Mutation point	Refseq ID	dbSNP rs no.			
HBOC	1	20s	*BRCA2*	c.(7435 + 1_7436-1) _(7805 + 1_7806-1)dup	NM_000059.3	NA	Breast		Surveillance
	2	30s	*BRCA2*	c.188T > A	NM_007294.3	rs80357086	Breast	Breast cancer, mother	Surveillance
	3	30s	*BRCA2*	c.5576_5579delTTAA (p.Ile1859Lysfs)	NM_000059.3	rs80359520	Breast		Surveillance
	4	30s	*BRCA2*	p.S1882X	NM_000059.3	NA	Breast		Surveillance
	5	30s	*BRCA1*	c.5154G > A (p.W1718X)	NM_007294.3	rs80357239	Breast	Breast cancer, mother	RRSO (7 months after genetic diagnosis)
	6	40s	*BRCA2*	c.2T > G (p.M1R)	NM_000059.3	rs80358547	None	Breast cancer, mother (BRCA2, case 14)	RRSO (6 months after genetic diagnosis)
	7	40s	*BRCA1*	c.3122C > G (p.Ser1041Ter)	NM_007294	rs397509035	Breast	Bilateral breast cancer, mother Breast cancer, aunt	Surveillance
	8	50s	*BRCA1*	Not available	—	—	Ovarian	Breast cancer, sister	Surveillance
	9	50s	*BRCA2*	c.9117G > A (p.P3039P)	NM_000059.3	rs28897756	Bilateral breast Gastric	Breast cancer, maternal side Ovarian cancer, many relatives	RRSO (7 months after genetic diagnosis)
	10	50s	*BRCA2*	c.5574_5577delAATT (p.Ile1859Lsyfs∗3)	NM_000059	rs80359520	Breast Sigmoid colon	Sigmoid colon cancer, father Gastric cancer, aunt Breast and colon cancer, paternal grandmother	Surveillance
	11	50s	*BRCA2*	c.5645C > A (p.Ser1882Ter)	NM_000059	rs80358785	Breast	Ovarian cancer, mother	RRSO (4 months after genetic diagnosis)
	12	50s	*BRCA2*	c.5645C > A (p.Ser1883Ter)	NM_000059	rs80358785	Breast	Breast and ovarian cancer, mother	RRSO (3 months after genetic diagnosis)
	13	60s	*BRCA2*	c.4085delA	NM_000059.3	rs431825315	Breast	Breast cancer, father Breast and ovarian cancer, sister	RRSO (8 months after genetic diagnosis)
	14	60s	*BRCA1*	c.2T > G (p.M1R)	NM_000059.3	rs80358547	Breast	Breast cancer, mother and sister Ovarian cancer, grandmother and aunt	RRSO (8 months after genetic diagnosis)
	15	60s	*BRCA1*	c.3122C > G (p.Ser1041Ter)	NM_007294	rs397509035	Breast	Breast cancer, sister Breast cancer, daughter (case 7)	BSO scheduled for ovarian cyst

Table 1: Continued.

	Case no.	Age at diagnosis	Genetic diagnosis				Cancer	Family history	Gynecological management
			Pathogenic mutation	Mutation point	Refseq ID	dbSNP rs no.			
Lynch syndrome	16	20s	*MSH2*	p.Q130X	NM_000251.2	rs1060501989	Colon	Unknown	Surveillance
	17	20s	*MSH6*	c.3663delGAinsT	NA	NA	None	Colon, endometrial, ovarian cancer, mother (MSH6)	Surveillance
	18	20s	*MLH1*	c.381_543del (p.Ala128Trpfs∗8)	NM_000249.3	NA	None	Colon cancer, father (MLH1)	Surveillance
	19	30s	*MLH1*	c.306+1G > A (p.K70_Q102del)	NM_0000249	rs267607734	Endometrial Breast	Breast, ovarian, colon cancer, mother	MPA
	20	40s	*MLH1*	g.IVS3+1G > A	NA	NA	Sigmoid colon AEH, ovarian	Colon cancer, father (MLH1) and grandmother Colon and endometrial cancer, sister	TAH + BSO
	21	70s	Not disclosed				Breast Colon Endometrial		TAH
Cowden syndrome	22	20s	*PTEN*	c.1026+1G > A	NM_000314	rs786201041	Bilateral breast Thyroid AEH	Father (*PTEN*)	D&C
	23	70s	Clinical diagnosis				Breast Thyroid	Bladder cancer, father Gastric and endometrial cancer, sister	Surveillance
Peutz–Jeghers syndrome	24	Teen	Clinical diagnosis	In house			Gastric		Surveillance
	25	50s	Clinical diagnosis	Others			None		Surveillance

HBOC: hereditary breast and ovarian cancer; RRSO: risk-reducing salpingo-oophorectomy; TNBC: triple negative breast cancer; AEH: atypical endometrial hyperplasia; TAH: total abdominal hysterectomy; BSO: bilateral salpingo-oophorectomy; D&C: dilatation and curettage; MPA: medroxyprogesterone acetate; *PTEN*: phosphatase and tensin homolog; and *BRCA*: breast cancer susceptibility gene.

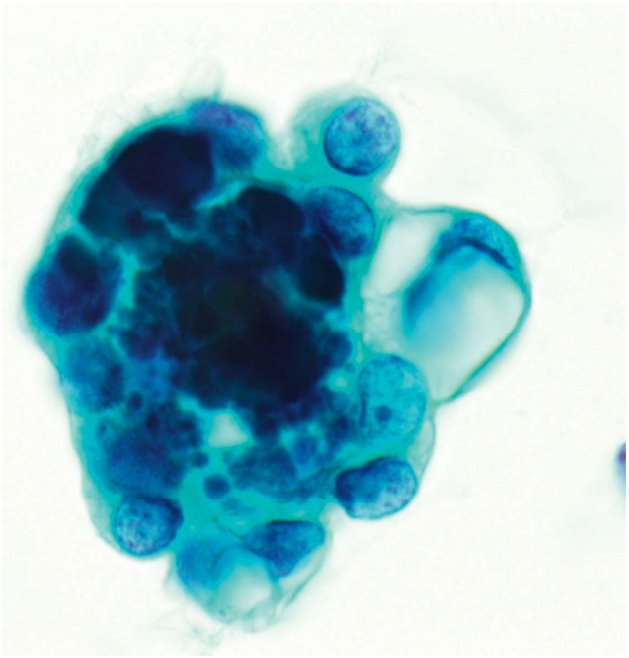


Figure 1: Ascites cytology after RRSO. An atypical cell cluster is seen. Atypical cells show unevenly distributed nuclei, overlapping nuclei, anisonucleosis, and prominent nucleoli. RRSO: risk-reducing salpingo-oophorectomy.

tensin homolog (*PTEN*) gene was identified. The patient had thyroid cancer in her 30s and had a thyroidectomy. The right breast cancer developed around the same time. She underwent breast-conserving surgery and was administered gonadotropin-releasing hormone (GnRH) agonist and tamoxifen therapy. She consulted our gynecological services department and requested to be included in cross-department surveillance at our hospital. Atypical endometrial hyperplasia was diagnosed 2 years later, and the tamoxifen was discontinued. The patient underwent dilation and curettage (D&C) every 6 months thereafter. No recurrence of AEH has been reported in the past 5 years.

3.1.4. Anxieties after Disclosure of a Pathogenic Mutation (Patient No. 5). The patient was seen in the Department of Breast Surgery with a lump she identified during a self-breast exam when she was in her 30s. Biopsy at that time revealed TNBC. The patient expressed her concerns about the possibility of hereditary cancer during a preoperative chemotherapy session. She was concerned because her mother had also been diagnosed with breast cancer. She was referred to the Department of Genetic Counseling. Total mastectomy of the right breast and sentinel lymph node dissection were performed. The patient had several postoperative counseling sessions and requested a genetic test 9 months after the first counseling session. After a pathogenic *BRCA1* mutation was found, the patient developed new anxieties as a result of the unexpected diagnosis and her history of the other type of cancer. Following multiple counseling sessions and surveillance by our gynecological services department, she underwent RRSO at 7 months after disclosure of the genetic test result. No pathological abnormalities were identified.

4. Discussion

The number of patients with genetically diagnosed hereditary cancer who were treated at our gynecological services department from 2012 to 2018 seemed small. In Ontario, where free *BRCA* screening services are provided by the municipal government/healthcare institutions, only 6.6% of patients with serous ovarian cancers were screened between 2001 and 2011 [3]. In our gynecological services department, outpatients with gynecological cancers were interviewed by genetic counselors from 2012 to 2016. Among patients with high risk of inherited cancer, 8.9% underwent genetic testing. Pathogenic variants were observed in 40% of patients who underwent genetic testing [1]. The reasons for not wanting to be tested included the following: testing would not cure the current disease (e.g., the test would not affect the current treatment); the patient wanted to concentrate on the current cancer treatment; the patient did not want to worry her family; and financial burden from treatments not covered by health insurance. Few patients named the lack of relevant laws such as the Genetic Information Nondiscrimination Act and the Health Insurance Portability and Accountability Act in the United States of America (U.S.A.) as a reason. The rationale for actively recommending genetic counseling was lacking because the incidence of hereditary gynecological cancer in Japan was previously unknown. However, the hereditary cancer incidence in Japan is now known to be comparable to that in the U.S.A. or Europe. This is based on recent studies which reported that the incidence of HBOC among ovarian cancer patients was 11.8%, the incidence of Lynch syndrome among ovarian cancer patients was 2.6%, and the incidence of Lynch syndrome among endometrial cancer patients was 4.4% [4, 5].

Current problems and challenges in managing hereditary cancer patients in the gynecological services department are discussed below.

4.1. Anxieties after Disclosure of Pathogenic Mutation. Genetic counseling for cancer generally eliminates the patient's anxieties [6]. We encouraged self-determination in many of our patients through multiple counseling sessions. As a result, most patients were able to take the subsequent treatment and surveillance in stride. However, it required a long time for Patient no. 5 to accept an unexpected genetic test result, which was administered after multiple counseling sessions held over a 9-month period. Although the overly anxious personality of this patient may have been a significant factor, her anxieties were multifaceted and vague, since elimination of the anxieties was the reason for the first consultation. The final course of treatment was undetermined at the time of the first consultation, at which point she was undergoing preoperative chemotherapy for breast cancer. The potential for good news was the primary reason for this patient to undergo counseling; receiving accurate information and deepening her knowledge did not

help eliminate her anxieties. The lack of a characteristic family history also increased her expectations of receiving good news. The patient was extremely shocked to learn that the genetic testing showed a pathogenic *BRCA1* mutation, which she had not expected. It was difficult for her to accept the test result. We recommended RRSO during outpatient surveillance, and the procedure was performed 7 months after the disclosure of the genetic test result.

Her case shows us that determining the patient's reason for consultation and the current disease and treatment are both fundamental and extremely important in genetic counseling. Although presenting the results of assessments made using risk prediction tools based on patients' family history is general practice, genetic counseling should be neutral irrespective of the assessment results. Thorough counseling on the possibility of unexpected results is important.

4.2. Management of HBOC Patients under 40. The ideal timing for RRSO to prevent ovarian cancer in patients with HBOC who are under the age of 40 is controversial. Until 2017, the National Comprehensive Cancer Network (NCCN) guidelines stated that the ideal age at which to undergo RRSO was from 35 to 40 years of age, after the patient has had children, regardless of *BRCA* status; however, the guidelines were revised and now RRSO can be delayed until the patient reaches 40 to 45 years old, since the age of ovarian cancer onset in *BRCA2*-positive patients is 8 to 10 years older than the mean age of onset in *BRCA1*-positive patients. The attending physicians should stay current with this type of information.

Hormone replacement after RRSO is acceptable based on a report on hormone replacement, in which the authors stated that hormone replacement therapy does not reverse the effects of RRSO as it reduces breast cancer risks. Short-term hormone therapy is acceptable as long as it is discontinued before the usual age of menopause. However, hormone therapy should be given carefully since no data are available from randomized controlled trials [7].

Prophylactic salpingectomy with delayed oophorectomy (PSDO) has also been considered as a possible treatment because more than 80% of ovarian cancers in *BRCA* mutation carriers are of the serous type, and more than 90% of serous ovarian cancers may be high-grade serous carcinoma (HGSC) originating from the tubal epithelium [8, 9]. The NCCN guidelines do not consider PSDO to be a standard treatment because its prophylactic effects are unproven. Problems with PSDO include the residual risk of ovarian cancer and the smaller effect of reducing breast cancer risk in premenopausal women compared with RRSO. A clinical study to evaluate the effects of PSDO is ongoing.

Five of our patients with HBOC included were under the age of 40 at the time of diagnosis (Table 2). The patients received thorough counseling on prophylactic surgery based on the NCCN guidelines and the previously mentioned studies. Only one of the 5 patients (Patient no. 5) was a pathogenic *BRCA1* mutation carrier. She underwent RRSO at the age of 38. The other 4 patients were carriers of pathogenic *BRCA2* mutations, with no third-degree relative having been diagnosed with ovarian cancer. These patients are under periodic surveillance without plans for prophylactic surgery.

4.3. Post-RRSO False-Positive Ascites Cytology. Intraoperative macroscopic findings during RRSO or histopathological examination of resected samples may unexpectedly suggest malignant or precancerous lesions. Detailed histopathology according to the sectioning and extensively examining the fimbriated end (SEE-FIM) protocol and postoperative evaluation by a gynecologic oncologist and a pathologist have been considered necessary. However, outcomes among patients with positive PWC (malignant or suspicious for malignancy) without pathologically identified malignancy or precancerous lesions have not been reported frequently. In the literature, 11 patients have had positive PWC with no macroscopic malignancies found during RRSO (Table 2) [10–14]. The frequency of cases with no macroscopic findings consistent with malignancy but with positive PWC is about 1%, which is not low. One patient with a tumor classified as suspicious for malignancy had endometriosis and endosalpingiosis and was followed after RRSO [11]. Microscopic malignancy or STIC was found in 5 patients. The other 5 patients had positive PWC without pathologically identified malignancies or precancerous lesions. Three of them received postoperative chemotherapy. Patient no. 6, who had positive PWC without macroscopic abnormality or evidence of pathological malignancy during RRSO, subsequently had negative PWC at 6 months and was followed closely.

4.4. Fertility Preserving Treatment in Patients with Lynch or Cowden Syndrome and Endometrial Cancer or Precancerous Lesion. Currently, fertility preservation is quite an important issue with respect to cancer treatment in young women. The American Society of Clinical Oncologists (ASCO) issued a recommendation for fertility preservation in cancer patients in 2006. The 2018 ASCO recommendation states, "People with cancer are interested in discussing fertility preservation. Health care providers caring for adult and pediatric patients with cancer should address the possibility of infertility as early as possible before treatment starts." In Japan, the Japanese Society for Fertility Preservation issued the "Guide for pregnancy, delivery, and reproduction medicine in breast cancer patients" in 2014, and the Japan Society of Clinical Oncology issued the "Treatment guidelines for fertility preservation in pediatric, adolescent, and young cancer patients" in 2017. Regional oncofertility networks are being established nationwide. In 2016, we established the Hyogo oncofertility network with the Center for Reproductive Medicine as the core institution that would actively advocate fertility preservation in young cancer patients. Fertility preservation in hereditary cancer patients brings many challenges. Few reports are available, especially as it relates to fertility preservation in patients with endometrial cancer.

Table 2: "Malignant" or "suspicious for malignancy" on peritoneal washing cytology without macroscopic abnormality.

	Period of RRSO	SEE-FIM protocol	PWC in RRSO	Malignancy in PWC (macroscopic cancer)	Histopathologic examination	Further examination and follow-up therapy
Colgan [10]	NA	No	35	Malignant	Early stromal invasion of the ovarian cortex	Chemotherapy, 2nd look
				Malignant	STIC	Observation
				Malignant	NED	Extensive additional sectioning, reviewed by additional 2 cytopathologists, negative cytology at 2nd look, chemotherapy and 2nd look surgery
Manchanda [11]	2005–2009	No	308	Malignant	STIC	Hysterectomy, OMT + LND
				Malignant	STIC	Hysterectomy, OMT + LND
				Suspicious for malignancy	STIC	Hysterectomy, OMT + LND
Landon [12]	2000–2012	Mostly	117	Malignant	NED	Reviewed by additional 4 cytopathologists, multiple levels of tissue blocks examined, chemotherapy, 2nd look surgery
				Suspicious for malignancy	Ovarian endometriosis and endosalpingiosis	Observation
Powell [13]	1995–2009	NA	405	Malignant	NED	Hysterectomy, OMT
				Malignant	NED	Hysterectomy, OMT followed by 6 cycles of chemotherapy
				Malignant	NED	Hysterectomy, OMT + LND followed by 6 cycles of chemotherapy
Blok [14]	2000–2014	Since 2006	267	None		
Total			1132	11 (1.0%)		

SEE-FIM: sectioning and extensively examining the fimbriated; PWC: peritoneal washing cytology; and STIC: serous tubal intraepithelial carcinoma.

Only one case of attempted fertility preservation with high-dose progestin in a young patient with endometrial cancer associated with Lynch syndrome has been reported [15]. Despite the strong family history of Lynch syndrome, the patient had not been genetically tested. A genetic diagnosis was made after fertility preservation treatment. One patient (no. 19) requested fertility preservation after receiving thorough information; however, whether to use MPA in a patient with endometrial cancer and confirmed Lynch syndrome should be considered carefully. Some prospective clinical studies of fertility preservation treatments in young patients with endometrial cancer excluded patients with suspected Lynch syndrome based on their family history [16]. One patient (no. 19) was diagnosed with a breast cancer after MPA therapy. The patient wished to preserve her fertility prior to starting preoperative chemotherapy. Lynch syndrome was suspected based on her family history and the onset of endometrial and breast cancer. Oocytes and unilateral ovarian tissue were cryopreserved after genetic testing to ensure that the ovarian tissue cryopreservation would be practical (to deny HBOC). The other ovary was preserved based on a study reporting no difference in ovarian cancer risk between young patients with endometrial cancer alone and those with concurrent Lynch syndrome [17]. Although no clinical report on the association between short-term high-dose progesterone therapy and the onset of breast cancer is available, the possibility of breast cancer should be taken into consideration when prescribing MPA therapy to patients with Lynch syndrome.

Complications with endometrioid adenocarcinoma and AEH have been reported, each occurring in one gynecological patient with Cowden syndrome and a pathogenic *PTEN* mutation [18, 19]. We found no reports on fertility preservation in patients with confirmed precancerous lesions. The lifetime risk of endometrial cancer in patients with Cowden syndrome and a pathogenic *PTEN* mutation is 28% [20]. We informed 1 patient (no. 22) of the risks involved in prior oral tamoxifen treatment after breast cancer surgery and with being a carrier of a pathogenic *PTEN* mutation. We explained that MPA therapy was impractical and hysterectomy might be considered. The patient wished to preserve her fertility after being thoroughly informed and having undergone counseling. Oral tamoxifen was discontinued, and complete D&Cs have been performed every 3 months for endometrial tissue examination. The patient has been receiving periodic examinations to date. The current examination frequency is every 6 months since the histopathology in the second and subsequent examinations all showed simple endometrial hyperplasia.

4.5. Surveillance in Patients with Lynch Syndrome. Early detection of ovarian cancer is difficult in the surveillance of patients with HBOC because of the peritoneal dissemination of HGSCs in the early stages. Møller et al. reported a 5-year survival rate of 88% in 19 Lynch syndrome patients with ovarian cancer onset during the surveillance period [21]. Woolderink et al. suggested that one of the reasons for the early detection of stage I or II ovarian cancer in 12 patients could be that an occult cancer was found during a hysterectomy for endometrial cancer or AEH [22]. Another reason for the early detection may be that HGSC is not a frequently occurring epithelial ovarian cancer associated with Lynch syndrome (endometrioid, 45%; high-grade serous, 28%; and low-grade serous, 13%) unlike most sporadic ovarian cancers or ovarian cancer associated with HBOC. The significance of Lynch syndrome surveillance can be seen in one patient (no. 20) who developed AEH after a 10-year surveillance period. At that time, she underwent hysterectomy during which low-grade and high-grade serous occult cancers were identified. She remained disease-free at 7 years after the hysterectomy.

While hereditary cancer treatments have advanced greatly in recent years, we often have a difficult time treating patients because little evidence is available from large-scale studies. Many of the patients are adolescents or young adults who need support with school, employment, or getting married in addition to medical care; however, regional support systems are often not available. The experience of our gynecologic services department, which we described in this paper, could provide useful information for managing patients with hereditary cancer.

A study on the role of genetic counselors in fertility preservation among patients with hereditary cancers has been submitted separately.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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臨床研究・症例報告

COL2A1 遺伝子新規突然変異による重症型先天性脊椎骨端異形成症の2例

谷口直子[※1] 皆川京子[※1] 折山恭子[※1] 三崎真生子[※1]
柴田暁男[※1] 川本久美[※1] 澤井英明[※2] 竹島泰弘[※1]

要旨

先天性脊椎骨端異形成症（spondyloepiphyseal dysplasia congenita，以下SEDC）は2型コラーゲン（*COL2A1*）遺伝子の変異が原因である。今回我々は，*COL2A1* 遺伝子新規突然変異による2例の重症型SEDCを経験した［c.1700G＞A（p.G567D）（症例1），c.1376G＞T（p.G459V）（症例2）］。出生前の画像解析において症例1は軟骨低発生症，症例2は軟骨無形成症が疑われたが，出生後重症型SEDCと診断された。重症型SEDCの予後は不良であるが，2例とも現在，それぞれ6歳，1歳で在宅人工呼吸管理により生存中である。胎児診断での予後予測は困難なこともあり，出生後に総合的な判断を行う必要がある。また，遺伝子型と重症度の関連は明らかでない点が多く，今後も遺伝子解析を含めて多症例の解析が必要である。

［小児科臨床　70：539，2017］

KEY WORDS　severe spondyloepiphyseal dysplasia congenita，*COL2A1*

はじめに

骨系統疾患は，従来，骨変化の類似性をもとにして各疾患単位を臨床的疾患グループにまとめて分類されていたが，近年，各疾患の遺伝子変異が明らかになっており，新分類では疾患グループを原因遺伝子に基づいて記載している。軟骨基質の主要成分である2型コラーゲン（*COL2A1*）遺伝子変異を原因とする疾患の臨床型は，先天性脊椎骨端異形成症（spondyloepiphyseal dysplasia congenita，以下SEDC）スペクトラム，Stickler骨異形成症，Kniest骨異形成症に分類される。SEDCスペクトラムの症状の幅は広く，重症から順に軟骨無発生症Ⅱ型，軟骨低発生症，SEDCの連続する臨床スペクトラムである。SEDCの臨床所見の特徴は脊椎椎体と骨端の骨化異常のため，四肢と体幹の短縮，短頸，樽状胸郭，鳩胸を呈する。脊椎後彎，腰椎前彎増強に加え，乳幼児期では脊椎洋梨状変形，成人では扁平椎をきたす。膝は外反，内反奇形をきたしやすく，軸椎歯突起の

※1：兵庫医科大学　小児科（〒663-8501　兵庫県西宮市武庫川町1-1）
※2：同　産婦人科

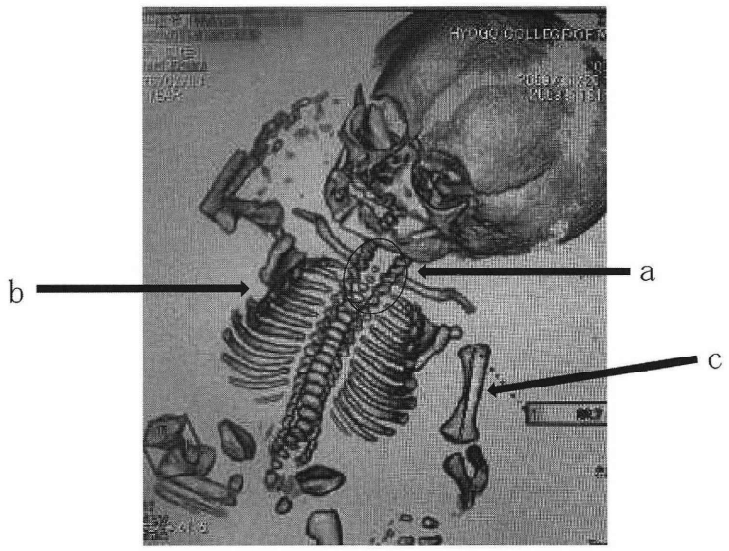


図1　症例1の胎児ヘリカルCT
頸椎椎体の乖離（a），胸郭低形成（b），長管骨の短縮（c）を認めた．

形成不全を認め，環軸椎亜脱臼は四肢麻痺や突然死の原因となりうる。また，眼異常（重症近視，硝子体網膜変性症），難聴を高率に合併する。発症の頻度は10万人に1人で，就学・就労可能な例から，呼吸不全をきたす，いわゆる重症型まで様々である[1)2)3)]。遺伝形式は常染色体優性遺伝であるが，症例の多くはde novoに発生しており，そのほとんどはグリシンからその他のアミノ酸に置き換わるミスセンス変異が原因である[4)]。しかし，遺伝子型と重症度の関連は明らかでない点が多い。今回我々は，呼吸不全が重度であったが，在宅人工呼吸管理が可能となった*COL2A1*遺伝子新規突然変異による2例の重症型SEDCを経験したので，文献的考察を加えて報告する。

症　例

〔**症例1**〕

母親34歳，未経妊未経産であり，自然妊娠成立後，妊娠30週より四肢短縮を指摘され，当院産婦人科を紹介受診した。胎児3Dヘリカル CT（在胎34週6日）で，頸椎椎体の乖離，胸郭低形成，長管骨の短縮を認め，軟骨低発生症が疑われていた（**図1**）。

在胎37週2日，アプガースコア4点（1分）4点（5分）で出生。出生時には啼泣はわずかで生後約10分で気管挿管を行い，肺低形成を疑い人工肺サーファクタントを投与し全身色は改善した。出生時体重 2,184 g（－1.5 SD），身長 34.5 cm（－6.1 SD），頭囲 35.5 cm（＋1.6 SD）と著明な低身長，四肢の短縮，筋緊張低下を認めた。全身X線所見で，胸郭低形成，扁平な肋骨，四肢の短縮，長管骨の短縮，大腿骨近位骨端の不整像，頸椎椎体の乖離を認めた（**図2**）。遺伝子検査により*COL2A1*遺伝子のエクソン26内に新規遺伝子変異 c.1700G>A (p.G567D) を認めた（PolyPhene-2：score 0.982）。両親には*COL2A1*遺伝子の変異を認めなかった。椎体の骨化不全が高度ではないため，軟骨低発生症ではなく重症型SEDCと診断した。現在6歳で，在宅で人工呼吸管理，胃瘻からの栄養を行っている。

〔**症例2**〕

母親28歳，未経妊未経産であり，自然妊娠成立後，妊娠22週より四肢の著明な短縮を指摘され，当院産婦人科を紹介受診した。胎児3Dヘリカル CT（在胎34週6日）で，長管骨の短縮，骨幹端部の拡大，弾丸状の椎体，顔面の低形成，頭蓋冠の拡大，腸骨翼の低形成を認め，軟骨無形成症が疑われていた（**図3A，B**）。

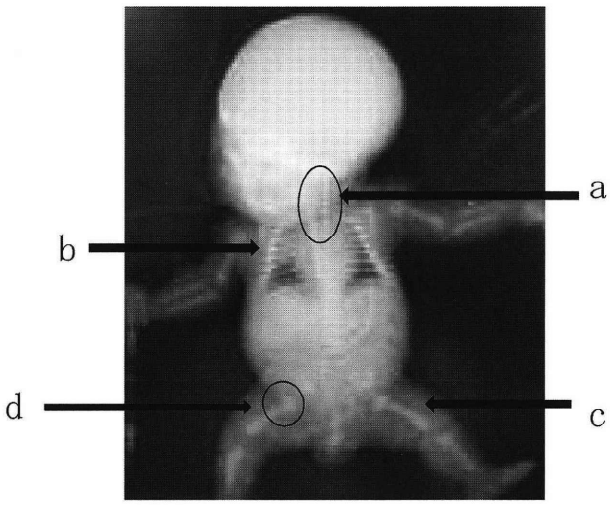


図2　症例1の出生時の全身X線
頸椎椎体の乖離（a），胸郭低形成（b），長管骨の短縮（c），大腿骨近位骨端の不整（d）を認めた．

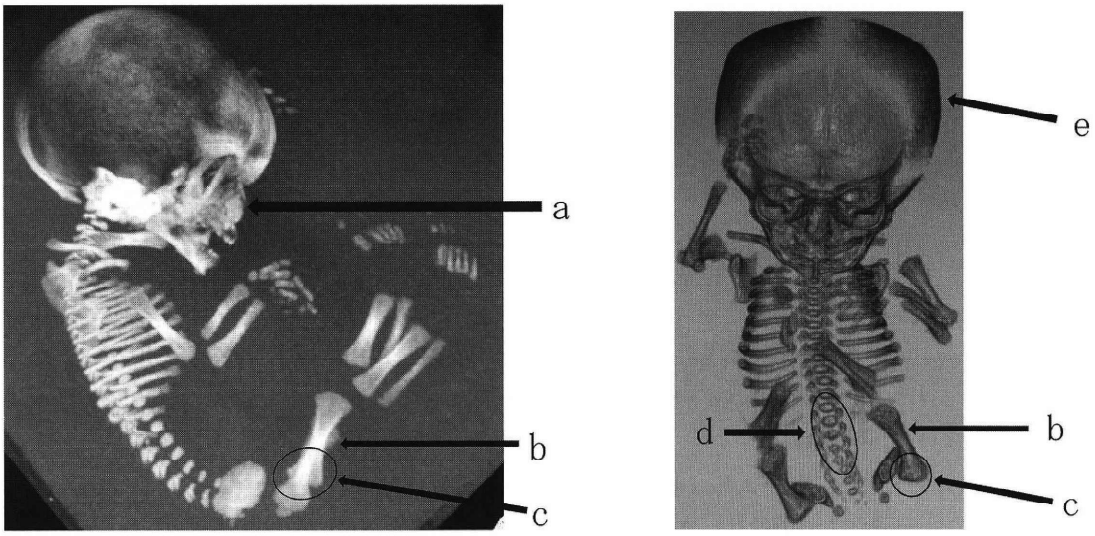
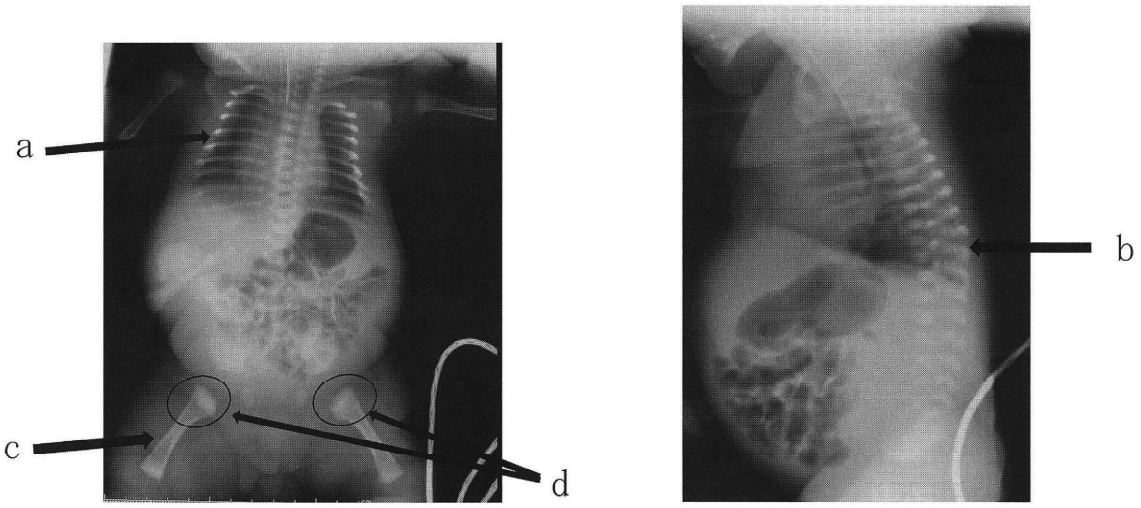


図3　症例2の胎児ヘリカルCT
顔面の低形成（a），長管骨の短縮（b），骨幹端部の拡大（c），弾丸状の椎体（d），頭蓋冠の拡大（e），腸骨翼の低形成を認めた．

在胎36週0日，アプガースコア7点（1分）8点（5分）で出生。出生時に啼泣減弱，全身チアノーゼ，筋緊張低下を認めた。マスクCPAP下で全身色は改善したが，徐々に陥没呼吸が増強したため，気管挿管し人工呼吸管理を行った。出生時体重 2,478 g（+0.1 SD），身長 37.0 cm（−3.2 SD），頭囲 36.0 cm（+2.8 SD）であり，全身所見で上腕と大腿の短縮，皮膚の余剰，胸郭低形成を認めた。全身X線所見で胸郭の低形成，ベル状の胸郭，長管骨の短縮，骨幹端のフレアリング，大腿骨近位骨端の不整像を認め（**図4A**），椎骨X線で洋梨状の椎体を認めた（**図4B**）。軟



A. 全身X線　　B. 椎骨X線

図4　症例2の出生時のX線
低形成でベル状の胸郭（a），洋梨状の椎体（b），長管骨の短縮（c），大腿骨近位骨端の不整像（d）を認めた．

骨無形成症の特徴である三尖手は認めなかった。これらの所見から重症型SEDCと診断した。遺伝子検査で，*COL2A1*遺伝子のエクソン22内に新規遺伝子変異c.1376G＞T（p.G459V）を認めた（PolyPhene-2：score 0.998）。両親は遺伝子検査を希望されなかった。最終的に，骨X線所見と遺伝子検査から重症型SEDCと診断した。現在1歳5カ月で，在宅で人工呼吸管理，胃瘻からの栄養を行っている。

考　察

Ⅱ型コラーゲン（*COL2A1*）遺伝子変異を原因とする疾患の臨床型は，SEDCスペクトラム，Stickler骨異形成症，Kniest骨異形成症に分類される。SEDCスペクトラムのほとんどの例はプロコラーゲンの三重鎖部のグリシン残基が他のアミノ酸に置換するミスセンス変異が原因である[5]。グリシン残基が側鎖のあるアミノ酸に置換されることでプロコラーゲンの三重鎖構造が破綻し，異常プロコラーゲンは細胞外に分泌されず細胞内に蓄積・変性し，優性阻害効果が生じると考えられている[2]。近年，骨系統疾患の原因遺伝子が次々に明らかになっており，骨系統疾患新分類では疾患グループが原因遺伝子に基づいて記載されている[4]。これまでNishimuraら，Huangら，Nakashimaら，Sangsinら，Kawanoらなどにより新規遺伝子変異の報告があるが，遺伝子型と表現型の相関は明確ではない[5)〜9)]。

今回，*COL2A1*遺伝子新規突然変異による2例の重症型SEDCを経験した。症例1はグリシンからアスパラギン酸，症例2はグリシンからバリンに置換して重症型SEDCとなった。

Nishimuraらの報告によると，グリシンがセリンに置換した症例の骨の表現型の重症度は様々であったが，グルタミン酸，アルギニン，アスパラギン酸，バリン，システイン等のセリン以外のアミノ酸に置換した症例の骨の表現型の重症度は重症であった[5]。分子量の大きいアミノ酸に置換すると，表現型が重症になると推測されている[5]。それぞれの分子量は，グリシン75.07，セリン105.09に対して，グルタミン酸147.13，アルギニン174.20，アスパラギン酸133.10，バリン117.15，システイン121.16と大きい。実際，症例1はアスパラギン酸，症例2はバリンへと分子量の大きいアミノ酸に置換した結果，重症型SEDCとなっており，同様の傾向がみられた。また，症例2と同じ部位のアミノ酸がアスパラギン酸に置換した症例が，身長33.5 cmで出生し，

生後4カ月で死亡した軟骨低発生症であったのに対して，症例2はアスパラギン酸より分子量の小さいバリンに置換した結果，身長 37.0 cm で出生し，重症型 SEDC の診断で生存中である。つまり，同じ部位のアミノ酸がより分子量の小さいアミノ酸に置換した場合，表現型はより軽症になった。一方で，同じ遺伝子変異をもつ SEDC の家族の報告によると，彼らは同じ遺伝子変異をもつにもかかわらず，表現型は様々であった。表現型を決める因子は，どのような遺伝子変異かだけではなく，力学的負荷などの環境因子[7]や修飾遺伝子なども関与するのではないかと推測される。

SEDC スペクトラム疾患の診断や重症度を超音波検査のみで診断するのは難しい。Schramm らの報告によると，軟骨低発生症6例中1例しか診断できなかった[10]。妊娠中に大腿骨の短縮傾向が出現する時期は SEDC では妊娠21～24週ごろ，軟骨低発生症では19～24週ごろで，近位肢節優位の四肢長管骨の短縮や骨幹端の拡大が特徴である。胎児3DCT では大後頭孔後部の骨化不全，扁平椎，骨幹端の拡大，頸椎，恥骨，踵骨の骨化欠如，近位肢節骨の短縮を認めるのが特徴である。出生前には症例1は軟骨低発生症，症例2は軟骨無形成症が疑われたが，出生後 SEDC と診断された。症例1に関して，軟骨低発生症と重症型 SEDC は連続するスペクトラムであり，鑑別は骨化不全の程度の差のみであるので，出生前での鑑別が難しかった。症例2に関して，実際出生後の呼吸障害が強く人工呼吸管理を要し，軟骨無形成症とは考えにくかった。軟骨無形成症は *FGFR3* 遺伝子の変異による疾患であり，遺伝子解析が鑑別診断に有用となった。出生前に想定していたよりも重症度が高かったため両親の受け入れに時間を要したが，現在は在宅移行となった。

SEDC の胎児診断は難しく，重症度，予後予測はさらに困難である[1]。出生前の診断が難しい理由として，骨異形成症は骨軟骨器官形成と恒常性維持に重要な遺伝子異常であるため，骨端の骨化遅延，不整骨化が出生後から顕在化してくることが理由として考えられる。よって，出生後に総合的な判断を行う必要がある。呼吸不全により予後不良と言われている重症型 SEDC も適切な診断，集中管理により，現在，在宅医療に移行して生存している。

結　語

COL2A1 遺伝子新規突然変異による2例の重症型 SEDC を経験した。遺伝子変異によりグリシンからより分子量の大きいアミノ酸に変異すると，より重症な表現型になる傾向があるが，表現型を決めるのは遺伝因子だけでなく環境因子も関与しているのではないかと考えられた。胎児診断は進歩しているが，重症度，予後予測は困難なこともあり，出生後に総合的な判断を行う必要がある。重症型 SEDC においても適切な診断，呼吸管理で長期生存が可能であるため，遺伝子型と表現型の関係をより明確にするために，今後，症例の蓄積が望まれる。

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Novel mutations in *COL2A1* in two cases of spondyloepiphyseal dysplasia congenita

Naoko Taniguchi[1)], Kyoko Minagawa[1)], Kyoko Oriyama[1)], Maiko Misaki[1)], Akio Shibata[1)], Kumi Kawamoto[1)], Hideaki Sawai[2)] and Yasuhiro Takeshima[1)]

[1)]Department of Pediatrics, Hyogo College of Medicine
[2)]Department of Obstetrics and Gynecology, Hyogo College of Medicine

Spondyloepiphyseal dysplasia congenita (SEDC) is caused by a mutation in the type 2 collagen gene (*COL2A1*). We report two cases of the severe form of SEDC involving novel *COL2A1* mutations. The patients in cases 1 and 2 were diagnosed with hypochondroplasia and achondroplasia, respectively, based on prenatal imaging, whereas they were definitively diagnosed with the severe form of SEDC after birth. Although the severe form of SEDC has a poor prognosis, both of our patients are receiving home mechanical ventilation and are still alive at the age of 6-years-old (case 1) and 1-year-old (case 2), respectively. In both of these cases, novel mutations, c.1700G＞A (p.G567D) (case 1) and c.1376G＞T (p.G459V) (case 2), were identified in *COL2A1*. Forecasting prognosis based on a fetal diagnosis is frequently difficult, so it is necessary to consider prognosis in a comprehensive way. In SEDC, the correlation between genotypes and severity is unclear ; therefore, further studies involving more cases are needed.

☆ ☆ ☆ ☆ ☆ ☆

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【症例報告】

遺伝性腫瘍女性3症例の妊孕性温存に対して認定遺伝カウンセラー®が果たした役割

鹿嶋見奈[1]，鍔本浩志[2]，上田真子[1,2]，脇本　裕[2]，岡田千穂[1]，覚道真理子[1]，今村美智子[3]，柴原浩章[2]，三好康雄[3]，渡　二郎[4]，佐藤智佳[1]，玉置知子[1,5]，澤井英明[1,2]

Role of certified genetic counselors for three cases of female patients with hereditary cancers for oncofertility therapy

Mina Kashima-Morii[1], Hiroshi Tsubamoto[2], Mako Ueda[1,2], Yu Wakimoto[2], Chiho Okada[1], Mariko Kakudo[1], Michiko Imamura[3], Hiroaki Shibahara[2], Yasuo Miyoshi[3], Jiro Watari[4], Chika Sato[1], Tomoko Hashimoto-Tamaoki[1,5], Hideaki Sawai[1,2]

【要旨】

本邦でもがん・生殖医療への取り組みが始まり，若年がん患者に対する生殖医療技術を用いた妊孕性温存が行われるようになってきた。しかし，若年がん患者には遺伝性腫瘍のリスクがあるが，がん・生殖医療における遺伝カウンセリングの報告は少ない。今回，遺伝性乳がん卵巣がん症候群（HBOC），Lynch症候群，Cowden症候群のがん治療および妊孕性温存に対して，関連科と連携を図りながら遺伝カウンセリングを行い，治療選択の支援を行った。若年女性と家族は，がん告知から治療までの短期間に妊孕性温存や遺伝学的検査などの問題に直面し迷いや不安を抱えていた。このような状況で，患者・家族の迷いや不安を傾聴し遺伝性腫瘍診療の立場から適切な治療選択を支援することは，認定遺伝カウンセラー®の重要な役割である。HBOC，Lynch症候群，Cowden症候群の3例のがん・生殖医療に認定遺伝カウンセラー®が果たした役割を報告する。

キーワード：がん生殖医療 oncofertility，遺伝性腫瘍症候群 hereditary cancer syndrome，認定遺伝カウンセラー® certified genetic counselor，遺伝カウンセリング genetic counseling

1）兵庫医科大学病院遺伝子医療部
Department of Genetics, Hyogo College of Medicine
2）兵庫医科大学産科婦人科学
Department of Obstetrics and Gynecology, Hyogo College of Medicine
3）兵庫医科大学乳腺・内分泌外科学
Department of Breast and Endocrine Surgery, Hyogo College of Medicine
4）兵庫医科大学内科学消化管科
Division of Gastroenterology, Department of Internal Medicine, Hyogo College of Medicine
5）高槻病院遺伝医療部門
Department of Clinical and Molecular Genetics, Takatsuki General Hospital

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伝子医療部に化学療法前の妊孕性温存が可能かどうかについての問い合わせがあった。当院遺伝子医療部と生殖医療専門医が協議した結果，患者はがん生殖外来の生殖医療専門医に初診となり，受精卵凍結，卵子凍結，卵巣凍結など様々な選択肢があることが説明された。認定遺伝カウンセラー®は初診時から診療に同席した。

1）遺伝カウンセリング

患者はすでに遺伝学的検査によりHBOCと確定診断されていたため，情報整理を目的として遺伝カウンセリングの場を設けた。家族歴に，実母が50歳で乳がん，母方祖母が70歳で乳がんの既往があった。がん生殖医療には様々な選択肢がある一方で，まだ実施症例数が少なく確定的な情報が乏しいため，患者は「受精卵凍結か卵子凍結にしようと思うが，どちらを選択するのが良いのか判断が難しい」と話した。そこで，がん生殖外来担当医からの説明内容をもとに，卵子凍結は受精卵凍結に比べ妊娠率は下がるが患者の同意のみで治療が可能であること，受精卵凍結では妊娠率は高まるがパートナーの同意が必要になることなど各治療のメリット・デメリットについて整理し，治療選択の支援を行った。

2）妊孕性温存の実施

術後化学療法の必要性が判明する前に，患者本人の妊孕性温存の希望を確認したうえで，排卵誘発し採卵を実施したが，採取できたのは未成熟卵子1個のみであった。そこで生殖医療専門医から再度の採卵も提案されたが，その時点で病理診断の結果により術後化学療法を行う可能性は低いことが判明したため，追加実施の希望はなかった。

(2) 症例2．Lynch症候群

当院初診時：31歳，未婚，未妊。

当院受診の経緯：31歳でB病院において子宮体がんと診断され，治療目的で当院産婦人科に紹介となった。患者は妊孕性温存を希望していた。婦人科腫瘍専門医からの依頼で産婦人科外来にて認定遺伝カウンセラー®が家族歴聴取を行ったところ，Lynch症候群が疑われる状況であった。そこで患者および本症候群患者と疑われる実母に対して，遺伝カウンセリングや遺伝学的検査についての情報提供を行った。しかし患者は子宮体がんの治療に専念したいという理由から，初診時には遺伝カウンセリングおよび遺伝学的検査の希望はなかった。その後，婦人科腫瘍専門医はLynch症候群が疑われること，Lynch症候群での子宮温存治療の報告は極めて乏しいため子宮温存治療による予後は不確実であることについて，患者に説明を行った。患者は子宮温存治療を希望し，高用量黄体ホルモン療法の結果，完全寛解を得た。

3年後の34歳時に片側乳がんを発症し，術前化学療法前の妊孕性温存および乳がん術式選択のために遺伝学的検査を考慮するとのことより，改めて当院乳腺専門医より遺伝カウンセリングの依頼があった。

1）遺伝カウンセリング

臨床遺伝専門医および認定遺伝カウンセラー®が遺伝カウンセリングを実施した。家族歴を再聴取したところ，実母が50歳代前半から60歳代前半にかけて，子宮体がん，卵巣がん，胃がん，大腸がんを発症，母方祖母が60歳代で大腸がん，70歳代で胃がんとなり死去していた。

患者の子宮体がんおよび乳がん発症年齢が若年であること，また家族歴があることからLynch症候群あるいはHBOCの可能性が疑われることが伝えられた。患者は妊孕性温存として卵巣凍結も希望していたため，遺伝学的検査が妊孕性温存の選択および乳がんの術式選択に対して重要な判断材料になることを説明した。

2）遺伝学的検査

Lynch症候群のみならずHBOCの可能性も考慮し，腫瘍関連遺伝子パネル検査を実施した結果，*MLH1*遺伝子に病的バリアントを認め，Lynch症候群と確定された。

患者は「遺伝学的検査でLynch症候群と診断がついて良かった」と話し，治療に対しても前向きに考える様子が見受けられた。一方で同席していた実母は「私から遺伝したと思うと申し訳ない」と話した。家族それぞれで結果の受け止め方が異なり，患者のみならず実母に対しても認定遺伝カウンセラー®が複数回にわたり，患者本人の外来の待ち時間や診察後の時間を利用し，本人や実母が抱える今後の治療や遺伝に対する迷いや不安な気持ちを聴き，関連科の主治医と情報共有を行いながら心理的負担を取り除けるようサポートを行った。

3）妊孕性温存の実施

Lynch症候群と確定診断後の術前化学療法前に，妊孕性温存の希望を確認した。そこで排卵誘発して採卵を実施し，未受精卵子19個の凍結保存を行った。さらに腹腔鏡下片側卵巣摘出術を実施し，卵巣凍結保存を行った。

(3) 症例3．Cowden症候群

当院初診時：31歳，未婚，未妊。

当院受診の経緯：10歳代後半に食道・胃の過形成ポリープ，20歳代前半に片側乳がんを発症し，C病院において乳がん手術および術後TAM治療を5年間受けた。30歳代前半に対側乳がんを発症し，同院において乳房全摘および術後TAM治療を受けていたが，経過中に甲状腺乳頭がんも判明したため，D病院において甲状腺全摘を受けた。その後，遺伝カウンセリングを目的に当院を受診した。

1）遺伝カウンセリング

遺伝子医療部において臨床遺伝専門医および認定遺伝カウンセラー®が遺伝カウンセリングを実施した。家族歴では，実父が50歳代で食道・胃の過形成ポリープ

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未発症家族にとって遺伝学的検査による確定診断は健康管理に有益な情報となる。しかし本邦では再発乳がん等での薬剤選択を考慮する特定の場合を除き，遺伝学的検査は自費診療であり，検診やリスク低減手術などの予防的処置に保険適用はない。よって経済的なハードルが高く，法が未整備であるなど問題点も多い。そのため，HBOCなど遺伝性腫瘍の病的バリアント保持者に対するサーベイランスや予防的処置への保険適用など法の整備が必要だと考える。また遺伝カウンセリングにより患者・家族の不安が解消されることが多い[13]と報告される一方で，患者・家族によっては遺伝学的検査の結果によって新たな不安が生じる状況も経験するため，個々人に合わせた対応が要求される。また情報は日々更新されるため，最新の情報を常に入手し内容を十分に理解・評価した上で，患者に適切な情報を提供し患者自身の選択を支援する必要がある。

若年遺伝性腫瘍患者・家族はがん告知・治療とほぼ同時期に遺伝学的検査，妊孕性温存，就労，予防的手術などの問題に直面し，短期間に選択を迫られることになるため，患者・家族に寄り添いながら十分な理解に基づく自己決定ができるよう支援することは，遺伝医学そして医療を理解している認定遺伝カウンセラー®の重要な役割である。がん告知・治療や複数科の診察など身体的・精神的負担を抱える患者・家族に対して，診療各科と密に連携を図り，可能な限り患者・家族の負担を軽減できるよう包括的診療体制の調整を行うことも重要である。

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編 集 後 記

兵庫医科大学産科婦人科学講座の礒島初代教授とは、退官される最後の１年に私が研究生として関わらせていただいた。短い期間であり、また、実際にお話しした時間はとても短かったが、真実は何か？を真摯に誠実に厳しく追及される姿勢が印象的であった。実際に研究に携わるようになり香山二代目教授に国内外の学会に連れて行ってもらうと、ホヤやヒトデの受精機構などが発表されており、受精を研究するのにヒトやウシ、ウマなど哺乳類に捕らわれず視野を広げることを学んだ。当講座の研究は生殖、周産期、腫瘍などそれぞれ分野は分かれているが、その根底にあるのは連綿と続く、科学者としての矜持である。

さて、論文発表の目的は、自分たちの新規知見を多くの医療者や研究者と共有することである。国際英文誌はより多くの目に触れる機会があり、自分たちの論文は将来にわたって残り誰かがPubMedなどで検索してくれる。解釈が変わることはあっても論文に記載された方法を行えば結果が再現される（もちろんlimitationもあるが)。個人的には、サイエンスを背景に創造性や新規性に富んだアートのような作品に惹かれるが、医療分野では具体的に社会に、より直接的には患者にどう還元されるかが要求されることも多い。これは、公的研究資金を得るためには必須の項目である。雑誌の編集長はインパクトファクターを高めることを目指し、医療者の中にも高インパクトファクターが業績として要求される場合があり、この点で和文紙は不利である。一方で、国内の医療状況に依存する課題に対して国内向けに情報発信するのであれば和文がふさわしいが、国内学会機関紙が関連する海外学会の英文誌に統一される傾向がある。

私が英文校正会社を初めて利用した時に、英語表記だけでなく論文構成や何が足りないかまでやり取りがあり、英文論文執筆の負担が大幅に軽減したことを覚えている。その会社はその後質が落ちてしまったが、現在はAIを用いて投稿先にふさわしい英文校正を行い論文作成しており、人の手を借りずに投稿しても特に不備はなく受理されるようになった。ここまで翻訳精度が上がると将来的に和文論文も容易に海外からアクセスできるようになるので、英文化を進めてきた国内学会機関紙が回りまわって日本語投稿を許容するようになるかもしれない（既にある学会では数年前に日本語投稿に変更された)。当然ながら論文作成の目的は「英語で書く」ことでは無いので、ここに時間を割くのは本末転倒である。ただ、参考文献は英語が多いので正確な日本語に翻訳する労力と英文で書く労力を天秤にかけて選択するように指導している。

若い先生は専門医取得のために論文が必要で、投稿から受理までの時間が早いことが現実的な投稿先の選択に関わってくる。投稿から受理、発刊まで迅速な雑誌は新規知見を発表するにも有利である一方で、査読者や編集者とのやり取りの中で質を高めていく作業が省略される。出版された後でerratumを出すこともあり注意が必要である。最近はオープンジャーナルによりインパクトファクターが高くなっている雑誌もあるが、オープンジャーナルは高額な出版費用が著者負担となり、投稿費用が現実的に投稿先を決める要因となっている。

研究内容や論文の書き方などは時代によって変わってきたが、研究のきっかけは礒島初代教授から変わらない。「なぜだろう？」という純粋な気持ちから論文発表になるよう指導していきたいと思う。

鍔本　浩志