p38 MAPK and p42/44 MAPK is involved in TGF-β1-induced increase in PAR2 expression in ESCs. In this context, it appears to be somewhat paradoxical that IL-1β and TNF-α did not increase PAR2 expression, although these molecules activate p38 MAPK and p42/44 MAPK in ESCs (Yoshino *et al.*, 2004). Presumably, the activation of p38 MAPK and p42/44 MAPK is necessary but not sufficient for increasing PAR2 expression in ESC, though the precise mechanism is unknown at present. Another notable point of our finding is that the effect of p38 MAPK and p42/44 MAPK inhibitors on PAR2 expression was not great, which indicates that other pathways may also have an effect on TGF-β1-induced PAR2 expression. In this sense, it is interesting that a PI3K inhibitor also inhibited TGF-β1-induced increase in PAR2 expression in ESCs. The PI3K pathway may play a complementary role to the MAPKs in the TGF-β1-induced response.

Since TGF-β stimulates disease progression, it should be a therapeutic target of endometriosis. A variety of agents that interfere with TGF-β signaling, including neutralizing antibodies, soluble receptors, antagonists and antisense nucleotides, have been developed to treat diseases in which TGF-β is a crucial pathogenic factor (Gordon and Blobe, 2008). Indeed, some of these drugs are undergoing clinical trials. The present study suggests that inhibitors of p38 MAPK and p42/44 MAPK could be candidate drugs for the treatment of endometriosis, given that they repress TGF-β1-induced PAR2 expression. This action by a p38 MAPK inhibitor may partly explain our previous finding that the inhibitor diminished endometriosis in a mouse model (Yoshino *et al.*, 2006).

In the present study, we evaluated the effect of TGF-β1, but neither TGF- β2 nor TGF- β3, on PAR2 expression in ESCs. As TGF- β2 and TGF- β3 have been reported to be increased in endometriotic tissues (Tamura *et al.*, 1999), it would be interesting also to study the effect of these molecules.

In summary, the present study demonstrated that TGF-β1 enhanced PAR2 expression and, as a consequence, increased PAR2-activation-induced IL-6 secretion from ESCs. In light of the multiple roles of PAR2 in promoting the development of endometriosis, TGF-β1 may accelerate disease progression by up-regulating PAR2 expression.

Authors' roles

A.S., Y.O. and O.Y. participated in study design and manuscript drafting. A.S., Y.O. and M.T. participated in execution and analysis. A.S., Y.O., T.H., Y.H., K.K., M.H., Y.T., T.Y. and Y.T. participated in critical discussion.

Acknowledgements

The authors thank Emi Nose for her technical assistance.

Funding

This study is partly supported by grants from the Ministry of Health, Labour and Welfare, and the Ministry of Education, Culture, Sports, Science and Technology.

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Myomectomy reduces endometrial T2 relaxation times

Magnetic resonance imaging was used to measure the endometrial T2 relaxation times of patients with infertility with fibroma. Although the location of fibromas did not influence the T2 relaxation times, we did observe a significant decrease in endometrial T2 relaxation times after myomectomy. (Fertil Steril® 2011;95:2781–3. ©2011 by American Society for Reproductive Medicine.)

Key Words: MRI, T2 relaxation time, fibroid, myomectomy, endometrium

Although uterine fibroids, which occur in 20% to 50% of women, are the most common type of solid pelvic tumor (1), the relationship between fibroids and infertility is not well established (2, 3). It has been reported that myomectomy can increase the pregnancy rate for patients with infertility (4). However, the mechanisms by which this occurs are not well understood. Several theories have been proposed. First, it is possible that fibroids alter uterine cavity contour, resulting in mechanical pressure. Alternatively, the fibroids may induce abnormal uterine contractility (5, 6). Finally, local inflammation associated with the presence of fibroids may give rise to a hostile endometrial environment that impairs sperm transport and embryo implantation (5). It has been reported that excessive concentrations of inflammatory cytokines have deleterious effects on embryonic development and implantation (7, 8). Inagaki et al. (9) demonstrated that uterine cavities containing fibroids exhibit a state of excess inflammation, with up-regulation of matrix metalloproteinases and inflammatory cytokines such as interleukin-1 and tumor necrosis factor α.

Magnetic resonance imaging (MRI) is a high-resolution method of differentiating soft tissues. In MRI, the nuclei of atoms in samples first are aligned along a static magnetic field, then are excited to a higher-energy state by a radiofrequency signal, and then return to a lower-energy equilibrium state. T2 relaxation time is a parameter that describes the relaxation to the equilibrium state once the radiofrequency signal is turned off. As an assessment of inflammatory status, T2 relaxation time is a useful way to detect the inflammatory status of rheumatoid disease (10, 11), dermatomyositis (12), and Graves' orbitopathy in Graves' disease (13, 14). In the present study, we investigated the endometrial T2 relaxation times of patients with infertility with fibroma. We compared T2 relaxation times before and after surgery to examine the effect of myomectomy on the endometrium of patients with uterine fibroids.

A total of 35 patients with uterine fibroids who desire pregnancy were examined by MRI between September 2008 and October 2010 at Takinogawa Clinic. Inclusion criteria were as follows. First, patients had intramural- or submucosal-type fibroid. Second, in advance of MRI all patients underwent screening for ovulation and corpus luteum function. Patients had regular menstrual cycles of approximately 28 days. Basal levels of serum FSH, LH, and PRL on menstrual cycle day 3 through 5 were within normal range (criteria: FSH 3.5–12.5 mIU/mL, LH 2.4–12.6 mIU/mL, and PRL 4.9–29.3 ng/mL). Serum E_2 and P concentration in midluteal phase were >100 pg/mL and 10 ng/mL, respectively. After the screening test, ovarian functional status was monitored by basal body temperature (BBT) chart. An analysis was performed of BBT graphs, in which a rise in temperature of at least 0.2°C above that of the preceding 6 days that was completed in <48 hours and sustained for at least 11 days would indicate the occurrence of ovulation (15). All patients included in this study showed unequivocal biphasic cycles in their BBT chart. We designated the first day showing elevated temperature of at least 0.2°C as luteal phase day 1. Third, MRI was performed during the time of implantation window (luteal phase day 5–day 9), judged retrospectively by BBT chart (judged by gynecologists O.Y. and H.T.).

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Received December 16, 2010; revised December 29, 2010; accepted January 7, 2011; published online February 11, 2011.
O.Y. has nothing to disclose. M.H. has nothing to disclose. Y.O. has nothing to disclose. T.H. has nothing to disclose. Y.S. has nothing to disclose. H.T. has nothing to disclose. O.N. has nothing to disclose. Y.T. has nothing to disclose.
Supported by Health and Labor Sciences Research Grants from the Ministry of Health, Labor, and Welfare of Japan and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology.
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By routine MRI study, the information retrieved included the location, number, and size of fibroids. Magnetic resonance studies were performed with use of a 1.5-T magnet unit (MRI machine from Siemens Japan, Shinagawa, Japan). Subsequently, conventional axial and sagittal T2–weighted images (repetition time [TR]/echo time [TE] = 4560–4720/107–111 milliseconds) and axial T1–weighted images (TR/TE = 550/8.5 milliseconds) were obtained with use of fast spin-echo techniques. T2 relaxation times of endometrium were measured on the same slice (350-mm field of view, 132 × 192 matrix, 3-mm slice thickness, bandwidth 362 Hz) with use of a spin-echo sequence. Eight images were acquired at each of the following TEs: 1.7, 23.4, 35.1, 46.8, 58.5, 70.2, 81.9, 93.6, 105.3, 117, and 128 milliseconds. The TR was 3 seconds, giving a total of 509 seconds acquisition time.

Ten out of 35 patients underwent myomectomy at Teikyo Mizonokuchi hospital. Among these 10 patients, 9 patients underwent laparoscopic-assisted myomectomy, and 1 patient underwent transcervical resection of fibroma. Four to 6 months after surgery, patients underwent a second MRI to evaluate T2 relaxation times during the implantation window. For statistical analysis, the Mann-Whitney *U* test was used for comparing between groups, and the paired *t*-test was used for comparing results before and after surgery.

T2 relaxation times in uterine endometrium obtained from patients with infertility who had intramural-type (n = 24) and submucosal-type (n = 12) fibroids were compared. We examined data from the midluteal phase. As shown in Figure 1A, the median value and minimum to maximum data of the two groups were 213 milliseconds (99–368 milliseconds) and 187 milliseconds (111–455 milliseconds) in intramural fibroids and submucosal fibroids, respectively. There was no statistical difference between groups (P=.9).

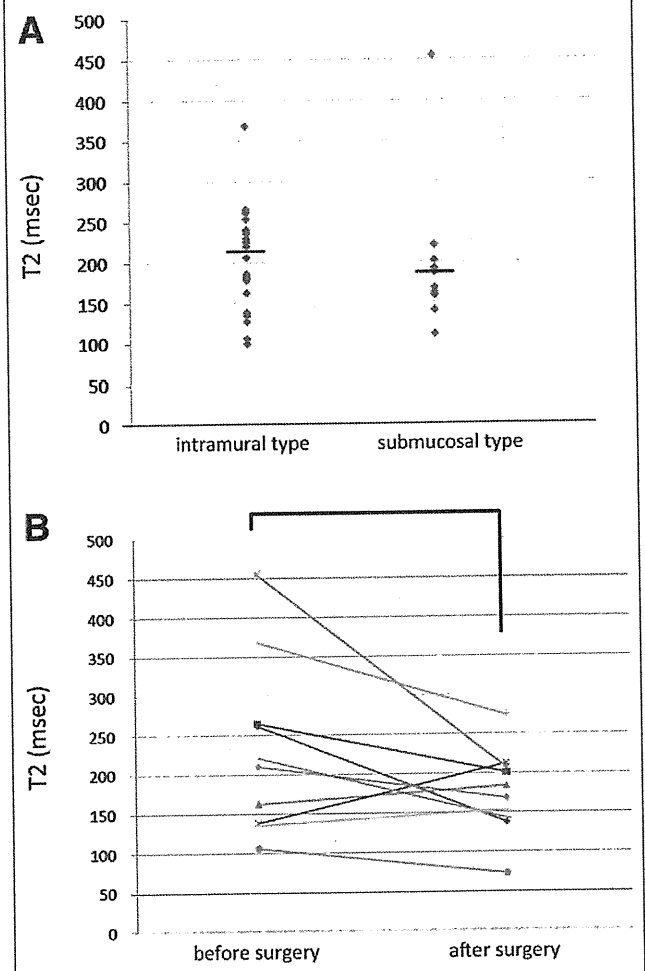
Because T2 relaxation times in the endometrium were comparable between intramural and submucosal fibroids (Fig. 1A), the data from both groups were combined in the subsequent study. After myomectomy, 10 patients underwent MRI at midluteal phase, and T2 relaxation times in the endometrium before and after surgery were compared. Of the 10 patients, 7 underwent surgery for intramural-type fibroids, and 3 underwent surgery for submucosal-type fibroids. As shown in Figure 1B, T2 relaxation times were decreased significantly after surgery (P=.03).

In the present study, we investigated the endometrial T2 relaxation times of patients with infertility with fibroma. We found that the endometrial T2 relaxation times were comparable regardless of the location of fibromas. Moreover, endometrial T2 relaxation times obtained after myomectomy were shortened significantly compared with the results before surgery.

Management of fibroids continues to present difficulties when used to treat infertility, because of a lack of understanding of the mechanisms by which fibroids impede pregnancy. Although myomectomy is recognized as a method to increase the rate of pregnancy (4), the precise mechanism of its contribution to fertility remains uncertain. It has been reported that the local inflammation associated with the presence of fibroids may result in a hostile endometrial environment that impairs fertility (7–9). Inagaki et al. (9) proved that the uterine cavities of patients with fibroids exhibited excessive inflammatory status. Accordingly, myomectomy might increase the fertility rate by decreasing the inflammatory status of the endometrium. In that study, 5 mL of saline solution was injected into the uterine cavity, and matrix metalloproteinase and cytokine levels of the fluid were measured to determine the inflammatory status directly (9). However, the volume of the uterine cavity can be decreased after removal of fibroids, making it difficult to compare precisely the inflammatory status before and after surgery. Therefore, it is necessary to develop less-invasive techniques that can estimate the inflammatory status of the uterine cavity. In the present study, we focused on T2 relaxation times obtained by MRI. This technique has proved useful in detecting the inflammatory activity of rheumatoid disease (10, 11), dermatomyositis (12), and Graves' orbitopathy in Graves' disease (13). Here, we observed a significant decrease in T2 relaxation times in patients examined after myomectomy. This suggests that myomectomy may suppress inflammatory activity in the endometrium.

FIGURE 1

(A) T2 relaxation times in uterine endometrium obtained from patients with infertility who had intramural-type (n = 24) and submucosal-type (n = 12) fibroids were compared. The data from the midluteal phase were examined. (B) Ten patients underwent myomectomy and received MRI examination at the midluteal phase before and after surgery. T2 relaxation times in the endometrium were compared P=0.03.



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T2 relaxation times in the human endometrium have been examined throughout the menstrual cycle. Varpula et al. (16) reported that a rapid increase in T2 relaxation times occurred during the proliferative phase, followed by little or no increase through the middle of the secretory phase. Hoad et al. (17) also reported that, during the periovulatory phase, T2 relaxation times were longer than in the other phases. They also observed that the variation in uterine tissue relaxation times between subjects was greater than the intrasubject cycle variation. Because of the large "normal" range, it might be very difficult to compare subjects or determine pathologic changes in the tissues from just a single measurement. However, because individuals exhibited similar increases and decreases over the menstrual cycle, the changes in T2 relaxation times within the same subject can be evaluated (17). Therefore, by comparing T2 relaxation times at the same menstrual phase obtained before and after myomectomy, the effect of surgery could be estimated. In our experiment, T2 relaxation times were measured during the "implantation window," the luteal phase day 5 to 9. We observed that there is no significant difference in T2 relaxation times between patients with fibroma and healthy volunteers (data not shown). Thus, measurement of T2 relaxation times would not be an effective way to detect uterine abnormalities, but it can be used to assess the success of myomectomy and is valuable in increasing our understanding of the pathophysiology of uterine fibroids in infertility. Other than inflammation (12, 14), iron content (18) is also known to increase T2 relaxation times. Therefore, further study is needed to confirm that T2 changes after myomectomy actually represent the change of inflammatory status in endometrium. This work represents a first step toward better understanding the relationship between T2 relaxation times and uterine fibroids in patients with infertility.

Acknowledgments: The authors thank Heather M. Martinez, Ph.D., for her helpful discussion and critical reading of the manuscript. They thank Yasufumi Shimizu, Ph.D., M.D., Kouji Motoyama, Ph.D., M.D., and Yasuhiro Kawamura, Ph.D., M.D. (Denentoshi Ladies' Clinic); Kenichi Tatsumi, Ph.D., M.D. (Umegaoka Women's Clinic); Susumu Tokuoka, Ph.D., M.D. (Tokuoka Women's Clinic); Ryo Matsuoka, Ph.D., M.D. (Tokyo Hitachi Hospital); and Takayoshi Ogawa, Ogawa, Ph.D., M.D. (Ogawa Clinic) for their supporting this study. They also thank Mr. Ryuji Nojiri and Mr. Yoshitsugu Funatsu (Takinogawa Clinic) for their technical assistance.

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High Mobility Group Box 1 (HMGB1) Levels in the Placenta and in Serum in Preeclampsia

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Keywords
Apoptosis, hypoxia, inflammation, oxidative stress, receptor for advanced glycation end products, toll-like receptors

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Submitted October 4, 2010;
accepted December 13, 2010.

Citation
Wang B, Koga K, Osuga Y, Hirata T, Saito A, Yoshino O, Hirota Y, Harada M, Takemura Y, Fujii T, Taketani Y. High mobility group Box 1 (HMGB1) levels in the placenta and in serum in preeclampsia. Am J Reprod Immunol 2011; 66: 143–148

doi:10.1111/j.1600-0897.2010.00975.x

Problem
Preeclampsia is a pregnancy disorder characterized by systemic inflammation. High mobility group box 1 (HMGB1) is a molecule known to act as a 'danger signal' by participating in various inflammatory processes, but data in regard to preeclampsia are sparse. The aim of this study was to analyze placental and serum HMGB1 levels in normal pregnancy and preeclampsia.

Method of study
Sera were collected from women with preeclampsia soon after the manifestation of the disease and before commencing any medication. Placental samples were collected immediately after delivery. Expressed isoforms of HMGB1 (28- and 30-kDa) in the placenta were evaluated by Western blot analysis. Serum HMGB1 concentrations were measured using enzyme-linked immunosorbent assays (ELISA).

Results
Two isoforms of HMGB1 are expressed by the human placenta. The 28- and 30-kDa HMGB1 isoforms were expressed highly in preeclamptic placental tissue; however, compared with normotensive control tissue, differences in detected expression levels did not reach statistical significance. No significant difference was observed in serum HMGB1 levels between control and preeclampsia.

Conclusion
Inflammation provoked by HMGB1 is likely to be involved in the proinflammatory process in preeclamptic placenta. Further studies are needed to elucidate the precise role of HMGB1 in preeclampsia.

Introduction

Preeclampsia is a placenta-originated disorder and affects 3–5% of all pregnancies. It remains as one of the leading contributors to maternal and fetal morbidity and mortality.[1] It is a disorder characterized by intravascular inflammation and endothelial cell dysfunction. Despite recent progress in research, the biology of preeclampsia is still poorly understood.[2]

High mobility group box 1 (HMGB1), a non-histone chromatin-associated protein, was discovered three decades ago as a nuclear protein that migrates quickly during electrophoresis and was named according to this property.[3] HMGB1 is released from damaged cells and acts as a 'danger signal' by

participating in various inflammatory processes, including maturation of immune cells, release of cytokines and other inflammatory mediators, and tissue remodeling.[4,5] HMGB1 mediates its inflammatory responses by signaling via receptors such as the receptor for advanced glycation end products (RAGE)[6] and toll-like receptor (TLR) 2 and TLR4.[7] Ligation for these receptors results in activation of nuclear factor kappa B (NFκB), which induces upregulation of leukocyte adhesion molecules and the production of proinflammatory cytokines in both hematopoietic and endothelial cells, thereby promoting inflammation.

It has been demonstrated that HMGB1 is involved in the pathogenesis of a variety of both infectious and non-infectious inflammatory conditions. Elevated levels of HMGB1 in serum and tissues are observed during infection and tissue injury, and targeting HMGB1 with specific antagonists can have protective effects in established inflammatory diseases. For instance, circulating HMGB1 levels are markedly increased during severe sepsis,[8] pneumonia,[9] systemic lupus erythematosis,[10] and in the synovial fluid of patients with rheumatoid arthritis.[11] Administration of HMGB1 antagonists has been reported to decrease organ damage and mortality in models of systemic inflammation such as sepsis,[12,13] brain infarction,[14] arthritis,[15] acute pancreatitis,[16] and lung inflammation.[17]

Preeclampsia is characterized by an inflammatory state that includes elevated levels of proinflammatory molecules in the placenta and maternal serum.[18] The expression of RAGE, one of the receptors for HMGB1, was reported to be significantly higher in preeclamptic placenta when compared with normal placental tissue.[19,20] TLR4, also a receptor for HMGB1, is expressed higher in trophoblasts from patients with preeclampsia compared to normal pregnancies.[21,22] As for HMGB1, Holmlund et al.[23] demonstrated its expression in the trophoblasts by immunohistochemistry. Further immunohistochemical analysis demonstrated higher expression levels of cytoplasmic HMGB1 in the decidua from women with preeclampsia compared with normal pregnancy, but the difference was not conclusive in trophoblasts.[23] The circulating level of HMGB1 in pregnant women has never been elucidated.

In this study, we measured HMGB1 levels in the placenta and serum in normal pregnancies and pregnancies complicated by preeclampsia to ascertain whether this molecule is involved in the pathogenesis of preeclampsia.

Materials and methods

Serum and Tissue Collection

The study was approved by the ethical committee of the University of Tokyo and Musashino Red Cross Hospital, and written informed consent was obtained from all women. Placentas and maternal venous blood were obtained from women with uncomplicated, normotensive pregnancies and pregnancies complicated by preeclampsia. Preeclampsia was diagnosed by the presence of hypertension (an absolute blood pressure ≥140 mmHg systolic and/or 90 mmHg diastolic after 20 weeks of gestation) with proteinuria (≥300 mg/24-hr). Patients with preeclampsia did not have any prior history of hypertension or renal disease. All women in control group did not show clinical or pathological signs of preeclampsia, infections, or any other maternal or placental disease.

Blood samples were collected from women with preeclampsia soon after the manifestation of the disease and before commencing any medication. Sera were separated by centrifugation and stored at −70°C before use. Placental samples were collected immediately after delivery. Placental tissue was taken from the middle part of the placenta to avoid amnion and decidual tissue contamination. All samples were stored at −70°C until assayed.

Western Blot Analysis

Placental tissues were homogenized and then sonicated in lysis buffer [10 mM Tris–HCl, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, (pH 7.0)] with protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was determined using a modified Bradford protein assay with bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) as a standard. Thirty micrograms of protein was separated on 12.5% sodium dodecyl sulfate polyacrylamide electrophoresis gel and then transferred onto polyvinylidene fluoride (PVDF) transfer membranes (Amersham Biosciences, Piscataway, NJ, USA). Protein extracted from human endometrium was used as a positive control.[24] The blots were blocked in tris-buffered saline – 0.1% Tween-20 containing 5% nonfat milk and then incubated with antibodies at 4°C overnight. The membranes were incubated with primary antibodies: anti-human HMGB1 antibody (final concentration 2 μg/mL; R & D

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systems, Minneapolis, MN, USA) or goat anti-human actin antibody (1/1000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) as a loading control. Normal mouse IgG2B (Amersham Biosciences, Little Chalfont, UK) was used as an isotype control. The secondary antibody was horseradish peroxidase-conjugated anti-mouse (1/1000; Amersham Biosciences) or anti-goat (1/5000; Santa Cruz) IgG, which was incubated for 1 hr at room temperature. Signals were developed using ECL Western blotting system (Amersham Biosciences). Densitometric analysis was performed using ImageJ Image Software (National Institutes of Health, Bethesda, MD, USA). Each HMGB1 band was normalized to the densitometric value obtained from the same lane by blotting for actin, the internal reference.

Enzyme-linked Immunosorbent Assay (ELISA) Measurement of HMGB1

The concentration of HMGB1 in serum was measured in duplicate by a specific ELISA kit (Shino-test Corporation, Kanagawa, Japan). The minimum detectable dose of HMGB1 was 1 ng/mL. The intra- and inter-assay coefficients of variation were all <10%.

Statistical Analysis

Data analysis was performed using the statistical software package SPSS for Windows (Chicago, IL, USA). All data were checked for their normal distribution by submission to the Kolmogorov–Smirnov test, and if significant, non-parametric statistical analysis was applied. Parametric variables underwent the Student's *t*-test. Statistical significance was considered as $P < 0.05$.

Results

We firstly analyzed HMGB1 expression in the placenta. Western blot analysis showed that the human term placenta expresses HMGB1 and is detected as a 28- and 30-kDa band corresponding to two distinct isoforms of the molecule (Fig. 1). The latter band corresponds to biologically active acetylated isoform.[5,24]

We then compared the placental expression levels of the two isoforms between normal pregnancy and pregnancy complicated by preeclampsia. Maternal age, gestational age, parity, and mode of in delivery were comparable in both groups (Table I). Compared

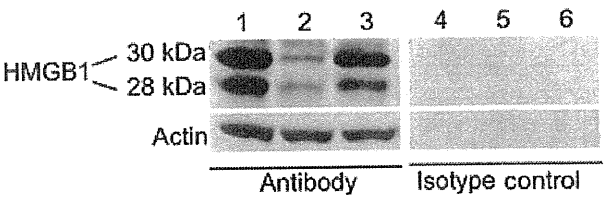

Fig. 1 A representative result of Western blot analysis in the placenta in preeclampsia (1, 4), normal pregnancy (2, 5) and human endometrium as a positive control (3, 6) for anti-high mobility group box 1 (HMGB1) or anti-actin antibody (1, 2, 3) and isotype negative control (4, 5, 6). Note there are two bands (28- and 30-kDa) specific for HMGB1.

Table I Clinical Backgrounds and Serum High Mobility Group Box 1 (HMGB1) Concentrations in Women With or Without Preeclampsia

	Normal pregnancy ($n = 32$)	Preeclampsia ($n = 35$)	P
Maternal age, year*	32.94 ± 3.58	33.49 ± 4.09	NS
Gestational age, week*	34.40 ± 4.90	33.50 ± 4.78	NS
HMGB1, ng/mL			
Median (IQR)	4.757 (2.592–6.861)	4.312 (2.451–6.011)	
Mean ± S.D.	5.119 ± 2.773	4.511 ± 2.537	NS

IQR, interquartile range.
*Data are presented as mean ± S.D.

to normal pregnancy, the level of 28- and 30-kDa HMGB1 expression was higher in preeclampsia, especially in the 28-kDa isoform (normal versus preeclampsia: 0.176 ± 0.112 versus 0.363 ± 0.296, 0.463 ± 0.332 versus 0.581 ± 0.379; 28-, 30-kDa, respectively: mean ± S.D.), although the difference did not reach statistical significance ($P = 0.087$, $P = 0.471$; 28-, 30-kDa, respectively) (Fig. 2, Table II).

Secondly, we measured the level of serum HMGB1 in normal pregnancy and pregnancy complicated with preeclampsia. As shown in Table II, maternal age and gestational age were comparable between the normal and preeclampsia group. When we compared serum HMGB1 concentrations, there was no significant difference between controls and women with preeclampsia.

Fig. 3 shows the correlation between serum HMGB1 concentration and gestational ages for both groups. There was no correlation between gestational

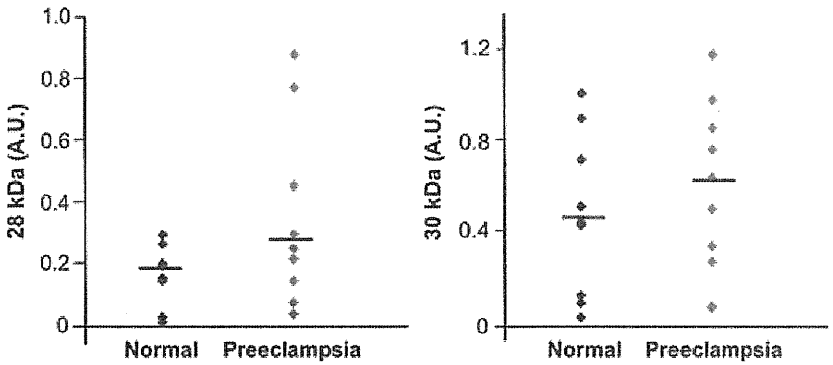

Fig. 2 A scatter plot of placental high mobility group box 1 (HMGB1) protein expression level in both 28- and 30-kDa bands in normal pregnancy and pregnancy complicated with preeclampsia. The data are presented as arbitrary densitometric units (A.U.). The horizontal bars indicate the mean. The expression level of HMGB1 protein in both isoforms was higher in preeclampsia, although the difference did not reach statistical significance ($P = 0.087$, $P = 0.471$; 28-, 30-kDa, respectively).

Table II. Clinical Backgrounds and Placental High Mobility Group Box 1 (HMGB1) Expression Levels in Women With or Without Preeclampsia

	Normal pregnancy ($n = 10$)	Preeclampsia ($n = 10$)	P
Primigravid (n)	7	7	NS[a]
Maternal age, year*	30.00 ± 5.14	33.40 ± 3.69	NS[b]
Gestational age, week*	38.81 ± 1.18	36.20 ± 3.26	NS[b]
Vaginal delivery (n)	7	3	NS[a]
HMGB1 28 kDa (A.U.)*	0.176 ± 0.112	0.363 ± 0.296	NS[b]
HMGB1 30 kDa (A.U.)*	0.463 ± 0.332	0.581 ± 0.379	NS[b]

*Data are presented as mean ± S.D.
[a]Fisher's Exact test.
[b]Student's t-test.

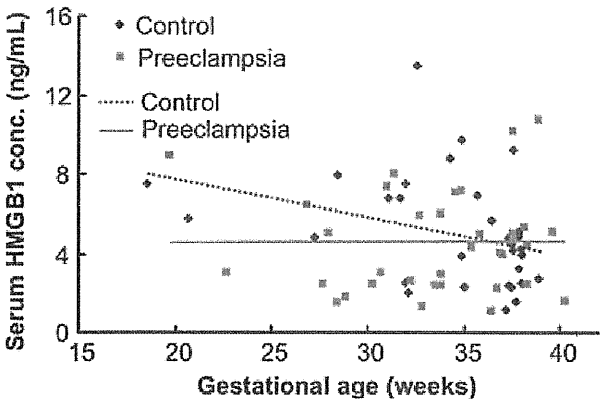

Fig. 3 A scatter plot of serum high mobility group box 1 (HMGB1) levels versus gestational age (weeks) in normal pregnancy (blue diamond dots) and women with preeclampsia (pink square dots). No statistically significant correlation was found between gestational age and serum HMGB1 level in either normal pregnancy (broken line; $r = -0.338$, $P = 0.058$) or women with preeclampsia (unbroken line; $r = 0.002$, $P = 0.993$).

age and serum HMGB1 level in normal pregnancies (Pearson correlation, $r = -0.338$, $P = 0.058$) or in women with preeclampsia ($r = 0.002$, $P = 0.993$).

Discussion

In the present study, we showed that the expression of HMGB1 in the placenta was higher in preeclampsia compared with normal pregnancy, although the difference did not reach statistical significance. There was no difference in serum HMGB1 levels between groups. These findings add to our understanding of the possible involvement of HMGB1 in the pathology of preeclampsia.

Firstly, a quantitative evaluation of HMGB1 expression in the placenta by Western blot demonstrated that the expression of 28- and 30-kDa isotypes in the placenta from women with preeclampsia was higher compared to healthy pregnancies, although the difference did not reach statistical significance. A variety of factors are reported to induce the expression of HMGB1 such as necrosis,[25] apoptosis,[26] oxidative stress,[27] and hypoxia,[15] which are all known to be enhanced in the placenta in preeclampsia. Therefore, we speculate that the expression of HMGB1 is increased in the damaged preeclamptic placenta as a 'danger signal', further enhancing the immune response.

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Given the observation that placental HMGB1 is higher in preeclampsia, together with the fact that its receptors, RAGE and TLR4, are upregulated in the placenta in preeclampsia,[19,20,22,28] we suggest that the proinflammatory axis provoked by HMGB1 is enhanced in the preeclamptic placenta. Indeed, changes that may be induced by HMGB1 include NFκB activation, followed by the production of pro-inflammatory cytokines such as TNF alpha,[29] IL-6,[30] and endothelin,[31] or induction of apoptosis[32,33] are all events observed in the preeclamptic placenta. Although other endogenous and exogenous factors besides HMGB1 may also bind to RAGE and TLRs, such as advanced glycation end products (AGE) to RAGE, lipopolysaccharides and heat-shock protein 70 to TLR4, or peptidoglycan to TLR2, our result suggests that HMGB1 is one of the contributors modulating the development of preeclampsia.

There are several explanations for the lack of significant difference in placental HMGB1 levels between preeclampsia and control. Firstly, the sample number in this study was so small that the statistical study was underpowered. It is also possible that Western blotting followed by densitometry analysis has a limitation in detecting subtle difference. Another explanation could be that even in the healthy condition, the placenta is exposed by a mild inflammation, which is a nature of normal pregnant uterine environment,[34] and HMGB1 is constitutively expressed regardless of whether healthy or preeclamptic condition.

We then measured the circulating levels of HMGB1 in pregnancy. Our observation that placental HMGB1 is slightly higher in preeclampsia, and given a greater amount of trophoblast fragments are detected in the maternal circulation in preeclampsia compared to normal pregnancy,[35] prompted us to hypothesize that the circulating level of HMGB1 is higher in preeclampsia. Contrary to our hypothesis, there was no difference in the serum level of HMGB1 between normal pregnancy and pregnancy complicated by preeclampsia. One explanation could be that the level of circulating HMGB1 does not reflect its release from the placenta. This is partially supported by our finding that serum HMGB1 levels did not positively correlate with gestational age, yet HMGB1 levels should be in proportion to placental size and the number of shedding trophoblasts entering the maternal circulation. It is also possible that some component present in serum may bind HMGB1 and interfere with the ELISA system, as reported by Urbonaviciute et al.[10] Indeed, this interference resulted in an underestimation of serum HMGB1 levels in rheumatoid arthritis.[36] In addition, soluble RAGE (sRAGE), which is reported to capture and eliminate circulating HMGB1,[37] may have affected our results because circulating sRAGE levels are known to be elevated in preeclampsia.[28] Therefore, our results do not exclude the possibility that circulating HMGB1 is elevated in preeclampsia and could be a therapeutic target for preeclampsia.

In summary, we have demonstrated that the levels of HMGB1 in the placenta were slightly higher in preeclampsia. Inflammation provoked by HMGB1 is likely to be involved in the proinflammatory event, which is a prominent feature found in preeclamptic placenta. Further studies are needed to elucidate the precise role of HMGB1 in preeclampsia.

Acknowledgments

The authors thank medical colleagues in the University of Tokyo Hospital, Musashino Red Cross Hospital, for their assistance in sample collection and Dr. Kate Hale for editing the manuscript. BW is a fellow of Japan China Sasakawa Medical Fellow for YO. This work was supported by grants from Ministry of health, Labor and Welfare and Ministry of Education, Culture, Sports, Science and Technology and by grants from Japan China Medical Association (BW), Yamaguchi Endocrine Research Foundation (KK), the Japan Society for the Study of Hypertension in Pregnancy (KK), and Inohana Alumni Association of Chiba University School of Medicine (KK).

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診 療

子宮筋腫により誘導される子宮内膜の異常蠕動様運動は妊娠率を低下させる

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子宮筋腫のうち，特に筋層内子宮筋腫が不妊症を引き起こす機序はいまだよくわかっておらず，その取り扱いに苦慮することが多い。正常子宮内膜は着床期に蠕動様運動を低下させることで胚の着床を促すことが知られている。動画MRIを用いて子宮内膜の蠕動様運動の評価を行い，筋層内子宮筋腫が着床期の子宮内膜蠕動様運動に与える影響と妊孕性への関与について検討を行った。筋層内子宮筋腫を合併し，かつ，卵管因子・重度の男性因子のない51例の挙児希望患者を対象とした。黄体期5～9日目に，動画MRI検査を行った。子宮内膜蠕動様運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，MRI撮影後4カ月間の妊娠率を前方視的に比較した。群分けの基準は，これまでの検討で子宮筋腫を有さない症例では子宮内膜蠕動様運動が3分間で2回未満であることによる。低頻度群は29名（57%），高頻度群は22名（43%）であり，子宮筋腫の個数および最大径に関して差を認めなかった。MRI撮影後，低頻度群は29名中10名（34%）が妊娠に至ったのに対し，高頻度群での妊娠例はなかった（0%，$p<0.005$）。本来では子宮内膜の蠕動様運動を認めない着床期において，筋層内子宮筋腫を合併する不妊患者の約4割に子宮内膜の異常蠕動様運動を認めた。子宮筋腫により誘導される子宮内膜の異常蠕動様運動は妊娠率を低下させる可能性が示唆された。

はじめに

子宮筋腫は生殖年齢女性の20～50%が罹患しており[1]，日常臨床においてよく遭遇する疾患であるが，大半の症例は特に症状がないことから治療を要しない[2]。しかし，特に不妊治療との関連となると，子宮筋腫の解剖学的位置，すなわち粘膜下，筋層内，漿膜下のいずれに存在するかより，その取り扱いは大きく異なる[3]。粘膜下子宮筋腫の場合，子宮内腔への突出率が高ければ，子宮鏡下に低侵襲手術を行うことができる。一方で，筋層内・漿膜下を手術する際，腹壁からのアプローチが必要となる[3]。特に筋層内子宮筋腫症例は，手術による正常筋層へのダメージを考慮し，子宮筋腫核出術後に不妊治療の一時中止および，分娩時には，子宮破裂のリスクを避けるために帝王切開が推奨されることが多く[2]，患者への負担が多いことが挙げられる。子宮筋腫の位置と妊娠率に関するメタアナリシスによると，粘膜下子宮筋腫は明らかに妊娠率を低下させ，漿膜下子宮筋腫は妊娠率を低下させないことが判明している一方で，筋層内子宮筋腫が妊娠そのものに与える影響は一定の見解が得られていない[2][3]。すなわち，筋層内子宮筋腫の不妊症治療という観点からの取り扱いには苦慮することが多い。そこで，われわれ

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は筋層内子宮筋腫が不妊症に与える影響について，そのメカニズムの観点から検討することとした。

これまで，筋層内子宮筋腫は，着床に悪影響を与えること[4]，その機序として子宮における異常収縮を誘導する可能性が提唱されてきた[2)5)]。しかし，この仮説を実際に検証する報告はこれまでなされていない。着床期，子宮内膜の蠕動様運動は減少することが知られており，このことが着床に重要な現象であると考えられている[6)~8)]。近年のMRI撮像技術の進歩により，数秒間隔での連続撮影が可能となった。同一部位で連続撮影を行うことで，動画画像（Cine MRI）を構築することができるようになり，心臓をはじめとする各種臓器での応用が始まっている。Togashiらは同法を用いて，子宮内膜の蠕動様運動が描出できることを報告しており，着床期における子宮内膜の蠕動様運動の頻度は3分間で0~1回と報告している[6)]。本研究は筋層内子宮筋腫が着床期の子宮内膜蠕動運動に与える影響とその妊孕性への関与について検討を行った。

1．方　法

本研究は各施設の倫理委員会の承認を得て行った。2008年9月から2009年10月までに子宮筋腫を有する不妊症例に対して4病院（帝京大学医学部附属溝口病院，慶應義塾大学病院，福井大学医学部附属病院，滝の川クリニック）にてMRI検査を受けた95名を対象とした。このうち，本研究の適応症例として，①粘膜下病変を有さない筋層内子宮筋腫症例，②子宮筋腫以外に不妊症原因のないこと。具体的には排卵，黄体機能，卵管，パートナーの精子所見に重度な異常を認めないこと，③月経周期が順調であり，基礎体温表でのフォローを行い，MRIを高温期の5~9日目，いわゆるimplantation windowに撮影した。95名のうち，この基準に合う症例は51名であった。通常のMRI検査を行い，子宮筋腫の数および最大径，子宮内膜の変形の有無，子宮内膜症病変の有無を検討した。その際のMRI撮影条件は以下のとおりである。なお，MRIは1.5テスラ装置を用いた。T1強調像：（TR/TE＝400~550/7.0~8.5 msec），T2強調像：（T2WIs，TR/TE＝4000~4720/90~111 msec）子宮内膜の動画MRIの条件はOrisakaらによって報告されている方法を用いた[9)]。通常の呼吸下に6秒ごとに3分間，計30枚の子宮内膜部位のMRIをsingle-shot fast spin-echo（SSFSE）法にて撮影した（TR/TE＝6000/78 msec，FOV＝240 mm，slice thickness＝10 mm，matrix＝256×256）。得られた画像はcineモードにて高速再生した。読映は放射線科医師1名が判定した。判定のポイントとして，①子宮内膜周囲に存在する正常筋層，いわゆるjunctional zoneに運動があるか否か，また運動がある場合は3分間における蠕動様運動の回数として表記，②子宮筋腫の個数とその位置，および③子宮内膜症の有無を主に子宮内膜症性卵巣嚢胞の有無にて判別した。動画MRI検査の結果により，子宮内膜蠕動様運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，MRI撮影後4カ月間の妊娠率を比較した。群分けの基準は，Togashiらの報告，およびわれわれのこれまでの検討で子宮筋腫を有さない症例では子宮内膜蠕動様運動が3分間で2回未満であることによる[6)]。MRI撮影後4カ月間，通常の不妊治療を行い，妊娠に関して前方視的に検討した。基本的に自然周期ないしclomiphene citrate（CC）による治療を2~3コース行い，妊娠に至らない場合はhuman menopausal gonadotropin（hMG）療法を2コース程度行った。卵胞の最大径が18 mmに成長した段階で，hCG注射（5,000単位）に切り替え排卵を誘導した。基本的に排卵時期を予測し，性交時期を指示したが，特にパートナーの運動精子濃度が20×10^6/m*l*以下の場合は人工授精を行った。黄体ホルモン補充療法は行わなかった。

子宮内膜蠕動様運動の低頻度群および高頻度群間の患者年齢，不妊期間，子宮筋腫の個数および最大径はmeans±SDで示し，両群間の比

表2 患者背景

	低頻度群	高頻度群	
患者数（人）	29	22	
年齢	36（29～41）	37（29～41）	中間値（最小～最高値） 有意差なし
不妊期間（月）	24（3～84）	24（4～108）	中間値（最小～最高値） 有意差なし
原発性・続発性不妊（患者数） 原発性 続発性	 20 9	 17 5	 有意差なし
体外受精の既往（患者数） なし あり	 24 5	 18 4	 有意差なし

子宮内膜蠕動運動が3分間で2回未満（低頻度群）および2回以上（高頻度群）の2群に分け，患者背景を比較した。

表1 蠕動様運動回数別の患者分布

蠕動様運動回数（/3 min）	患者数（全51名）
0	19
1	10
2	1
3	6
4	10
5	3
6	2

動画MRI法（Cine MRI法）を用いて子宮内膜の蠕動様運動を計測した。3分間当たりの蠕動様運動回数別による患者分布。これまでの報告から，3分間で2回以上の蠕動様運動は高頻度と考えられる。

較はMann-Whitney's U-test(Statcel software)を用いた。その他の指標については患者数で示し，χ^2検定にて2群間の比較を行った。

2．結　果

MRI検査を受けた子宮筋腫を有する不妊症95例のうち，上記条件に適合した対象患者は51名であった。51名を子宮内膜の蠕動様運動回数別に分類した（表1）。3分間に蠕動様運動を0ないし1回認めた低頻度群，および2回以上を認めた高頻度群の症例数はそれぞれ，29例(57%）と22例（43%）であった。両群の患者背景を表2に示す。患者年齢，不妊期間，原発性不妊と続発性不妊の割合，体外受精既往の割合に差を認めなかった。MRI検査で得られた所見に関して表3に示す。子宮内膜症の罹患率，子宮筋腫の個数および最大径，子宮筋腫により子宮内膜の変形をきたしている症例数の割合は，両群間で差を認めなかった（表3)。子宮筋腫の個数と最大径はそれぞれ，低頻度群で2.8個，53 mm（中間値），高頻度群では3.5個，58 mm（中間値）であった。なお，子宮筋腫の位置は子宮体部もしくは子宮底部であり，子宮狭部や頸部筋腫症例は認めなかった。低頻度群29名中6名，および高頻度群22名中6名がHMG療法を受け，残りは自然排卵もしくはクロミフェン療法を受けた。また，パートナーの精液所見が不良（運動精子$<20\times10^6$/m*l*）により，低頻度群29名中9名，および高頻度群22名中4名が人工授精を受けた（表4)。MRI撮影後の4カ月間，低頻度群29名中10名（34%）が妊娠したのに対し，高頻度群22名中妊娠例は0名であった（表3）（0%，$p<0.005$)。

妊娠症例10名の内訳は，7例が自然排卵および3例がクロミフェン療法を受けていた。また，10例中，1例が人工授精による妊娠であった(表4)。

表3 両群間の MRI 所見と妊娠率

	低頻度群	高頻度群	
患者数（人）	29	22	
子宮内膜症 なし あり	 22 7	 16 6	 有意差なし
子宮筋腫の個数	2.8±2.8	3.5±3.0	有意差なし
子宮筋腫の最大径（mm）	53±17	58±21	有意差なし
子宮内腔の変形をきたした症例 なし あり	 14 15	 12 10	 有意差なし
MRI 後の妊娠例 症例数（%）	 10（34%）	 0（0%）	 p<0.005

子宮内膜蠕動様運動が３分間で２回未満（低頻度群）および２回以上（高頻度群）の２群に分け，MRI 所見および MRI 撮影後４カ月間に妊娠した症例を示した。
N. S.: not significant

表4 MRI 撮影後の不妊治療の詳細と妊娠症例数

低頻度群			
排卵誘発法		患者数	妊娠例
自然	タイミング指導	14	7
	人工授精	5	0
クロミフェン	タイミング指導	2	2
	人工授精	2	1
hMG	タイミング指導	4	0
	人工授精	2	0
高頻度群			
排卵誘発法		患者数	妊娠例
自然	タイミング指導	11	0
	人工授精	3	0
クロミフェン	タイミング指導	2	0
	人工授精	0	0
hMG	タイミング指導	5	0
	人工授精	1	0

3．考　察

子宮内膜は蠕動様運動を呈し，この運動の頻度および方向は月経周期によって大きく変動することが知られている[8]。特に排卵期および月経期に子宮内膜蠕動様運動の頻度が大きい。排卵期は子宮頸部から底部に向かって運動することで，精子を汲み上げる働きをしていることが考えられている。また，月経期には子宮底部から頸部方向に運動することで，月経血を子宮内腔より排出する作用があると考えられている。一方で着床期には，子宮内膜の蠕動様運動はほとんどみられなくなる[6)9)]。そして運動を抑制することで，胚の子宮内膜への着床を促していることが予想されている[10]。これら子宮内膜の蠕動様運動は女性ホルモンにより制御されており，エストロゲンは運動亢進を，プロゲステロンは運動抑制に寄与することが知られている[11]。

Fanchin らは子宮に異常を認めない不妊症患者を対象に超音波断層装置を用いた検討を報告している。彼らは IVF-ET 患者の胚移植時の子宮内膜蠕動様運動の回数と妊娠率を比較し，蠕動様運動回数が少ないほど妊娠率が高くなるとしている[7)8)]。彼らの報告は，implantation window（黄体期 day 5～day 9）時点での検討ではなく，黄体期２日目での検討ではあるが，子宮内膜の蠕動様運動頻度が高いと妊娠率が低下することの理由として，同運動が胚を子宮腔から

押し出すことが推測される[7]。Orisakaらはcine-MRIを用いた検討で，子宮に異常を認めない正常コントロールでは着床期に子宮内膜蠕動様運動を認めなかったのに対し，筋層内子宮筋腫を有する患者の中には異常運動を呈する症例があることを報告している[9]。

子宮筋腫，特に筋層内子宮筋腫が不妊症に与える影響はよくわかっておらず，また不妊症の観点からは，その取り扱いに苦慮することが多い。われわれは子宮筋腫により誘導される子宮内膜の異常蠕動様運動に着目し，これらが不妊症患者に対し悪影響を与えているかを検討した。MRI検査を着床期に行ったところ，51例中22例（43.1%）と半数以下の症例で，本来は子宮内膜に蠕動様運動を認めない時期に異常運動を認めた。さらに，MRI検査後に前方視的に妊娠について調査を行ったところ，興味深いことに蠕動様運動を高頻度に認めた22例中に妊娠症例を認めなかった（0%）のに対し，低頻度群では29例中10例（34.5%）に妊娠を認めた。低頻度群と高頻度群を比較したところ，子宮筋腫の個数，最大径，内腔の変形をきたしている患者の割合に差がなかった（表3）。また，妊娠例（10例）と非妊娠例（41例）に分けて同様に比較したところ，子宮筋腫の個数，最大径，内腔の変形をきたしている患者の割合に差を認めなかった（データは示さず）。

今回の検討で，どの条件の筋層内子宮筋腫が子宮内膜の異常蠕動様運動を引き起こすかについて，子宮内腔を変形させるタイプ，個数，最大径などの条件では明らかにすることはできなかった。上述したようにエストロゲンは子宮内膜の蠕動様運動を亢進させる作用がある[11]。子宮筋腫にはエストロゲン産生に重要な酵素であるアロマターゼが発現している[12]ことから，子宮筋腫によっては局所のエストロゲン濃度が亢進することで，蠕動様運動を誘導しているのかもしれない。さらなる検討が必要である。

今回，われわれはMRIを用いて子宮内膜の蠕動様運動の評価を行った。超音波断層法では，子宮筋腫の存在により，子宮内膜を正確に描出することが困難なことが多い。また，経腟プローブはその刺激により子宮収縮を誘導することから，この分野の検討には不向きだとされている[13]。すなわち，特に子宮筋腫を有する患者の子宮内膜の運動評価にはMRI法が優れているといえる。

本研究において，われわれは子宮筋腫により誘導される子宮内膜の異常蠕動様運動が妊娠を妨げている一要因になりうる可能性を示した。

おわりに

本研究は不妊症の観点から子宮筋腫の手術を受けるべきか悩んでいる患者の一助になる可能性があり現在，子宮筋腫核出術が異常蠕動様運動の改善につながる否かについて検討中である。また，同法は着床の評価法としても有用であるかもしれない。例えば，一般的に体外受精において採卵周期よりも自然周期のほうが，着床率が高いことが知られている。この理由として採卵周期の高エストロゲン状態が子宮内膜の異常蠕動様運動を誘導することで，着床を妨げているのかもしれない。今後，同法を用いることで，不妊症の評価が発展することが期待される。

謝辞：本研究を遂行するにあたり，田園都市レディースクリニック 清水康史先生，許山浩司先生，河村寿宏先生，梅ヶ丘産婦人科 辰巳賢一先生，とくおかレディースクリニック 徳岡晋先生，小川クリニック 小川隆吉先生，東京日立病院 松岡良先生，滝野川内科クリニック 野尻龍二氏，船津嘉二氏，帝京溝口病院 佐渡島陽子先生，土屋裕子先生，原子満氏に多大な御協力をいただきました。深く御礼を申し上げます。

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学会案内

第20回日本婦人科がん検診学会総会・学術集会

日時 日時　平成23年11月19日（土）

会場 ベルサール飯田橋駅前（JR飯田橋駅より徒歩約2分）
〒102-0072 東京都千代田区飯田橋3-8-5　住友不動産飯田橋駅前ビル1・2F

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参加費 6,000円

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Reprod Med Biol (2012) 11:69–72
DOI 10.1007/s12522-011-0107-4

ORIGINAL ARTICLE

Alternative strategies to in vitro fertilization/intracytoplasmic sperm injection treatment for aged infertile women

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Received: 23 May 2011 / Accepted: 20 July 2011 / Published online: 13 August 2011

Abstract
Purpose This study aimed to maximize the chance of pregnancy and provide an optimal protocol for infertile female patients of advanced reproductive age as an alternative to in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) treatment.
Methods We retrospectively analyzed medical records of 432 infertile women aged ≥38 at the beginning of the treatment. Stepwise non-IVF/ICSI treatments using timed intercourse or intrauterine insemination, with or without controlled ovarian stimulation, were adopted for all patients. In this population, we extracted 8 representative infertility factors and examined these effects on fertility rate by calculating clinical pregnancy rate.
Results The prognosis for infertile women possessing at least one of the three factors, 'advanced female age (≥42 years)', 'endometriosis/adenomyosis', and 'tubal infertility' was apparently poor because only 5 out of 155 women were able to conceive (1.02% per cycle). In contrast, 95 patients without the four factors, 'advanced female age', 'endometriosis/adenomyosis', 'tubal infertility', and 'male infertility', were more likely to conceive (9.14% per cycle).
Conclusions Fertility centers can offer appropriate protocols for non-IVF/ICSI treatment and establish guidelines for aged infertile patients by examining infertility factors and considering their combinations.

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Keywords Advanced reproductive age · Clinical pregnancy rate · ICSI · Infertility · IVF

Introduction

Although age-related fertility decline is widely accepted [1], recent trends have led to alterations in the pattern of childbearing in developed countries, with more people now delaying reproduction into their late 30s and 40s [2]. Historical observations show that fertility decreases by nearly 50% at the age of 40 compared with females younger than 25 and reaches 0% by the age of 47–48 [3]. Considering the steady and irreversible decline in ovarian reserve, it seems appropriate that older patients are often counseled to undergo in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) to maximize the per-cycle chances of pregnancy; however, many couples may not have access to these technologies for financial, social, psychological or other reasons, or may not wish to pursue them [2, 4]. As an alternative to IVF/ICSI, the stepwise approach using ovarian stimulation with clomiphene citrate (CC) or human menopausal gonadotropin (hMG) combined with timed intercourse (TI) or intrauterine insemination (IUI) has been commonly utilized as a means to increase the fertility rate of subfertile couples with patent fallopian tubes. This stepwise non-IVF/ICSI treatment is possibly efficient because the observed clinical pregnancy rate (CPR) per cycle is 4–9% using CC [5], and 8–23% using hMG [6]. However, the efficacy of non-IVF/ICSI is postulated to be questionable for aged patients since the likelihood of fertility decreases significantly after the age of 37 [5, 7, 8]. In addition, the duration of non-IVF/ICSI treatment is important for successful IVF/ICSI, because female age is the most significant factor affecting the success of IVF/ICSI [3, 9].

The objective of this retrospective study is to determine the crucial factors that affect the age-related decline in CPR, and to optimize the treatment protocols for infertile advanced reproductive age women whether these women necessitate IVF/ICSI treatment or non-IVF/ICSI treatment according to the evidence-based policies [10].

Materials and methods

Collection of data

The medical records of 372 infertile female patients who had undergone a total of 1392 non-IVF/ICSI treatment cycles from 1 January 2000 to 28 February 2010 at The University of Tokyo Hospital were reviewed retrospectively. During this period, general trends of treatment strategy for infertile couples remained unchanged. Of 432 women aged ≥38 at the start of the infertility treatment cycle, we excluded 60 patients: 39 were lost during the follow-up period and 21 were excluded for unsuccessfully treated recurrent fetal loss. Our research covered the process of achieving first pregnancy after the beginning of infertility treatment and we registered the number of cycles for each ovarian stimulation method with TI or IUI. This study was reviewed and approved by the Human Ethical Committee of The University of Tokyo Hospital.

Outcome measures

The endpoint, clinical pregnancy, was defined as the detection of a gestational sac by transvaginal ultrasound. To detect the factors that affect CPR in these aged patients, we considered 8 representative factors that are generally examined prior to the infertility treatment. These include (1) 'advanced female age' defined as ≥42 years at the time of the first visit, in view of the report that CPR per embryo transfer decreased from 26% to 13% from age 40 to 44 [11] ($n = 75$), (2) 'endometriosis/adenomyosis' defined as a past or present history of endometriosis and/or adenomyosis, based on clinical images ($n = 57$), (3) 'tubal infertility' defined as an obstructive fallopian tube, based on hysterosalpingography, hysterofiberscope, or past tubal surgery ($n = 49$), (4) 'male infertility' defined as sperm abnormality, based on semen analysis [12] ($n = 208$), (5) 'decrease in ovarian reserve' defined as serum follicle-stimulating hormone level >10 mIU/mL in the early follicular phase ($n = 147$), (6) 'primigravida' ($n = 213$), (7) 'uterine fibroid' defined as a past or present history of uterine fibroid, based on clinical images ($n = 131$), and (8) 'positive serum Chlamydia antibodies' defined as serum Chlamydia antibody immunoglobulin (Ig)A and/or IgG > 0.9 cut-off index ($n = 64$). Eighteen females, including 70 cycles, had none of these 8 factors. We stratified these 8 groups into 24 subgroups by three methods of ovarian stimulation, namely, unstimulated, CC, and hMG cycle. After identifying these factors, we further selected all patterns of their combination to identify the patients' characteristics that are most difficult to conceive by calculating CPR. In this process, the difference of ovarian stimulation methods was not considered and we measured the overall CPR.

Statistical analysis

Categorical variables were compared using chi-squared test, and multivariate logistic regression analysis was used to test for correlations between the 8 factors and the occurrence of pregnancy since various factors overlapped in some cases. Calculations were performed using JMP version 5.1.

Results

The overall outcomes of the 8 groups

The 1392 cycles of non-IVF/ICSI treatment yielded 54 pregnancies and overall CPR per cycle was 3.88%. Of these 54 pregnancies, a live birth rate per cycle was 2.16% (30/1392) and 5 patients were lost during the follow-up period in the first trimester. The CPR of the 24 subgroups is shown in Table 1. There was no successful pregnancy in the 4 subgroups, (1) 'advanced female age; treatment, CC-TI/IUI', (2) 'advanced female age; treatment, hMG-TI/IUI', (3) 'endometriosis/adenomyosis; treatment, hMG-TI/IUI', and (4) 'tubal infertility; treatment, hMG-TI/IUI'.

The overall outcomes of the 8 groups, including the number of patients who had the 8 factors, CPR per cycle and the number of pregnant patients in each group with *P* values are summarized in Table 2. We found that the 4 groups, 'advanced female age', 'endometriosis/adenomyosis', 'tubal infertility' and 'male infertility', exhibited extremely poor outcomes because the CPR per cycle was less than 2%. However, among the 4 factors, 'endometriosis/adenomyosis' and 'tubal infertility' were not significantly different by multivariate analysis. Only 2 females were able to conceive after non-IVF/ICSI treatment in the 3 groups, 'advanced female age', 'endometriosis/adenomyosis' and 'tubal infertility', although the treatment cycle in each group was >100 cycles. On the other hand, the factor 'decrease in ovarian reserve' showed a significant difference, although CPR per cycle was 2.55%.

CPR in each combination of the 4 factors

To indicate the efficacy and predict the outcomes at the beginning of non-IVF/ICSI treatment, each combination of

Table 1 CPR per cycle of 24 subgroups divided by ovarian stimulation protocols

		Age	EM/AM	Tube	Male	Ovary	Primigravida	Fibroid	Chlamydia
No	CPR	2.22% (2/90)	1.18% (1/85)	1.28% (1/78)	1.70% (7/411)	1.65% (4/243)	2.00% (9/451)	1.74% (5/287)	6.61% (8/121)
	P value[a]	0.56	0.25	0.30	0.0061	0.082	0.012	0.061	0.077
CC	CPR	0% (0/97)	1.12% (1/89)	2.13% (1/47)	0.75% (2/268)	2.65% (6/226)	4.36% (13/296)	6.59% (12/182)	7.23% (6/83)
	P value[b]	0.014	0.075	0.37	<0.0001	0.044	0.60	0.16	0.53
hMG	CPR	0% (0/46)	0% (0/24)	0% (0/21)	5.15% (5/97)	4.94% (4/81)	3.70% (3/81)	3.92% (2/51)	8.33% (2/24)
	P value[c]	0.0037	0.31	0.34	0.22	0.38	0.079	0.90	0.19

We stratified 392 patients (1392 cycles) into 24 subgroups by 8 infertile factors and 3 methods of ovulation stimulation. Remarkably, there was no successful pregnancy in the 4 subgroups

No no stimulation, *Age* advanced female age, *EM/AM* past or present history of endometriosis/adenomyosis, *Tube* tubal infertility, *Male* male infertility, *Ovary* decrease in ovarian reserve, *Fibroid* uterine fibroid, *Chlamydia* positive serum Chlamydia antibodies

[a, b, c] *P* value was calculated using chi-squared test. In each subgroup, we compared the patients with above listed factor with the patients without the factor

Table 2 Overall CPR per cycle in the 8 groups

Infertility factors	CPR with the following factor	CPR without the following factor	*P* value in univariate analysis	*P* value in multivariate analysis
Women aged ≥42	0.86% (2/233)	4.49% (52/1159)	0.0088	0.013
Endometriosis/adenomyosis	1.01% (2/198)	4.36% (52/1194)	0.024	0.11
Tubal infertility	1.37% (2/146)	4.17% (52/1246)	0.097	0.19
Male infertility	1.80% (14/776)	6.49% (40/616)	<0.001	<0.001
Decrease in ovarian reserve	2.55% (14/550)	4.75% (40/842)	0.037	0.048
Primigravida	3.01% (25/830)	5.16% (29/562)	0.043	0.13
Uterine fibroid	3.65% (19/520)	4.01% (35/872)	0.74	0.93
Serum Chlamydia antibodies	7.02% (16/228)	3.26% (38/1164)	0.045	0.059

The effects of 8 infertile factors on CPR were evaluated by univariate and multivariate analysis. Four groups exhibited extremely poor outcome, <2% CPR per cycle, and three groups showed significant statistical differences

Table 3 Cumulative CPR in each combination of the 4 factors

Analyzed factors	Patients with at least one factor in the following combination			Patients without any factor in the following combination		
	Number of pregnancies	CPR per cycle	CPR per patient	Number of pregnancies	CPR per cycle	CPR per patient
A/E	4	1.02%/391	3.25%/123	50	5.00%/1001	20.1%/249
A/T	4	1.14%/351	3.54%/113	50	4.80%/1041	19.3%/259
A/M	16	1.80%/887	6.58%/243	38	7.52%/505	29.5%/129
E/T	3	0.96%/311	3.06%/98	51	4.72%/1081	18.6%/274
E/M	15	1.74%/862	6.58%/228	39	7.36%/530	27.1%/144
T/M	16	1.93%/829	6.93%/231	38	6.75%/563	27.0%/141
A/E/T	5	1.02%/488	3.23%/155	49	5.42%/904	22.6%/217
A/E/M	17	1.77%/962	6.51%/261	37	8.60%/430	33.3%/111
A/T/M	18	1.93%/931	6.90%/261	36	7.81%/461	32.4%/111
E/T/M	16	1.78%/901	6.45%/248	38	7.74%/491	30.6%/124
A/E/T/M	18	1.80%/988	6.50%/277	36	9.14%/394	37.9%/95

To analyze each combination of the 4 factors that showed extremely poor outcome for CPR, we divided the patients into two groups: patients with at least one of the analyzed factors and patients with none of the analyzed factors. Four groups with only female factors exhibited approximately 1% CPR per cycle

A advanced female age, *E* past or present history of endometriosis/adenomyosis, *T* tubal infertility, *M* male infertility