37°C. After being blocked with blocking solution, the sections were incubated with an anti-DIG, alkaline phosphatase-conjugated antibody (1:500; Roche, Basel, Switzerland) for 1 hr at room temperature. Color development was carried out by overlaying them with 4-nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche), and they were incubated in a humidified container in the dark for 12 hr. Sense probe hybridization was used as a control for background level.

Measurements of Estradiol, Progesterone and MK in FF

Concentrations of estradiol in FF were measured using an SR1 analyzer (Biochem ImmunoSystems Italia, Rome, Italy). For the intra-assay and inter-assay, coefficients of variation were <10% for this assay. Concentrations of progesterone in FF were measured using a specific enzyme immunoassay (Progesterone EIA Kit; Cayman Chemical, Ann Arbor, MI, USA). The sensitivity limit was 10 pg/mL and the intra-assay and inter-assay coefficients of variation were <10% for this assay. Concentrations of MK in FF were determined using an enzyme-linked immunosorbent assay (ELISA), kindly provided by Cell Signals. The sensitivity limit of this assay was 0.1 ng/mL per sample.

Treatment of LGC with MK and Cell Proliferation Assay

Isolated LGC were suspended in DMEM/Ham's F-12 medium containing 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 μg/mL amphotericin B, then plated in 24-well plates at a density of 5×10^4 cells/well and kept at 37°C in a humidified 5% CO_2/95% air environment. After 24 hr of incubation, FBS-free media were replaced, and the cells were cultured for another 24 hr. To examine the effect of MK, the cells were further cultured in FBS-free media containing recombinant MK, which was kindly provided by Cell Signals, for an additional 24 hr.

The cell proliferation assay was performed as we have reported previously.[14] The effect of MK on the proliferation of LGC was examined by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA using the Biotrak cell proliferation ELISA system (Amersham Biosciences) according to the manufacturer's instructions.

Statistical Analysis

The data of MK in FF were described as median and interquartile range (IQR). Correlations between the concentrations of MK and those of oxygen, estradiol, and progesterone in FF were calculated by linear regression analysis. Student's *t*-test was used for the analysis of BrdU incorporation of the cultured cells. $P < 0.05$ was accepted as statistically significant.

Results

Presence of MK Protein in FF

Using Western blotting, we demonstrated the presence of MK protein in FF as a band at about 13 kDa, which was the same size as recombinant MK. MK in FF bound to heparin-sepharose was eluted in a 0.8 M NaCl fraction (Fig. 1).

Expressions of MK mRNA and its Receptor LRP1 mRNA in LGC

RT-PCR analysis demonstrated the expressions of MK mRNA and its receptor LRP1 mRNA in LGC as a clear band at 320 and 240 bp, respectively (Fig. 2).

In vivo, Expression of MK mRNA in Human Follicles

Human MK mRNA was detected in both granulosa cells and theca cells of large follicles by *in situ* hybridization (Fig. 3). No specific hybridization products were observed when using the sense riboprobes.

Correlation Between Concentrations of MK and those of Oxygen and Ovarian Steroid

The median concentrations of MK in FF were 217 ng/mL (IQR, 160–313). As depicted in Fig. 4a,b,

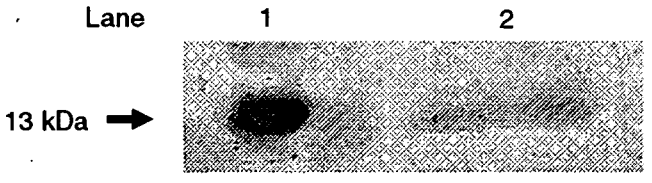

Fig. 1 Midkine (MK) protein in human follicular fluid (FF). MK protein expression was examined by Western blotting in human FF. Several fractions were separated from crude FF by heparin-sepharose chromatography. Recombinant MK was used as a positive control band. Lanes 1, recombinant MK; lane 2, separated fractions eluted by 0.8 M NaCl.

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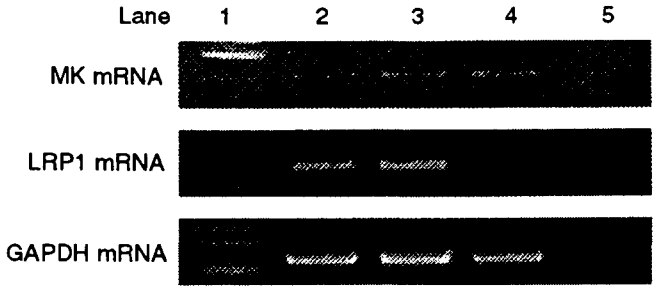

Fig. 2 Expression of midkine (MK) mRNA and low density lipoprotein receptor-related protein 1 (LRP1) mRNA in human luteinized granulosa cells (LGC). Amplification of GAPDH was used to ensure RNA quality and amounts. Lane 1, Marker; lanes 2, 3 and 4, three different samples of LGC; lane 5, negative control with water.

simple regression analysis demonstrated a significant negative correlation between the concentrations of oxygen and those of MK in FF ($r = -0.6661$, $P = 0.0013$) and a significant positive correlation between the concentration of estradiol and MK ($r = 0.6362$, $P = 0.0019$). No correlation was detected between the concentration of progesterone and MK ($r = 0.00949$, $P = 0.9676$).

Effect of MK on the Proliferation of LGC

The effect of MK on DNA synthesis in LGC is shown in Fig. 5. MK at 100 ng/mL significantly increased BrdU incorporation to 140% of that of the control ($P < 0.005$).

Discussion

In the present study, we demonstrated the expression of MK in the human ovarian follicle. The concentrations of MK in FF were negatively correlated with those of oxygen and positively correlated with those of estradiol. *In vitro*, study showed the mitogenic activity of MK on human granulosa cells.

The present study verified that concentrations of MK in FF were relatively high as reported in our previous study.[9] A possible explanation of the high concentration is a local production of MK in the follicle. The notion is supported by our finding that MK mRNA was expressed in both granulosa and theca cell layers of the growing follicle. Interestingly, local gene expression of MK in the follicle has also been observed in the rat ovarian follicle although the expression is exclusively in granulosa cells.[7,8]

Vasculature of the follicle is limited to the thecal layer outside the basement membrane, and the oxygen tension tends to decline inside. Accordingly, angiogenesis is an essential event for a growing follicle to supply oxygen to cells comprising a follicle.[15] Indeed, several angiogenic factors have been suggested to play roles in the follicular development. It is generally known that hypoxic condition stimulate angiogenesis by upregulating various angiogenic substances. We previously reported that the volume of FF is negatively correlated with oxygen tension and positively correlated with the concentration of IL-8.[4]

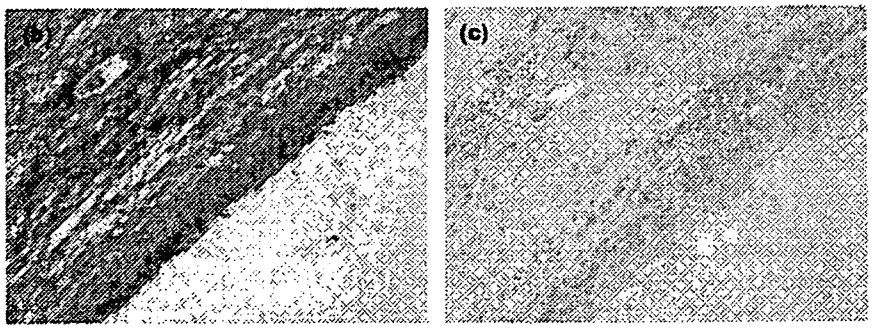

Fig. 3 *In situ* hybridization for midkine (MK) in the human ovarian follicle. Sections of ovarian tissue with large follicles were stained with hematoxylin and eosin (HE) (a) Slide sections were hybridized with digoxigenin (DIG)-labeled antisense (b) or sense (c) riboprobes. MK mRNA was strongly expressed in a human large follicle. Magnification, ×100. The section represents four samples from different women stained with the same procedure.

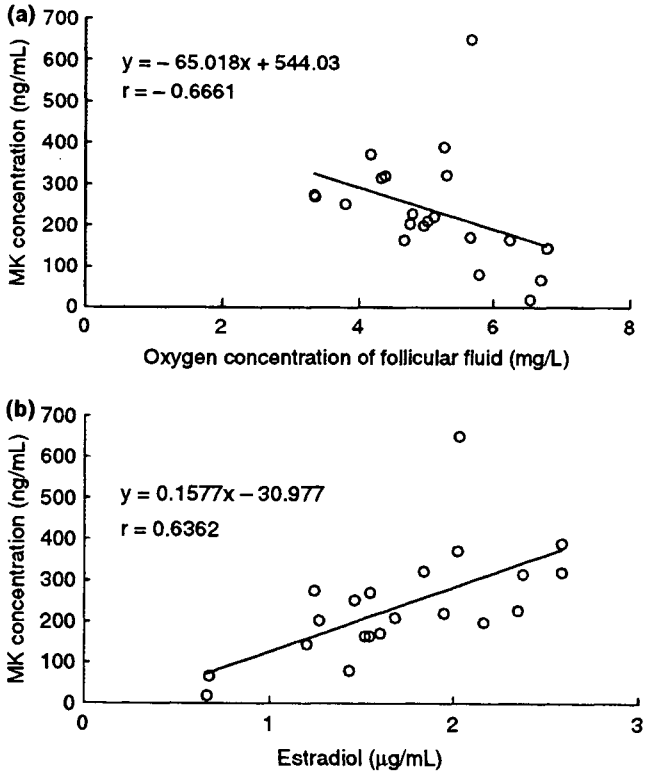

Fig. 4 Correlation between concentrations of midkine (MK) and those of oxygen and estradiol in follicular fluid (FF). (a) There was a significant negative correlation between the concentrations of oxygen and those of MK in FF ($r = -0.6661$, $P = 0.0013$). (b) There was a significant positive correlation between concentrations of estradiol and those of MK in FF ($r = 0.6362$, $P = 0.0019$).

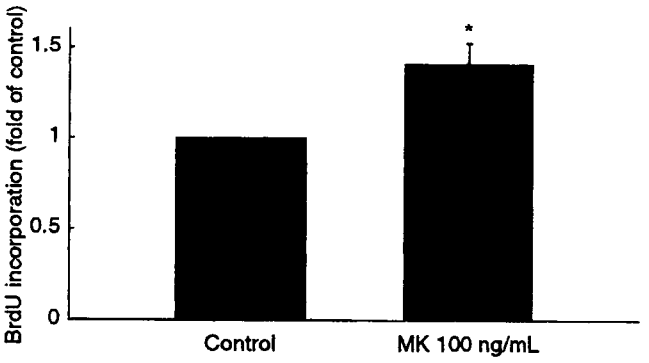

Fig. 5 Midkine (MK) stimulated the proliferation of luteinized granulosa cells (LGC). The effect of MK on the proliferation of LGC was examined by measuring 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA using the cell proliferation enzyme-linked immunosorbent assay (ELISA). LGC were treated with fetal bovine serum (FBS)-free media containing recombinant MK for 24 hr. As a control, FBS-free medium was used. Values are the means ± S.E.M. of pentaplicate cultures. The data shown is representative of three experiments using different LGC preparations. Student's *t*-test was used for comparison of the BrdU incorporation. $^*P < 0.005$ (versus control).

In the present study, concentrations of MK were negatively correlated with those of oxygen in FF. Given that MK is upregulated by hypoxia[16], our finding imply that MK is involved in angiogenesis of the developing follicle exposed to hypoxic environment.

Angiogenic factors that have hitherto been demonstrated in the FF include vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), angiogenin, and IL-8.[1,3,4,17] Interestingly, concentrations of VEGF, HGF, and angiogenin are all positively correlated with those of progesterone in FF, and not with those of estradiol. Thus, it seems quite unique that concentrations of MK are positively correlated with those of estradiol but not progesterone. It may be speculated that MK possesses specific feature in its effect on follicular steroid production, being different from other angiogenic factors in FF.

Midkine is known to have mitogenic activity in several cells.[18–20] We have previously shown that MK promoted the proliferation of endometriotic stromal cells.[9] In the present study, MK stimulated the proliferation of LGC. The expression of LRP1 in LGC suggests the presence of molecular complex of MK receptor that mediate the mitogenic function. Considering that granulosa cell proliferation promote follicular development and estradiol production, it is plausible that higher concentrations of MK are correlated with higher levels of estradiol in FF.

Pleiotrophin (PTN) has similar sequence and functions to those of MK, and the receptor and signaling systems of the two factors are closely related.[5] Recently, mice doubly deficient in genes of MK and PTN have been developed.[21] Interestingly, most of the female double deficient mice were infertile with reduced mature follicles. Given the possible importance of MK in human follicles, MK deficiency might be associated with human infertility.

In summary, the present study suggested that MK functions as a local regulator of follicular growth in the human ovary.

Funding

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Human round spermatids from azoospermic men exhibit oocyte-activation and Ca^{2+} oscillation-inducing activities

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Summary

During mammalian fertilization, intracellular Ca^{2+} oscillations are important for both oocyte activation and embryonic development. As the ability of round spermatids (ROS) to induce Ca^{2+} oscillations and oocyte activation is different between species, we examined Ca^{2+} oscillation- and oocyte activation-inducing abilities of human ROS originating from patients with non-obstructive azoospermia. Human ROS from 11 non-obstructive azoospermic patients were collected during their TESE–ICSI cycles. Following injection into mature unfertilized mouse oocytes, we examined the oocyte-activating and Ca^{2+} oscillation-inducing activities of ROS by using Ca^{2+} imaging and confocal laser scanning microscopy (mouse test). In these 11 cases, clinical TESE–ICSI using mature testicular spermatozoa was successful, with the exception of one case in which only one sperm-injected oocyte was not fertilized. The mean fertilization rate was 70.1% (40–100%); the mean cleavage rate was 97.9% (46/47). Two pregnancies were established from 10 transfer cycles (PR; 20%). When the ROS from these patients were injected into mouse oocytes, the ROS from all patients induced at least some intracellular Ca^{2+} oscillations (25–100%). In all patients, 40 out of 82 oocytes injected with ROS exhibited normal oscillation patterns of $[Ca^{2+}]i$.

Human spermatogenetic cells acquired oocyte-activating and Ca^{2+} oscillation-inducing abilities at the round spermatid stage, an earlier stage than found for rodent cells. These data indicate that human ROS might be useful for clinical treatments of non-obstructive azoospermic patients exhibiting mature spermatozoa in biopsied specimens.

Keywords: Calcium oscillation, Human, Non-obstructive azoospermia, Oocyte activation, Round spermatid

Introduction

Spermatids are spermatogenetic cells that have just completed meiosis and possess a haploid set of chromosomes, making them genetically equivalent to mature spermatozoa. Recently, human round spermatids (ROS) have been used as a clinical treatment for non-obstructive azoospermic patients (Tesarik *et al.*, 1996; Vanderzwalmen *et al.*, 1997; Sousa *et al.*, 1999). As the pregnancy rates reported using ROS are poor in comparison with those using testicular or ejaculated spermatozoa, it is likely that functional immaturities of round spermatids, such as an insufficient activity of sperm-borne oocyte-activating factor (SOAF), result in inefficient fertilization.

In previous studies using experimental animal models, mouse ROS lack oocyte activation-inducing ability (Kimura & Yanagimachi, 1995, Yazawa *et al.*, 2000). At least in mice, SOAF becomes biologically active during spermiogenesis. In contrast, ROS from hamsters and rabbits exhibit oocyte-activating ability. These cells cannot, however, induce Ca^{2+} oscillations,

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the repetitive spikes in intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), only producing several transient rises in $[Ca^{2+}]i$ in mouse oocytes (Yazawa *et al.*, 2000). We presumed that the total absence of SOAF resulted in no activation and no Ca^{2+} responses in injected mouse oocytes, while insufficient SOAF activity could induce oocyte activation, but did not promote the oscillatory increases in $[Ca^{2+}]i$, only several transient rises of $[Ca^{2+}]i$ as seen with the ROS of hamsters and rabbits. Recently, Ogonuki *et al.* (2001) demonstrated that ROS from monkeys could induce oocyte activation and Ca^{2+} oscillations in a pattern similar to that of normal fertilization. It is possible that ROS from monkeys possess similar levels of SOAF activity as mature spermatozoa. Thus, the timing at which SOAF becomes biologically active during spermatogenesis and spermiogenesis differs among species.

To examine the basic mechanisms of spermatogenesis, we sought to determine if human ROS from non-obstructive azoospermic men have sufficient SOAF activity by examining oocyte activation and Ca^{2+} responses following injection into mouse oocytes. As a clinical investigation, we discussed the indications for ROS injection and the usefulness of this procedure in the treatment of non-obstructive azoospermia.

Material and methods

Preparation of mouse oocytes

B6D2F1 female mice (6–8 weeks old) were superovulated with an i.p. injection of 8 IU of pregnant mare's serum gonadotrophin (PMSG; Teikokuzouki Co.), followed by 8 IU human chorionic gonadotrophin (hCG; Mochida Pharmaceutical Co.) after 48 h. Oocyte–cumulus complexes, obtained from oviducts approximately 16 h after hCG injection, were treated with HEPES-buffered human tubal fluid medium (mHTF; Irvine Scientific) containing 0.1% hyaluronidase (from bovine testis; 825 IU/mg; Sigma) to dissociate the cumulus cells. Cumulus-free oocytes were rinsed thoroughly and incubated in human tubal fluid medium (HTF; Irvine Scientific) with 10% synthetic serum substitute (SSS; Irvine Scientific) at 37°C under 5% CO_2, 5% O_2 and 90% N_2 for up to 2 h before spermatid injection.

Patients

We examined ROS from 11 patients with non-obstructive azoospermia. These patients underwent intracytoplasmic sperm injection (ICSI) treatment using testicular sperm extracts (TESE) at Fukushima Medical University (FMU) Hospital. In all 11 male patients, preliminary pathological diagnosis of testicular biopsies indicated hypospermatogenesis. Normal karyotypes and no defects of DAZ and SRY were detected by blood sampling. No female-related infertility factors were identified in these 11 couples. During the treatment cycle, all 11 patients produced spermatozoa upon testicular extraction, allowing successful TESE–ICSI. Prior to beginning experimentation, informed consent was obtained from all patients. The agreement of the ethical committee at FMU was also obtained.

Controlled ovarian hyperstimulation and oocytes retrieval

Controlled ovarian hyperstimulation was performed using the combination of a gonadotrophin-releasing hormone (GnRH) analogue (buserelin acetate, Suprecure; Hoechst Japan Co.), follicle-stimulating hormone (FSH, Fertinom P; Serono Japan Co.), human menopausal gonadotrophin (HMG, Pergonal; Teikokuzouki Co.) and human chorionic gonadotrophin (HCG; Mochida Pharmaceutical Co.). GnRHa was administrated at 600 μg/day from day 21 of the previous cycle; FSH was injected on days 3 and 4 (300 IU) and 5 and 6 (150 IU) of the treated cycle, with 150 IU HMG given daily beginning on day 7 until the maturation of follicles. When the two largest follicles reached mean diameters of 18 mm, 5000–10 000 IU HCG were administrated. Oocyte retrieval was carried out under transvaginal ultrasound 35 h after HCG administration.

Procedure of testicular sperm extraction

Testicular sperm extraction (TESE) was performed on the same day as oocyte retrieval. After confirmation of the recovery of greater than two oocytes, TESE was performed using the open excisional method, which is similar to a diagnostic testicular biopsy. Briefly, a small incision was made in the skin of the scrotum under local anesthesia. In addition, after cutting the tunica albuginea, a small piece of testicular tissue, including seminiferous tubule, was excised. The sample isolated in the TESE procedure was washed thoroughly, placed in 0.5 ml mHTF and cut into small pieces with a pair of scissors. Repeated gentle pipetting was used to release the spermatogenic cells from the tubular fragments and disperse the cells into the medium. If spermatozoa could not be identified by microscopic examination, an additional excision was performed from a different site of testis. A 3 μl droplet of the spermatozoa-containing suspension was placed in a plastic Petri dish (chamber for microinjection), covered with mineral oil and incubated at 37°C for 2–3 h before injection.

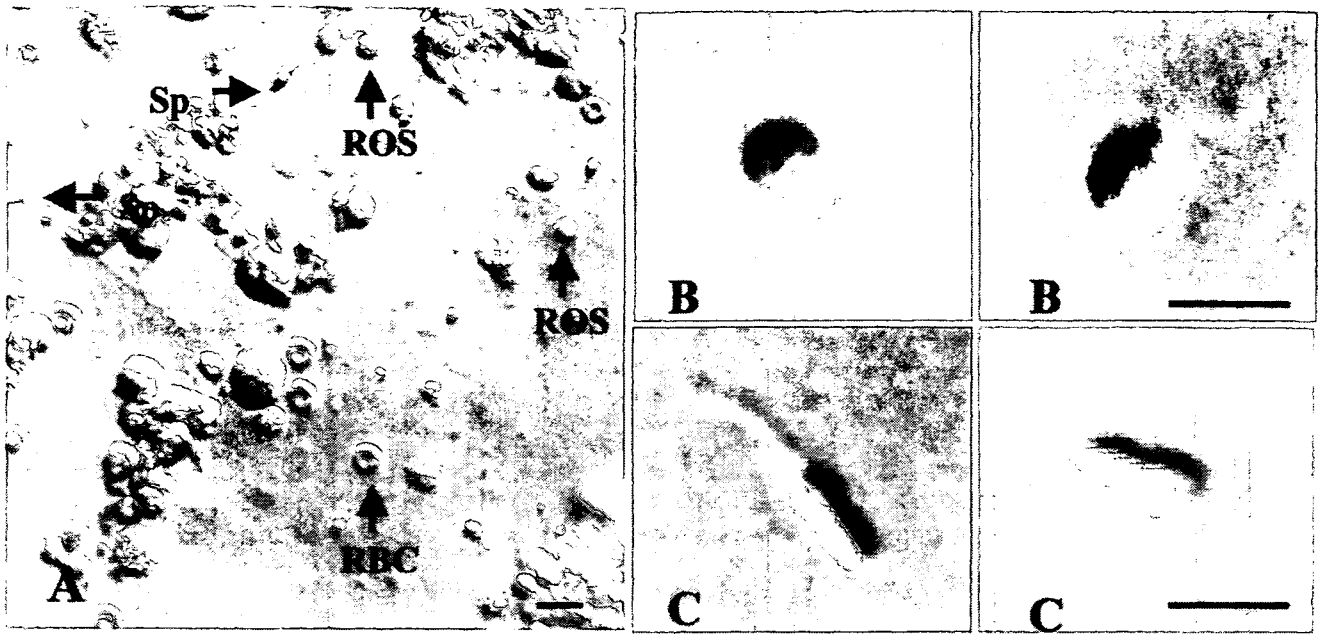


Figure 1 Wet preparation of a human testicular extraction isolated from a non-obstructive azoospermic patient (*A*). Isolated round spermatids (*B*) and isolated elongated spermatids (*C*) were suspended in medium. In (*A*), ROS, Sp and RBC (arrow) indicate round spermatids, testicular spermatozoa and red blood cells, respectively. Scale bar 10 μm.

Preparation of human round spermatids

This suspension containing spermatogenic cells was mixed thoroughly with an equal volume of 0.9% NaCl containing 10% polyvinylpyrrolidone (PVP-360; Sigma). A 3 μl droplet of this mixed suspension was kept for less than 2 h before the injection of the round spermatids into mouse oocytes. The chamber was mounted onto the stage of an inverted microscope equipped with a microinjection system (Fig. 1*A*).

Microinjection of testicular spermatozoa as a clinical treatment

After identification of motile spermatozoa, intracytoplasmic testicular sperm injection was performed using a micromanipulator with piezo-electric elements (model PMM-MB-A; Prime Tech Ltd) in a manner similar to that used for clinical treatments (Kimura & Yangimachi, 1995a: Yanagimachi, 1998). A single spermatozoon was sucked into an injection pipette (about 5 μm inner diameter at the tip); application of a piezo pulse damaged the plasma membrane and immobilized the spermatozoa. A mature unfertilized oocyte (metaphase II) was secured by a holding pipette with the first polar body at the 12 or 6 o'clock positions. The zona pellucida was penetrated at the 3 o'clock position by applying several piezo pulses. After the tip of the needle was advanced into the ooplasm, the oolemma was punctured with one piezo pulse; the spermatozoon was then slowly expelled into the ooplasm before gently withdrawing the pipette.

All the procedures of intracytoplasmic sperm injection were performed in 3 μl of mHTF on the stage of microscope warmed to 37°C. After injection, oocytes were washed three times in HTF and incubated under 5% CO_2, 5% O_2 and 90% N_2 at 37°C.

Culture and transfer of embryos

After ICSI, oocytes were cultured for approximately 18 h. Normal fertilization was confirmed by possession of a second polar body and two pronuclei. Normally fertilized oocytes were cultured for another 48 h before assessing their quality according to the classification system by Veeck (1991). Up to two of the best embryos were then transferred into the uterus of a female partner. In some cases, fertilized oocytes were cultured for approximately 72 h after confirmation of fertilization, and then transferred into the uterus at the blastocyst stage. Luteal support (Progehormon® 50 mg/day, Mochida Pharmaceutical Co. Ltd) was administrated for 14 days, beginning on the day of oocyte retrieval. Pregnancy was confirmed by detection of increased urine HCG concentrations 14 days after embryo transfer.

Microinjection of human round spermatids into mouse oocytes (mouse oocyte activation assay; mouse test)

Round spermatid injection was performed using a micromanipulator with piezo-electric elements in a

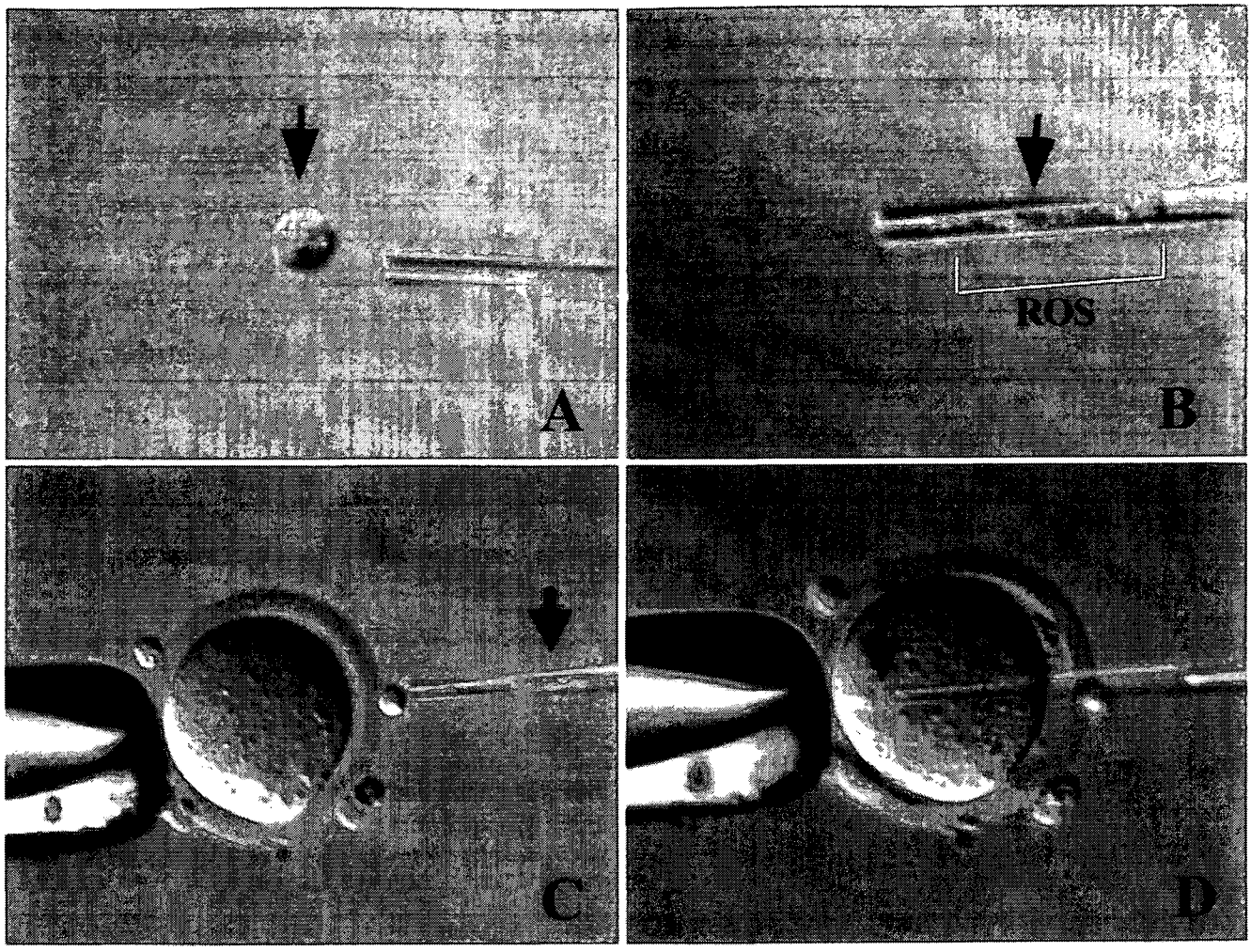


Figure 2 Human round spermatid injection into mouse oocytes using a piezo micromanipulator (mouse test). An isolated human round spermatid (ROS) was transferred into an injection pipette (*A, B*). The entire round spermatid was then injected into the ooplasm of unfertilized mouse oocyte (*C, D*).

similar manner as that used for testicular sperm injection. Human round spermatids (Sa1) can easily be distinguished from other spermatogenic and somatic cells by their size (about 7–8 mm in diameter, slightly larger than a red blood cell) and the presence of a round nucleus with a centrally located nucleolus (Fig. 1*A, B*) (Ogura & Yanagimachi, 1993; Aslam *et al.*, 1998; Sousa *et al.*, 1999). We performed round spermatid microinjection as previously described (Yazawa *et al.*, 2000). Briefly, a round spermatid was transferred into an injection pipette (about 6 μm inner diameter at the tip); its plasma membrane was damaged by the application of a piezo pulse (Fig. 2*A, B*). A mature unfertilized oocyte isolated from a B6D2F1 mouse was secured by a holding pipette with the metaphase II spindle at the 12 or 6 o'clock position. The entire round spermatid was injected into the ooplasm (Fig. 2*C, D*). All intracytoplasmic injection procedures were performed in 3 μl of mHTF on the stage of a microscope cooled to 17–18°C (Kimura & Yanagimachi, 1995; Yazawa *et al.*, 2001). After injection, oocytes were held at room temperature for 10 min, washed three times in HTF and incubated under 5% CO_2, 5% O_2 and 90% N_2 at 37°C. During these injection procedures, we were careful to avoid any additional procedures capable of inducing oocyte activation, such as vigorous cytoplasmic aspiration.

As a control, mature spermatozoa originating from fertile men, elongated spermatids (ELS, Fig. 1C) isolated from testicular biopsies of azoospermic patients and human tubal fluid medium lacking spermatid/spermatozoa were injected into oocytes.

Examination of oocyte activation

After a 5 h incubation, spermatid-injected oocytes were placed between a slide and a coverslip, fixed and stained with acetocarmine to examine the chromatin configuration of the spermatid and oocyte chromosomes. Oocytes with a second polar body and

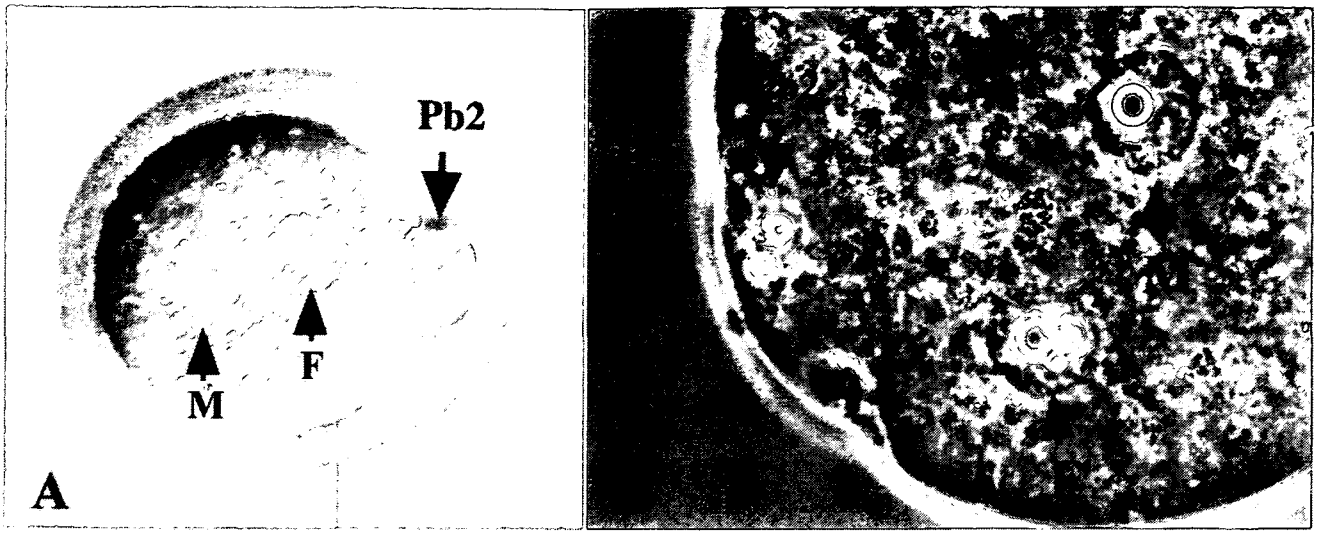


Figure 3 A normally fertilized mouse oocyte shown at 4 hours after injection with a human round spermatid. The oocyte was normally activated, forming a second polar body and two pronuclei – male pronucleus (M) and female pronucleus (F). Fresh zygote (*A*) and fixed, stained zygote (*B*).

two pronuclei (male and female) were considered to be activated (Fig. 3*A*, *B*).

Measurement of $[Ca^{2+}]i$ of spermatid-injected oocytes

We examined the Ca^{2+} responses of spermatid-injected oocytes using Ca^{2+}-imaging on a confocal laser scanning microscope (Bio-Rad MRC-600, Nippon Bio-Rad Ltd). Prior to injection, oocytes were loaded for 30 min with the Ca^{2+}-sensitive fluorescent dye fluo-3 acetoxymethyl ester (Fluo-3/AM, Molecular Probes Inc.) in dimethylsulfoxide (final concentration 44 μM in HTF) with 0.02% Pluronic F-127 at 37°C. Loaded oocytes were washed thoroughly and placed in a 3 μl droplet of mHTF on a chambered coverglass (Lab-Tek, Nunc Inc.) covered with mineral oil. Intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) responses of injected oocytes were measured after the chamber was mounted on the stage of a phase-contrast inverted microscope equipped with an image processor. For the majority of oocytes, measurements of Ca^{2+} responses were initiated 15–20 min after injection and continued at 20 s intervals for approximately 60 min.

Results

In this study, we examined the clinical outcome of TESE–ICSI for 11 patients with nonobstructive azoospermia. We also investigated the ability of ROS isolated from testicular biopsies of these patients to promote oocyte activation and Ca^{2+} oscillations.

The mean age of the male patients was 36.5 (range 27–40), while that of the female partners was 32.6 (range 25–37). All 11 male patients had normal karyotypes (46,XY); no defects in the *DAZ* and *SRY* genes were detected. All 11 female partners were free of any obvious causes of infertility.

Results of clinical TESE–ICSI

Table 1 summarizes the clinical results of TESE–ICSI cycles for these 11 couples. For one case (case h), although two oocytes were retrieved, one of them was a metaphase I oocyte; therefore, only one oocyte could be injected with testicular spermatozoa. As the injected oocyte was not fertilized, embryo transfer could not be performed. Except for this case, greater than two oocytes were fertilized for each couple. The mean fertilization rate was 70.1% (47 of 67 injected oocytes were fertilized). Most of the fertilized oocytes cleaved; the mean rate of cleavage for all cases was 97.9% (46 of 47 fertilized oocytes cleaved). In six cases (b–d, g, j, k), embryo transfer was performed on day 3 of fertilization at the 8-cell stage of cleavage. In four cases (a, e, f, i), embryo transfer was performed on day 5 of fertilization at the blastocyst stage. In all cases, two embryos could be transferred into the uterus. Out of 10 transferred cycles, pregnancies were confirmed in two cases 14 days after transfer; a single gestational sac with fetal heart movement was detected later in both cases. The pregnancy rate was 20% and the implantation rate was 10% for all 10 cases.

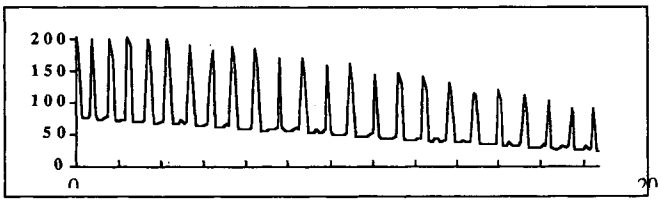
Results of human ROS injection into mouse oocytes (mouse oocyte activation assay)

We examined ability of ROS from 11 non-obstructive azoospermic patients to induce Ca^{2+} oscillations

Table 1 Results of clinical TESE/ICSI

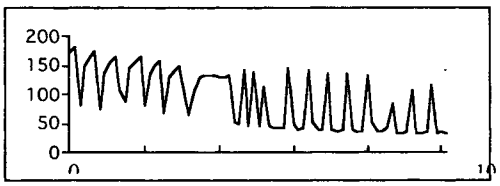
Case	No. of oocytes injected	No. of oocytes survived	No. of oocytes fertilized (%)[a]	No. of oocytes cleaved (%)[b]	No. of oocytes transfered	Pregnancy
a	9	6	6 (100)	6 (100)	3 (bl)	–
b	3	3	2 (67)	2 (100)	2	–
c	6	6	6 (100)	6 (100)	2	–
d	6	5	2 (40)	2 (100)	2	–
e	13	12	9 (75)	8 (89)	2 (bl)[c]	–
f	11	10	5 (50)	5 (100)	2 (bl)[c]	+
g	4	4	4 (100)	4 (100)	2	–
h	2	1	0 (0)	0 (0)	0[d]	+
i	10	10	5 (50)	5 (100)	2 (bl)[c]	–
j	5	4	2 (50)	2 (100)	2	–
k	6	6	6 (100)	6 (100)	2	–

[a]Oocytes as percentage of survivied oocytes.
[b]Oocytes as percentage of fertilized oocytes.
[c]Blastocyst transfer was performed on day 5 of fertilization.
[d]Embryo transfer could not be performed because no cleavage embryos were obtained.

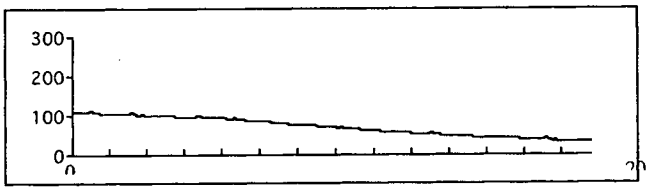


Type A: Normal oscillation pattern

Type C: Transient pattern



Type B: Atypical oscillation pattern



Type D: No-response pattern

Figure 4 Patterns of intracellular Ca^{2+} concentration changes in oocytes injected with human ROS. Type A: the normal oscillation pattern consisted of regular repetitive spike-shaped peaks in $[Ca^{2+}]i$ at intervals of 2–10 min. Type B: the atypical oscillation pattern exhibited irregular peaks of $[Ca^{2+}]i$ in an oscillatory pattern. Type C: the transient pattern was composed of several (1–4) transient peaks in $[Ca^{2+}]i$. Type D: the no-response pattern lacked any $[Ca^{2+}]i$ peaks.

in mouse oocytes (mouse test). We previously described the classification of the $[Ca^{2+}]i$ patterns of spermatid/sperm-injected oocytes into four groups (Yazawa *et al.*, 2000). In this study, the classification was modified slightly. A normal oscillation pattern (Type A) consisted of repetitive spike-shape Ca^{2+} rises at intervals of 2–10 min. An atypical oscillation pattern (Type B) consisted of irregular rises in $[Ca^{2+}]i$ with an oscillatory pattern. The transient pattern (Type C) exhibited only several (1–4) transient rises in $[Ca^{2+}]i$. The no-response pattern (Type D) lacked any $[Ca^{2+}]i$ rises during the observation period (Fig. 4).

Eighty-two mouse oocytes were injected with human ROS from the 11 patients to examine $[Ca^{2+}]i$ oscillations (Table 2). The ROS of all 11 patients exhibited some Ca^{2+} oscillation-inducing ability, ranging from 25–100%. Of the ROS-injected oocytes examined in this study, we observed a normal oscillation pattern, transient pattern and no-response pattern in 48.8% (40/82), 13.4% (11/82) and 37.8% (31/82) of the oocytes examined, respectively; none of the oocytes exhibited an atypical oscillation pattern (Type B). We also examined the oocyte-activating activity of human ROS using a subset of the samples isolated by testicular extraction. A total of 119 oocytes were injected with human ROS from cases e–k; 100 survived the injection and 56 oocytes were activated (with a second polar body and more than two pronuclei) and 45 oocytes (45%) were normally activated (with a second polar body and two pronuclei). The rates of normal activation for these

Table 2 Results of intracellular carcium concentration ($[Ca^{2+}]i$) patterns and activation of human ROS injected oocytes

Case	No. of oocytes	Normal oscillation examined for $[Ca^{2+}]i$ (Type A) (%)	Atypical oscillation pattern (Type B) (%)	Transient pattern (Type C) (%)	No response pattern (Type D) (%)	No. of oocytes injected for activation	No. of oocytes survived	No. of oocytes arrested at MII	No. of oocytes activated	No. of 2PN eggs[a] (%)
a	4	1 (25)	0	0 (0)	3	–	–	–	–	–
b	6	2 (33)	0	0 (0)	4	–	–	–	–	–
c	7	4 (57)	0	2 (29)	1	–	–	–	–	–
d	5	5 (100)	0	0 (0)	0	–	–	–	–	–
e	8	4 (50)	0	2 (25)	2	12	11	5	6	6 (55)
f	8	5 (63)	0	2 (25)	1	12	10	4	6	5 (50)
g	9	4 (44)	0	1 (11)	4	21	14	3	11	9 (64)
h	7	3 (43)	0	1 (14)	3	7	7	4	3	3 (43)
i	9	3 (33)	0	1 (11)	5	20	18	7	10	8 (44)
j	9	6 (66)	0	0 (0)	3	25	21	18	7	6 (29)
k	10	3 (39)	0	2 (20)	5	22	19	6	13	8 (42)
Total	82	40 (49)	0	11 (14)	31 (38)	119	100	47	56	45 (45)

[a]Eggs as percentage of survived oocytes.

Table 3 Results of intracellular carcium concentration ($[Ca^{2+}]i$) patterns and activation of human sperm, ELS injected oocytes and sham operation (control)

Material injected	No. of oocytes	Normal oscillation pattern (Type A) (%)	Atypical oscillation pattern (Type B) (%)	Transient pattern (Type C) (%)	No response pattern (Type D) (%)	No. of oocytes injected for activation	No. of oocytes survived	No. of oocytes arrested at MII (%)[a]	No. of oocytes activated (%)[a]	No. of 2PN eggs (%)[a]
Sperm[b]	40	36 (90)	0 (0)	0 (0)	4 (10)	39	30 (77)	1 (3.3)	29 (97)	29 (97)
ELS[c]	17	11 (65)	3 (18)	0 (0)	3 (18)	22	18 (82)	4 (22)	14 (78)	12 (67)
Medium[d] (sham)	14	0 (0)	0 (0)	0 (0)	14 (100)	49	46 (94)	46 (100)	0 (0)	0 (0)

[a]Eggs as percentage of survived oocytes.
[b]Each oocyte was injected with mature spermatozoa originating from fertile men.
[c]Each oocyte was injected with elongated spermatid originating from testicular biopsies of some cases.
[d]Each oocyte was injected with a bolus (5 μl) of HTF medium without spermatozoa/spermatids.

seven cases ranged from 29 to 64%. Approximately half of human ROS-injected mouse oocytes exhibited a normal $[Ca^{2+}]i$ oscillation pattern and were normally activated. We evaluated from these data that human ROS had already acquired the oocyte-activating and Ca^{2+} oscillation-inducing abilities.

Results of human sperm and ELS injection and sham operation

When mature human spermatozoa isolated from fertile men and ELS from testicular biopsies of non-obstructive azoospermic patients were injected into mouse oocytes (Table 3), the rates of normal activation were 97% and 67%, respectively; oscillations were observed in 90 and 82% of examined oocytes, respectively. When human tubal fluid medium lacking sperm/spermatozoa were injected into mouse oocytes (sham operation), neither activation nor Ca^{2+} oscillation was observed.

Discussion

We previously reported that the oocyte-activating and Ca^{2+} oscillation-inducing abilities of ROS differed among species (mouse, hamster, rat and rabbit were examined). ELS from those experimental animals had the ability to induce oocyte activation and Ca^{2+} oscillations in a mouse oocyte activation assay (Yazawa *et al.*, 2000). Sousa *et al.* (1996) demonstrated that injection of human ROS into human oocytes with a Ca^{2+}-ionophore activated the oocyte and induced Ca^{2+} oscillations. Ogonuki *et al.* (2001) recently demonstrated that greater than 90% of mouse oocytes were activated and intracellular Ca^{2+} oscillations were

induced in 64% of mouse oocytes following injection of cynomolgus monkey ROS. These results suggest that ROS of primates are more mature than those of rodents; the oocyte-activating Ca^{2+} oscillation-inducing factor (OA-COIF; maybe the same as SOAF or sperm factor) of primates appears in the earlier stages of spermiogenesis than seen in other animals.

In this study, we examined the oocyte activation and Ca^{2+} oscillation-inducing abilities of human ROS originating from azoospermic patients during their TESE/ICSI treatment cycles to determine if human ROS exhibit OA-COIF activity. We confirmed that human ROS already had the activity of OA-COIF (SOAF or sperm factor), despite the levels being less when compared with ELS or mature spermatozoa.

The possibility of using ROS injection as a clinical treatment for patients with non-obstructive azoospermia with maturation arrest during spermiogenesis was first described by Edwards *et al.* (1994). Prior to the clinical application of spermatid injection, normal fertilization and the delivery of offspring using ROS were reported in mice (Ogura & Yanagimachi, 1993; Ogura *et al.*, 1993, 1994; Kimura & Yanagimachi, 1995b) and rabbits (Sofikitis *et al.*, 1994). The first fertilization with human spermatids was reported by Vanderzwalmen *et al.* (1995), following injection of one late-stage spermatid obtained from testicular biopsy into a human oocyte. The late-stage spermatid used was oval shaped, which suggested that it was an elongating or elongated spermatid. The first pregnancy using an elongated spermatid (Sd1) was reported by Fishel *et al.* (1995). The first births of healthy babies following round spermatid injection were reported by Tesarik *et al.* (1995, 1996). These procedures utilized ROS from ejaculates for microinjection. This procedure was the first birth to be successful after the transfer of embryos obtained by injection of ROS from azoospermic patients. Since this first report, successful births using ROS or ELS from testicular biopsies have been achieved by several centres (Mansour *et al.*, 1996; Vanderzvalmen *et al.*, 1997; Barak *et al.*, 1998; Barros *et al.*, 1998; Bernabeu *et al.*, 1998; Kahraman *et al.*, 1998). The efficacies of spermatid injections for fertilization and implantation have been discussed in detail, with special concern placed on the developmental stage of the injected spermatids and the histopathology of testicular biopsy.

Although maturation arrest may occur at any stage of spermatogenesis, according to Aslam *et al.* (1998), spermatocyte arrest is most common. Arrest at the spermatid level is slightly less frequent, while spermatogonial arrest is least common. It is unclear if maturational arrest at the ROS stage exists. In their examination of the histology of testes from 125 patients with maturation arrest, Silber & Johnson (1998) found that maturation arrest always occurred in meiosis. No round spermatids were found, with the exception of those cases in which elongated spermatids and spermatozoa were also identified. Whenever round spermatids were observed in an area of maturation arrest, elongated spermatids or mature spermatozoa were also observed (Shilber *et al.*, 1996). Thus, failure of ROS to develop into mature spermatozoa (spermiogenic failure) was never observed in patients with maturation arrest.

A number of authors, however, have reported that round spermatids were identified within testicular biopsy specimens, at places at which neither elongated spermatids nor mature spermatozoa could be found. Round spermatid injection (ROSI) was attempted as a clinical treatment for patients with such a situation; the results of these attempts were compared with those of elongated spermatid injection (ELSI) or mature spermatozoa injection (ICSI) (Vanderzwalmen *et al.*, 1997, 1998; Sousa, *et al.*, 1999; Lavran, *et al.*, 2000). According to Lavran *et al.*, out of 18 non-obstructive azoospermic patients who underwent TESE, mature spermatozoa were recovered from six (33%), round spermatids alone were identified in eight (44%) specimens and neither spermatids nor mature spermatozoa could be identified in the remaining four patients (22%). The fertilization and cleavage rates of ROSI (44.9 and 59.2%, respectively) were significantly lower than those seen following TESE/ICSI (69 and 91.8%, respectively). While the implantation and clinical pregnancy rates were 16.6 and 50%, respectively, in TESE/ICSI cycles, no transferred embryos were implanted in ROSI cycles. Similar results were obtained by Vanderzwalmen *et al.* (1997, 1998). In 42 (30.7%) of 137 non-obstructive azoospermic patients who underwent TESE, only spermatids could be identified; no mature spermatozoa could be identified in the biopsied specimens (complete spermiogenesis failure) (Vanderzwalmen *et al.*, 1997). Ninety-five (69%) of the 137 patients exhibited mature spermatozoa (partial spermiogenesis failure). The rates of fertilization and good quality embryo generation were lower in spermatid-injection cycles than those of TESE/ICSI cycles. Comparison of the resulting rates of fertilization, cleavage and pregnancy among elongated, elongating and round spermatids revealed that ROSI cycles were significantly less effective than elongated/elongating spermatid-injected cycles. The fertilization rate of spermatids isolated from patients with complete spermiogenesis failure was lower than that using spermatids isolated from patients with partial spermiogenesis failure (27 versus 8%) (Vanderzwalmen *et al.*, 1998). Fertilization and pregnancy rates of ROSI were significantly affected by the proportion of tubules demonstrating spermatozoa in previous testicular biopsies. When no spermatozoa could be identified in all tubules of previous testicular biopsies, the fertilization rate of ROSI decreased to 11% and no pregnancies could be established. If

spermatozoa could be identified in previous testicular biopsies, the fertilization rates ranged from 32 to 37% according to the percentage of tubules exhibiting spermatozoa.

What are the indications for ROS injection in clinical treatment?

ROS originating from severely defective testes, as seen in patients with complete spermiogenesis failure, might lack the ability to develop to term in part due to cytogenetic causes (Benkhalifa *et al.*, 2004). Clinical application of ROS injection might be appropriate for azoospermic patients who have exhibited spermatozoa in previous testicular biopsies, but lack spermatozoa in the specimens biopsied during treatment cycles; these are patients who do not exhibit complete spermiogenesis failure in previous diagnostic testicular biopsies. According to Vanderzwalmen *et al.* (1998), no spermatozoa could be identified in 25% of TESE/ICSI treatment cycles, despite the presence of spermatozoa in previous biopsies.

To date, many studies have been conducted to identify the oocyte-activating and Ca^{2+} oscillation-inducing factor (SOAF or sperm factor) and several proteins were put forward as candidates, such as a 33 kDa glucosamine-6-phosphate deaminase (Parrington *et al.*, 1996) and a truncated c-*kit* transmembrane receptor (Sette *et al.*, 1997). However, these were disputed later. In addition, more recently, the sperm-specific zeta isoform of phospholipase C, named PLCζ, has been identified and demonstrated as a powerful candidate for a sperm factor (Cox *et al.*, 2002; Saunders *et al.*, 2002). Microinjection of PLCζ cRNA triggered Ca^{2+} oscillations similar to those observed at fertilization, mouse oocytes injected with PLCζ content of a single sperm developed normally into blastocysts and mouse eggs microinjected with anti-PLCζ antibody-treated sperm extract exhibited no Ca^{2+} responses. These findings and several supporting studies indicated that PLCζ was a reliable candidate for being a sperm factor and this proposition seems to have been now validated (Fujimoto *et al.*, 2004; Roger *et al.*, 2004; Yoda *et al.*, 2004; Coward *et al.*, 2005; Sone *et al.*, 2005).

This study demonstrated that ROS from all of the patients examined exhibited some Ca^{2+} oscillation-inducing activity, but the rates at which ROS-injected oocytes displayed a normal oscillation pattern varied from 25 to 100% among patients. From our clinical and experimental data, we could not discern an obvious relationship between the rate of Ca^{2+} oscillation induction in the mouse assay and the clinical results. In cases in which pregnancies were achieved by TESE–ICSI treatment (cases f and i), the rates of Ca^{2+} oscillation induction by the mouse test were not always high (63 and 33%, respectively) in comparison with the other cases. We also reported that Ca^{2+} oscillation-inducing ability was acquired at later stages of spermiogenesis than for oocyte-activating ability. Ca^{2+} oscillation is important in embryonic development until the blastocyst stage, but is not essential for embryonic development into normal offspring (Yazawa *et al.*, 2001). It is likely that, if the ROS of non-obstructive azoospermic patient could be restored for Ca^{2+} oscillation-inducing ability, these ROS have sufficient SOAF to allow the injected oocyte to develop normally into offspring.

In these experiments, biopsied specimens from all patients possessed testicular spermatozoa within their testicular tissue; we did not examine the activity of ROS from patients with complete spermiogenesis failure. As the clinical results of ROSI for patients with complete spermiogenesis failure are poor, it is possible that the ROS of these cases are lacking in any ability to induce Ca^{2+} oscillations; it may be necessary to perform such experiments in the future to confirm this defect and analyse potential therapies to reverse it.

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第

生殖補助医療と看護の役割

生殖補助医療の基礎知識

不妊因子からみたARTの成績
－重症男性不妊症例に焦点をあてて－

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●要旨
ARTには人工授精(AIH)，体外受精(IVF)，顕微授精(ICSI)がある。乏精子症や精子無力症が存在するとき，AIHが選択される。それで妊娠しなければIVFが選択される。精液パラメーターで捉えるとAIHとIVFの精液パラメーターの守備範囲は極めて似ている。ある程度以下のレベルの精液パラメーターではAIHでもIVFでも有効性が少ない。真に重症男性不妊症例が治療できるのはICSIである。しかし，遺伝的安全性が十分に確認されていないのが現状である。

●Key Words
人工授精，体外受精，顕微授精，卵細胞質内精子注入法，男性不妊症

●はじめに

不妊原因の中で主要なものは女性側の卵管因子で，外来でも多いという印象がある。本稿のテーマは男性不妊症例の生殖補助医療(ART)の成績であり，その男性因子は全不妊原因の30～45％を占め，不妊男性因子の治療を行わずして不妊治療を行うことはできないのが現状である。男性因子がクローズアップされるようになったのはARTの導入が契機であり，それ以前にはそれほど注目されていたようには記憶していない。1978年の体外受精・胚移植の成功[1]，そして，1992年の顕微授精(卵細胞質内精子注入法)の成功[2]が男性不妊の存在を改めて知らしめたように思われる。それは，それまで著効する男性不妊症の治療がなかったこと，そして体外受精あるいは顕微授精でそれらの治療に活路が開けたこと，そしてそれらの治療でも有効性が期待できない重症男性不妊症が存在していることなど，男性不妊症の病態がより鮮明に捉えられるようになったからである。

●不妊症の中の男性不妊症

不妊症夫婦の割合は10～15％とされている。不妊症の原因には男性不妊症と卵管性，卵巣性(排卵性，ある

表１○男性不妊症の原因

1. **造精機能障害**
   1) 特発性
   2) 精索静脈瘤
   3) 染色体異常
   4) 停溜精巣
   5) 薬剤性
   6) 発熱性疾患
   7) その他
2. **輸精路通過障害**
   1) 精管欠損症
   2) 炎症性
3. **副性器機能障害**
4. **射精障害**
5. **抗精子抗体**
6. **その他**

いは内分泌学的)，子宮性，頸管性，免疫学的などの原因を有する女性因子がある。女性因子に対する比率は，女性因子：男性因子で７：６ともいわれる。男性不妊症の原因を表１にあげたが，種々の原因がある。しかし，視点を変えれば男性の性機能の目的は父親の遺伝情報を子(受精卵)に伝えることであるので，男性の妊孕能(にんようのう)は受精できる精子がどの程度存在するかで評価される。したがって，男性不妊症の治療には精液検査による精液パラメーターの評価が重要な検査となる。

通常の診療の中で行いうる男性妊孕能の検査は，精液検査以外ないといっても過言ではない。本来ならば精子が受精を成立させるために必要な機能，例えば先体反応を起こすこと，精子の運動性が超活性化(hyperactivation)すること，精子－卵子融合することなどの機能が重要なのであって，精子濃度や運動率が男性の妊孕能を直接表現しているのではないことを承知しておかなければならない。精子濃度や精子運動率だけで男性の妊孕能を評価できる基準値があれば，治療法の選択がクリアカットに行えて有益である。しかし，実際はそうではなく，いわゆるグレーゾーンがやや広く，臨床家の経験的判断によって評価がなされることが多々ある。男性不妊で重症と形容される場合，前述した曖昧さと同様にその定義は不明である。男性不妊症の治療には薬物療法，手術療法および生殖補助医療がある。一般的に薬物療法の奏効率は低く，手術療法では実施できる施設が限られ

表２○WHO精液パラメーターの正常値

精液量	2ml 以上
精子濃度	20×10^6/ml 以上
総精子数	40×10^6/ml 以上
精子運動率	前進性精子50%以上または高速前進精子25%以上
精子形態	正常形態 15%以上
精子生存率	75%以上
白血球数	1×10^6/ml 以下

(WHO laboratory manual for the examination of human semen and semen-cervical mucusinteraction, 3rd ed, Published on behalf of the World Health Orgnization, Cambridge University Press, Cambridge, UK, 1999. より引用)

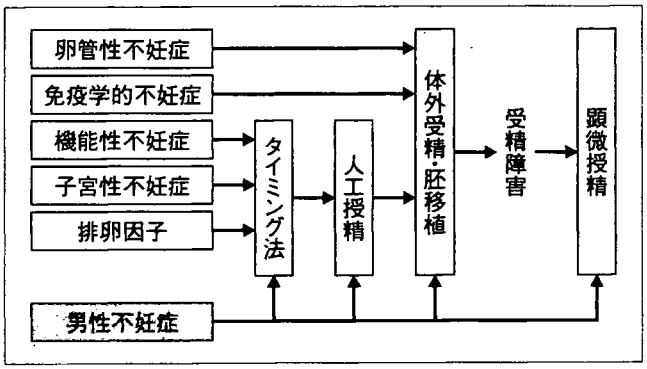

図１○不妊症治療の概要

ていることなどの制約が入り，その恩恵にあずかることができるクライアントは限られることになる。そして，やはり生殖補助医療がどちらかというと頼りにされている治療という位置づけにあるのが現状である。ただし，医療の第一の目的である本来の機能を回復させることからはやや遠ざかる治療となっている。

●精液パラメーターと男性不妊症の治療方針

重症男性不妊症とは人工授精を実施しても妊娠に至らず，体外受精にステップアップするケースとも捉えられる。しかし，人工授精が有効と考えられる精液パラメーターと体外受精が有効と考えられる精液パラメーターはほとんど同じレベルであるというのが実状である。参考までに表２にWHOによる精液パラメーターの基準値を示した。

●不妊治療の中の男性不妊治療

不妊治療の基本的方針はタイミング法，人工授精，体外受精そして顕微授精である。不妊症全体をみた場合の，それぞれの不妊因子の治療の流れを図1に示した。不妊原因は卵管性，免疫学的，子宮性，排卵因子，原因不明そして男性因子をあげた。女性側因子に対する治療法はタイミング法，人工授精，体外受精の順に選択され進行する。原因によっては，卵管因子などのようにタイミング法や人工授精を跳ばして，最初から体外受精を選択する場合がある。一方，男性因子は精子濃度や運動率からの評価によりタイミング法，あるいは人工授精，体外受精そして顕微授精がダイレクトに選択される。もちろん，選択された治療法で妊娠に至らなかった場合には，治療がステップアップされる。

●重症男性不妊症の治療成績

1) 人工授精

AIHが適応となる精液パラメーターについては，子宮内腔に注入される運動精子数が2.0×10^6個にて妊娠例が報告されているが，一般的に10.0×10^6個以上が望ましい。

実際にAIHを行った症例での精液検査の平均値は，精子濃度が63.0×10^6/ml ±51.6(SD)，精子運動率が47.8%±2.7(SD)であった[3]。またWHOの診断基準によって精液パラメーターを評価すると，精液所見が正常な症例は全体の45%，乏精子症が5%，乏精子無力症が11%，精子無力症が39%となっており，精子無力症が多い。

妊娠率に関して，文献報告を集めると(表3)，対周期妊娠率は7.5%，対症例妊娠率が23.8%となった[4]。また，自験例の分析では，精子濃度が5×10^6/mlまたは運動率が10%以下では妊娠例を認めず，精液パラメーターが正常所見群と異常所見群の比較では妊娠率(3.7% vs. 2.4%)，および妊娠に要したAIH実施回数(4.0回 vs. 5.4回)に有意差を認めなかった[3]。

2) 体外受精

IVFが適応となる精液パラメーターは媒精濃度が10×10^5/ml ～20×10^5/mlであるので，この濃度が調整できればよいことになる。原精液の精液パラメーターでいうと，やはり10×10^6/ml以上の精子濃度が必要であり，真に重症な男性因子例には有効性が低いと考えられる。

体外受精・胚移植(IVF)の適応は卵管性不妊症，乏精子症，免疫性不妊症，原因不明不妊症などである。日本産科婦人科学会の2004年(平成16年)度倫理委員会・登録・調査小委員会報告(日産婦，2006)によると，平成16年分の体外受精・胚移植の臨床実施成績は，表4の結果となっている[5]。胚移植あたりの妊娠率は25%を超えるが，移植あたりの生産率は17.9%である。妊娠を十分に期待できる実施回数4回であり，原因別に検討すると卵管因子が4回，男性因子が4回，原因不明が2回となっている(吉村ら，2001)[3]。平均受精率では男性因子が卵管因子よりも有意に低値であり，当然のことといえた。適応別の成績については，男性因子例では卵管性不妊症と比較して低い傾向を認めた(16.4% vs. 11.8%)。反復実施に伴う累積妊娠率の評価では，男性因子例では反復実施5回目まででほとんどの妊娠が得られ，それ以降では妊娠例がなかった[3]。しかし，この成績は他の原因と比較して特徴的なものではない。

3) 顕微授精

現在実施されている顕微授精は，卵細胞質内精子注入法(Intracytoplasmic sperm injection；ICSI)である。これは1個の精子を卵子内に注入する方法であるので，精液パラメーターがきわめて劣悪な男性因子例にも有効である。重症男性因子例に真に有効な方法がICSIであると考えられる。

顕微授精の適応は「難治性の受精障害で，これ以外の治療によっては妊娠の見込みがないか極めて少ないと判断される場合」で，IVFの受精障害や受精障害が予測されるような重症男性因子例(精子減少症，精子無力症，精子奇形症，無精子症など)に適応される。自験例からでは，精子濃度が5×10^6/ml未満ではICSIの適応と，また5×10^6/ml ～10×10^6/mlではグレーゾーンと考えている。

表３　人工授精の成績に関する主要論文報告(会議録を含む)

著　者 (施設名)	報告年	症例数	実　施 周期数	対症例妊娠率(%)	対周期妊娠率(%)	症例当たりの 平均施行回数	妊娠例の平均 施行回数
後藤ら[3](京　都)	1991	40	75	7.5	4.0	—	
藤本ら[4](京都府立)	1992	154	336	15.6	7.4	2.2	
松岡ら[5](大阪市立)	1992	151	325	14.6	6.8	2.2	
武田ら[6](愛　媛)	1993	56	135	17.8	7.4	2.4	1.6
飯田ら[7](順天堂)	1993	268	700	20.9	8.0	2.6	
石原ら[8](埼　玉)	1993	122	410	20.5	6.1	3.4	6.3
森　ら[9](札　幌)	1995	91	552	38.5	6.3	6.1	
桑原ら[10](徳　島)	1996	56	266	23.2	4.9	4.8	
朱亜ら[11](広　島)	1997	62	311	22.6	4.5	5.0	
廣瀬ら[12](滋　賀)	1998	1311	3279	24.3	9.7	—	
保條ら[13](愛　知)	1999	88	651	29.5	4.0	7.4	
小原ら[14](自　治)	2000	382	1493	38.0	9.7	3.9	
著者ら(福島県立)	2004	279	1223	12.9	2.9	4.7	4.6

〔吉村泰典，柳田薫，他：配偶子・胚提供を含む統合的生殖補助技術のシステム構築に関する研究，不妊原因に応じた最適な不妊治療の選択指針の確立に関する研究，その２(吉村泰典班)．平成15年度厚生科学研究費補助金(子ども家庭総合研究事業)分担研究報告書，p 90-106，2004．より引用〕

平成16年度の体外受精・胚移植などの臨床実施成績報告(日本産科婦人科学会倫理委員会・登録・調査小委員会)によれば，射精精子を用いたICSI(子宮腔内移植)では，採卵あたり17.8%，移植あたり26.0%の妊娠率であった[5]。移植あたりの生産率が16.7%で，流産率が22.2%である。同報告では無精子症という分類がされていないが，「その他の採精精子」という分類には無精子症が主体と考えられる。この分類での成績は，採卵あたり18.6%，移植あたり25.2%の妊娠率であった。移植あたりの生産率が17.8%で，流産率が19.1%であった。この成績は，射精精子のものとまったく同等のものであり，ICSIでは男性因子の重症度に依存しない一定の成績が得られることがわかる[5]。ただし，無精子症であっても精巣精子回収法(TESE)によって精子が回収されることが前提となる。無精子症の場合，TESEで精子が回収できる確率は約70%である[6]。マイクロダイセクションTESEを行うと約90%で精子が回収できるといわれる。累積妊娠率を調べると，６回目以降で妊娠率の低下が明かとなり，５回目での累積妊娠率が約90%となることから，ICSIが有効と考えられるのは実施回数５回以内であった(吉村ら，2001)[3]。

表４　平成17年度の日本産科婦人科学会倫理委員会報告(平成16年の成績)

	IVF-ET	ICSI
患者総数	29,023	26,835
治療周期数	41,328	40,548
採卵総回数	39,397	39,822
移植回数	28,858	27,172
採卵あたり妊娠率	21.6%	17.8%
移植あたり妊娠率	29.5%	26.0%
妊娠あたり流産率	21.3%	22.2%
妊娠あたり多胎妊娠率	18.0%	15.7%
多胎妊娠あたり超多胎妊娠率	7.4%	5.9%
出生児数	6,686	5,373
移植あたり生産率	19.3%	16.7%

前述したように，無精子症でもその治療成績は通常のICSIと同じように期待できるが，不動精子しか得られないケースでは成績の低下が予測される。その理由は，

不動精子では死滅している精子が含まれており，ICSIにそれらの死滅精子を選択して顕微注入に用いられる場合があるからである。

①精子死滅症

Vital stain (eosin 染色，Live/Dead Fertilite stain) によって，すべての精子が死滅している。死滅精子では当然，退行性変化が起きており，DNA も傷害されるので，ICSI の成績はまったく期待できない。この場合には TESE を行い生存精子が存在すればその精子を用いる。

②不動精子しか得られない症例

不動精子しか得られない症例では，vital stain によって生存精子が認められる。この場合では，生存精子を hypoosmotic swelling test(HOST)を行って選択する[6]。この方法が困難な場合には TESE にて精子を回収する。

●おわりに

男性因子はその原因として原因が不明な場合が多い。そのためか薬物療法の効果が得られにくい。そこで，AIH や IVF などの生殖補助医療による治療がなされる。乏精子症や精子無力症では程度が軽ければ，AIH や IVF でも治療できるが，重症の場合では ICSI が適応となる。ICSI は IVF より，より侵襲が大きい治療で，妊娠し誕生した子どもへの遺伝的リスクが昨今問題になっている。つまり，ICSI では出生児の *de novo* の染色体異常が1.1～2.9%，遺伝性異常が0.5～1.1%に認められ，一般児よりも高いことが報告されている。また，性染色体異常の頻度も増加するようである[7)-9)]。さらに，造精機能関連遺伝子異常が男児へ遺伝するリスクも存在する。したがって，安易な ICSI の実施は避けたほうがよいといえる。しかし，すでに述べたように重症男性因子例では ICSI に頼らざるを得ないのが現状であり，リスクの有無を明確にすることが今後の課題である。

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顕微授精での受精障害

Fertilization failure in ICSI

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◎顕微授精では体外受精での受精障害も適応となり，受精の成立を強力に図る．不妊治療のなかでは受精成立にもっとも信頼がおける治療方法である．この方法をもってしても1～8%の頻度で受精障害が発生してしまう．その場合の原因は卵子側と精子側とに存在する．現時点である程度解析されている原因としては，精子がもっている卵活性化因子の異常があり，受精障害例の約50%にその原因が考えられる．その場合には卵活性化因子の作用を補完する意味で，卵活性化処理を行う臨床試験が試みられている．現時点ではカルシウムイオノフォア処理，電気刺激処理，ストロンチウム処理などによる妊娠分娩例が報告されている．この卵活性化併用の顕微授精は過去の顕微授精で受精障害となったケースに適応となるが，遺伝的安全性はいまだ十分に確立されていない．

Key word　顕微授精，卵細胞質内精子注入法，受精障害

体外受精の受精障害は約10%の頻度で認められるが，顕微授精では約3%程度とまれである．しかし，受精障害が発生したときの対応策がなく，難治性不妊症といえる．本稿では，受精障害の原因と対策について考察する．

顕微授精のなかでも，ここでは卵細胞質内精子注入法(intracytoplasmic sperm injection：ICSI)での受精障害について述べる．ICSIの受精率は生殖補助医療のなかではもっとも高い受精率が得られる．しかし，なかにはまれであるが，ICSIを行っても受精卵が得られない症例が存在する．受精の成立は精子と卵子の相互作用によって成立するので，精子と卵子のそれぞれに原因の存在した可能性がある．また，受精障害例への対処も今後の課題である．

受精障害にはtotal(complete)fertilization failureとlow fertilization rateとがある．前者はICSIを行った卵子すべてに受精が成立しなかった場合で，後者は受精率が10～25%以下の低率である場合である．ここでは前者の場合の受精障害について述べる．

ICSIでの受精障害の現状

ICSIの結果を調べると，ICSIの治療周期当り7.9%(2,066治療周期，2006.1～2007.12，山王病院リプロダクションセンター)に認められた．2,066治療周期での受精率を分布図で示すと図1のようになり，受精率100%の例が57.0%を占めていた．また，ICSIを行った卵子数も受精率と密接な関連がある．ICSIを行った卵子が1個の場合，受精障害の頻度は13.3%となり，受精障害のリスクがきわめて高い．2個では3.1%，3個で0.9%，4個で1.3%となり，3個以上ではほぼ1%以下で，6個以上での受精障害はなかった(図2)．平均ICSI卵数は3.7個であった．自験例の既報と比べると数値は異なるが[1]，傾向は同一で，1個ICSIでの受精障害のリスクはきわめて高い．その原因は不明であり，卵巣刺激法の種類などの分析が必要であるが，卵巣刺激に対して低反応であったことは事実で，そのような状況下で採卵された卵子の質が不良であったこと，技術的なこと(最初のICSI後に，ICSI関連機器の不調があれば再調整が行われるなども含まれる)が推測される．