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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.

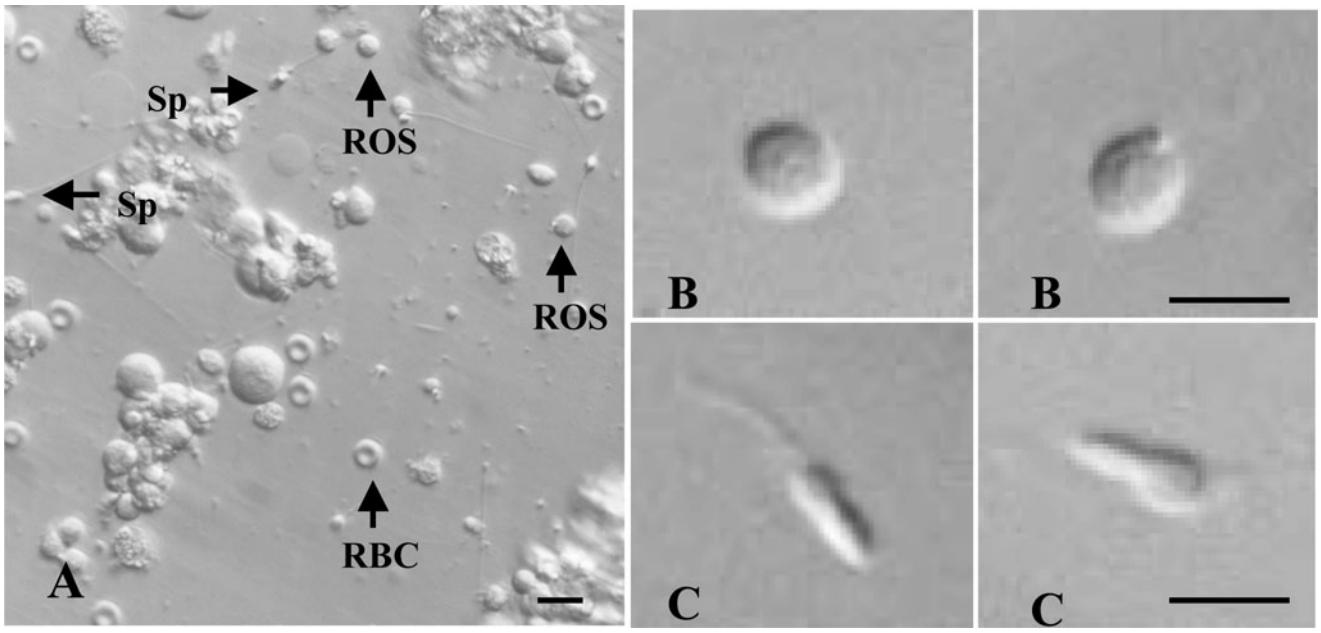

Figure 1 Wet preparation of a human testicular extraction isolated from a non-obstructive azoospermic patient (*A*). Isolated round spermatids (*B*) and isolated elongated spermatids (*C*) were suspended in medium. In (*A*), ROS, Sp and RBC (arrow) indicate round spermatids, testicular spermatozoa and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

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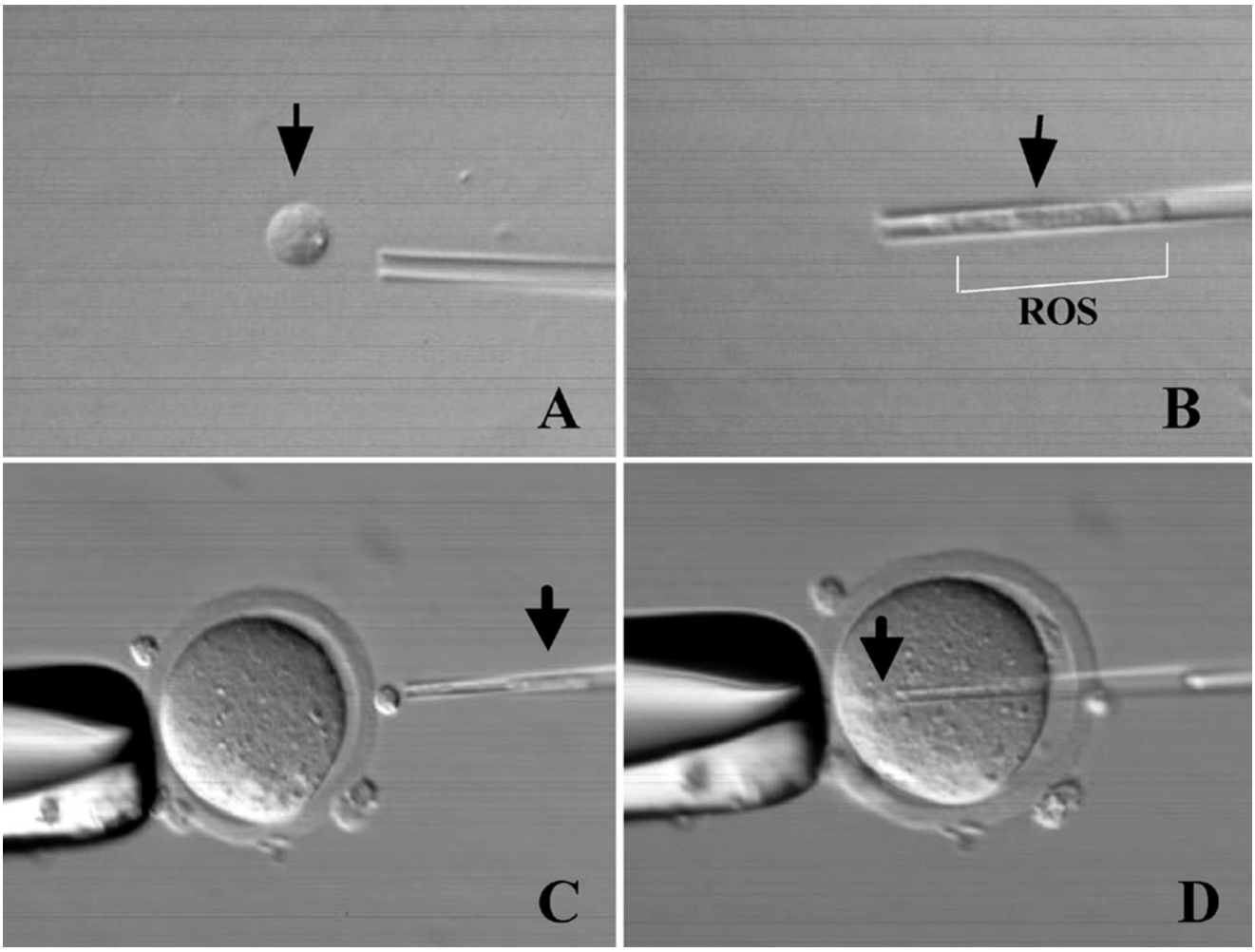

Figure 2 Human round spermatid injection into mouse oocytes using a piezo micromanipulator (mouse test). An isolated human round spermatid (ROS) was transferred into an injection pipette (*A*, *B*). The entire round spermatid was then injected into the ooplasm of unfertilized mouse oocyte (*C*, *D*).

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

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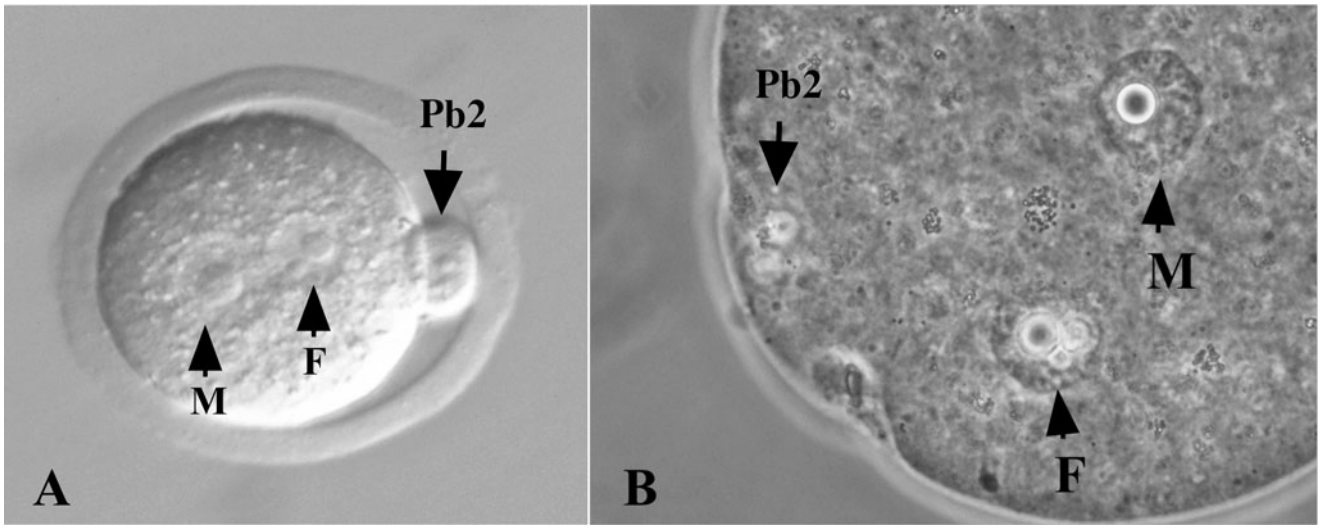

Figure 3 A normally fertilized mouse oocyte shown at 4 hours after injection with a human round spermatid. The oocyte was normally activated, forming a second polar body and two pronuclei – male pronucleus (M) and female pronucleus (F). Fresh zygote (*A*) and fixed, stained zygote (*B*).

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

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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.

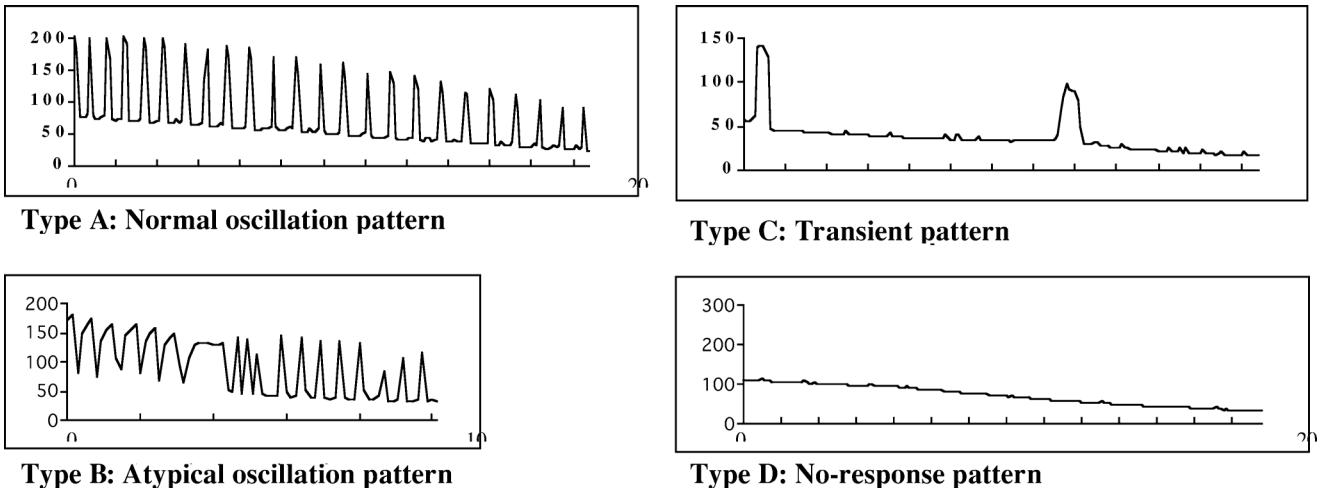

Figure 4 Patterns of intracellular Ca^{2+} concentration changes in oocytes injected with human ROS. Type A: the normal oscillation pattern consisted of regular repetitive spike-shaped peaks in $[Ca^{2+}]$i at intervals of 2–10 min. Type B: the atypical oscillation pattern exhibited irregular peaks of $[Ca^{2+}]$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

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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

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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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