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The oocyte activation and Ca^{2+} oscillation-inducing abilities of mouse and human dead (sonicated) spermatozoa

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Summary

In ICSI procedures, it is well known that the selection of viable (live) spermatozoa and certain types of immobilization prior to injection is very important for obtaining successful results, but unfortunately there are rare situations when only immotile spermatozoa are available (such as in severe asthenozoospermia or necrozoospermia). In such cases, failure of oocyte activation after ICSI often occurs and may be due to the lack of SOAF (sperm-borne oocyte activating factor) activity. In order to investigate the SOAF activities of dead spermatozoa, mouse and human spermatozoa were immobilized (killed by sonication), maintained in THF medium for varying time intervals (up to 72 h) and then injected into mature unfertilized mouse oocytes. Injected mouse oocytes were examined for their activation, development into blastocysts and Ca^{2+} responses by imaging and confocal laser scanning microscope. The rates of oocyte activation, blastocyst development and normal patterns of Ca^{2+} oscillation from the killed-sperm-injected oocytes decreased gradually in accordance with the maintenance interval between sonication and injection. For injection with mouse sonicated spermatozoa, the rate of normal Ca^{2+} oscillations declined first (after a 3 h maintenance interval) and then blastocyst development was gradually obstructed (after approx. 10 h). The oocyte activation-inducing ability of dead spermatozoa was maintained for a relatively long period, but began to decline after 20 h. The activation rates and Ca^{2+} response of the oocytes that were injected with human sonicated spermatozoa decreased earlier than those injected with mouse spermatozoa. Although the oocyte activation-inducing ability was maintained for a relatively long time after the death of the spermatozoa, embryo development into blastocysts and the rate of normal Ca^{2+} oscillations declined after a short maintenance interval between sonication and injection. The Ca^{2+} response seemed to be the most sensitive indicator for the evaluating the SOAF activity of dead (killed) spermatozoa.

Keywords: Ca^{2+} oscillation, Dead sperm, ICSI, Oocyte activation, Sonication

Introduction

It is well known that oocyte activation is induced when fertilizing spermatozoa inactivate metaphase-promoting factor (MPF), which blocks the oocyte cell cycle during metaphase of the second meiotic division (MII) and then initiates normal embryonic development (including exocytosis of cortical granules for blocking polyspermic fertilization), pronucleus formation and cleavage division progression. In all mammalian species studied to date, it is also known that repetitive intracellular $[Ca^{2+}]i$ elevations (called Ca^{2+} oscillation), which are released from the oocyte's endoplasmic reticulum, occur. This Ca^{2+} oscillation is

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not essential for normal embryonic development to term, but plays an important role in effective embryonic development (Yazawa *et al.*, 2001).

In previous studies using microinjection of immature spermatogenic cells from experimental animals and human (Yazawa *et al.*, 2000, 2001, 2007; Ogonuki *et al.*, 2001), it was demonstrated that sperm-borne oocyte-activating factor (SOAF) becomes biologically active during the maturation of spermatogenic cells, but the timing of the acquisition of SOAF activity differed among species. For example, mouse round spermatids (ROSs) do not have any SOAF activity, hamster and rabbit ROSs have intermediate levels of SOAF activity and monkey and human ROSs have some SOAF activity. Moreover, except for rare cases of fertilization failure with ICSI in mice and humans (Tesarik & Sousa, 1995; Yanagida *et al.*, 1999, 2006; Elder-Geva *et al.*, 2003), live mature spermatozoa possess sufficient SOAF activity.

To examine the fertilizing ability of immobilized (killed) mouse and human spermatozoa, we sought to determine when and how their SOAF activity declined after spermatozoa immobilization by analyzing their ability to activate oocytes and induce Ca^{2+} responses following injection into mouse oocytes.

Materials and methods

Preparation of mouse oocytes

Six- to 8-week-old B6D2F1 female mice were superovulated by consecutive i.p. injections (48 h apart) of 8 IU pregnant mare's serum gonadotropin (PMSG; Teikokuzouki Co.) and 8 IU human chorionic gonadotropin (HCG; Mochida Pharmaceutical Co.). Mature oocytes were collected from the oviducts 15–16 h after hCG injection and freed from cumulus cells by treatment with 0.1% hyaluronidase in HEPES-buffered human tubal fluid medium (mHTF; Irvine Scientific) supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific). The cumulus-free oocytes were rinsed thoroughly, placed in drops of human tubal fluid medium (HTF; Irvine Scientific) containing 10% SSS and covered with mineral oil for up to 2 h at 37°C under 5% CO_2, 5%O_2 and 90% N_2 before ICSI.

Preparation of mature spermatozoa of mouse and human

Preparation of mouse epididymal spermatozoa

The cauda epididymis was isolated from a mature 8–10-week-old male B6D2F1 mouse. Mature spermatozoa were obtained by puncturing the epididymal tubes and incubating them in 3 ml of mHTF for 10–15 min at 25°C, to allow the spermatozoa to disperse evenly in the medium. Next, this suspension was lightly sonicated for 5 s at 5 W power output using an ultrasonic sonicator (Microson; Misonic Inc.) and washed using HTF medium by centrifugation for 5 min at 1000 *g*. With this treatment, >95% of spermatozoa were immobilized and decapitated. All isolated sperm heads were diagnosed as 'dead' after assessment with a sperm viability kit (Live/Dead Fertilight Sperm Viability Kit; Molecular Probes Inc.) (Kuretake *et al.*, 1996). As a control, the sonicated (dead) spermatozoa were injected immediately into mouse oocytes. For the experiments, the sonicated spermatozoa were kept in HTF medium for up to 72 h at 37°C under 5% CO_2, 5% O_2 and 90% N_2 before injection. After varying maintenance intervals (1.5–72 h), the sonicated/incubated spermatozoa were injected into mouse oocytes to examine their oocyte activating and Ca^{2+} oscillation-inducing abilities and the developmental capacities of those injected oocytes.

Preparation of human spermatozoa

Human spermatozoa were obtained from volunteers with proven fertility. Semen samples were allowed to liquefy for 30 min at room temperature and spermatozoa were collected by the swim-up method using HTF medium. This suspension was then lightly sonicated in the same manner as described above. This sonicated sperm were kept in HTF medium for up to 30 h at 37°C under 5% CO_2, 5% O_2 and 90% N_2 before injection (Fig. 1*a*).

Microinjection of mouse and human sonicated (dead) spermatozoa into mouse oocytes

Mouse and human sperm injections were preformed using a micromanipulator with piezo-electric elements (Kimura & Yanagimachi, 1995; Yanagimachi, 1998). The method of intracytoplasmic sperm injection (ICSI) has been described previously (Yazawa *et al.*, 2000, 2001). Briefly, a single spermatozoon was sucked into an injection pipette (5–7 μm inner diameter at the tip) that was attached to a piezo-electric driving unit (model PMM-MB-A; Prime Tech Ltd) (Fig. 1*b*). A mature unfertilized mouse oocyte was stabilized using a holding pipette and its zona pellucida was penetrated by applying several piezo pulses (Fig. 1*c*, *d*). When the needle had advanced deep enough into the ooplasm, the oolemma was punctured with a single piezo pulse and the spermatozoa were slowly released into the ooplasm, before the pipette was gently removed (Fig. 1*e*, *f*). All ICSI procedures were performed in 3 μl of mHTF on a stage cooled to 17–18°C (Kimura & Yanagimachi, 1995a; Yazawa *et al.*, 2000). 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.

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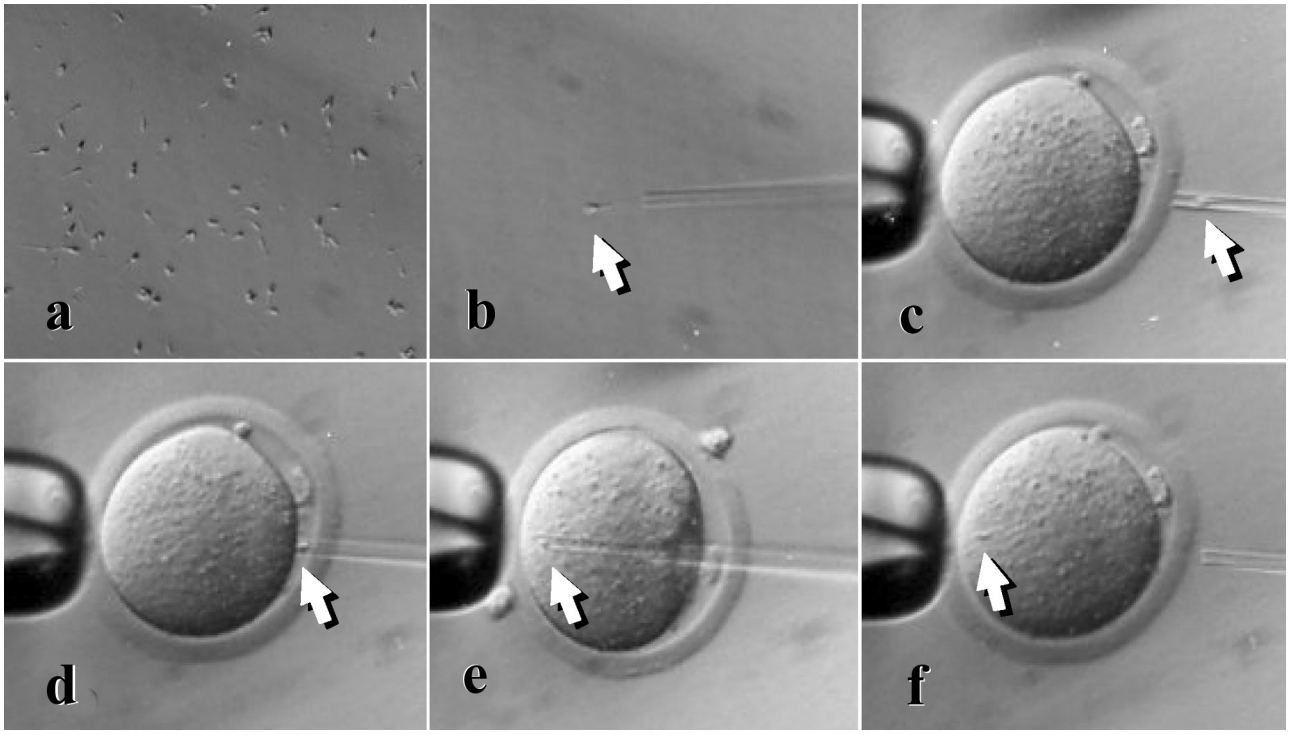

Figure 1 Human sonicated sperm injection into mouse oocytes using a piezo micromanipulator. Sonicated and decapitated human spermatozoa are suspended in medium (*a*). A sonicated human spermatozoon was sucked into an injection pipette attached to a piezo-electric driving unit (*b*). A mature unfertilized mouse oocyte was stabilized using a holding pipette and its zona pellucida was penetrated by applying several piezo pulses (*c*, *d*). When the needle had advanced deep enough into the ooplasm, the oolemma was punctured with a single piezo pulse and the spermatozoon was slowly released into the ooplasm (*e*), before the pipette was gently removed (*f*). Arrows indicate the positions of spermatozoa.

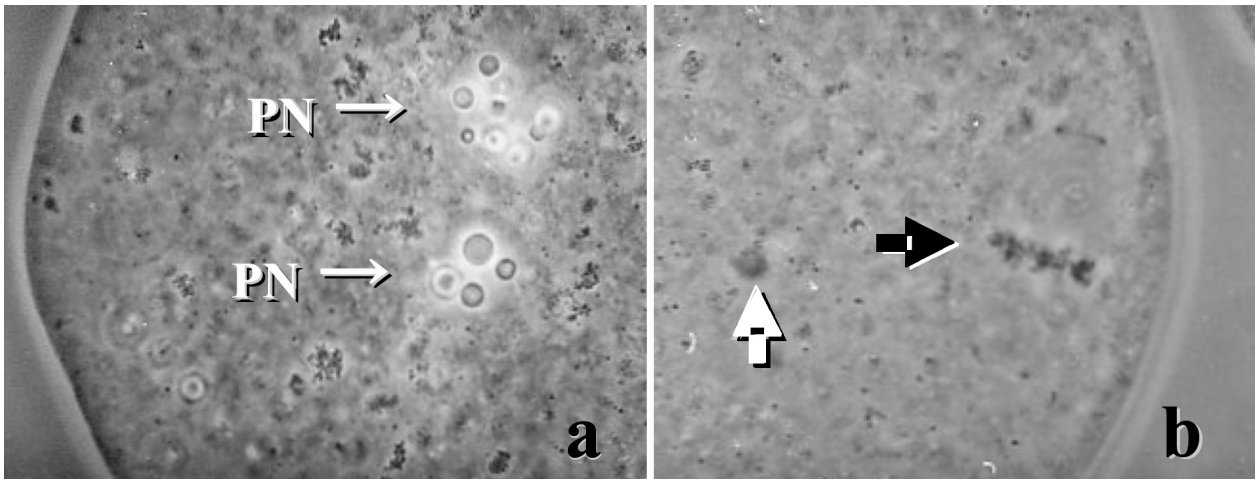

Figure 2 The sonicated sperm-injected oocytes were fixed and stained to examine their oocyte activating ability. Normally activated oocyte with two pronuclei (PN) (*a*) and an unactivated oocyte with decondensed sperm head (white arrow) and MII plate of an oocyte (black arrow) (*b*).

Examination of oocyte activation and embryo development

After 4–5 h incubation, the sonicated sperm-injected oocytes were placed on a slide coverslip and fixed and stained with acetocarmine to examine the chromatin configurations of the sperm and oocytes chromosomes (Yanagida *et al*., 1991) (Fig. 2). Oocytes with a second polar body and two pronuclei were considered to be 'normally activated'. In other experiments, the

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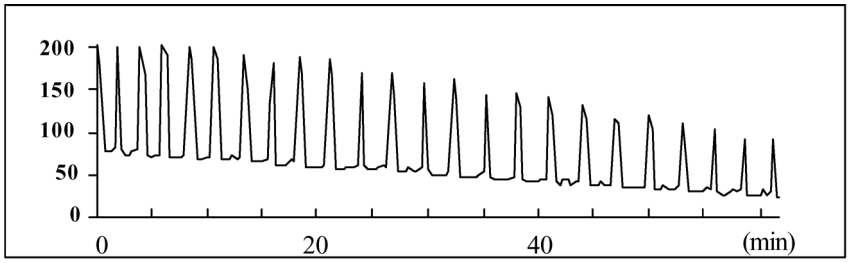
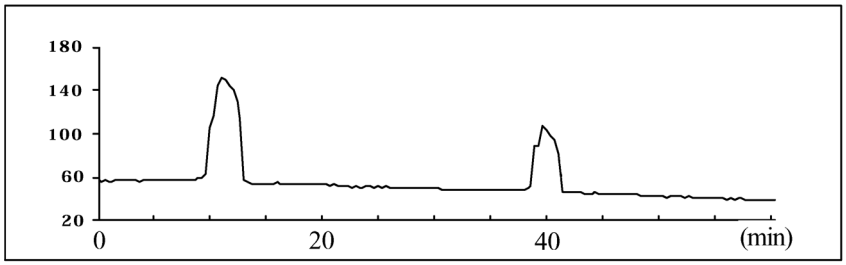
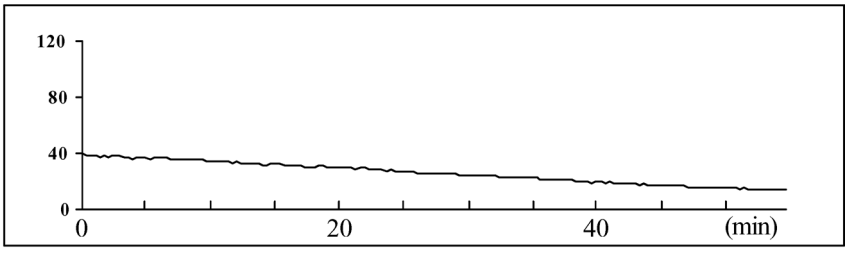


(a) Normal oscillation pattern



(b) Transient pattern



(c) No response pattern

Figure 3 Intracellular calcium ([Ca^{2+}]i) patterns of sperm-injected oocytes. The normal oscillation pattern (*a*) consisted of regular repetitive spike-shaped peaks in [Ca^{2+}]i at intervals of 2–10 min. The transient pattern (*b*) was composed of several (1–4) transient peaks in [Ca^{2+}]i. The no-response pattern (*c*) lacked any [Ca^{2+}]i peaks. The atypical patterns had irregular rises in [Ca^{2+}]i that did not fit into any of the above three patterns.

sonicated mouse sperm injected oocytes were cultured continuously in THF (up to 96 h) to examine embryo development to the morula and blastocyst stages.

Measurement of [Ca^{2+}]i of sperm injected oocytes

The Ca^{2+} responses of sonicated sperm injected oocytes were investigated using a Ca^{2+}-imaging method and a confocal laser scanning microscope system (Bio-Rad MRC-600, Nippon Bio-Rad Laboratories). Before injection, oocytes were loaded with the Ca^{2+}-sensitive fluorescent dye fluo-3-acetoxymethyl ester (Fluo-3/AM; Molecular Probes Inc.) in dimethyl sulfoxide (final concentration 44 μmol/ml in HTF) with 0.02% Pluronic F-127 for 30 min at 37°C. The loaded and injected oocytes were placed in a droplet (approx. 3 μl) of mHTF and covered with mineral oil on the chambered coverglass (Lab-Tec, Nunc Inc.). The dish was mounted on the stage of a phase-contrast inverted microscope equipped with a confocal laser scanning microscope system and Ca^{2+} changes were sampled at 20 s intervals for approx. 60 min.

We previously classified the Ca^{2+} response patterns of sperm-injected oocytes into four groups (Yazawa *et al.*, 2000). In this study, the classification was modified slightly (Fig. 3). A normal oscillation pattern consisted of repetitive spike-shaped [Ca^{2+}]i rises. A transient pattern had only a few transient rises [Ca^{2+}]i (1–4/60 min). The no-response pattern did not have any [Ca^{2+}]i rises during the observation period. Atypical

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Table 1 The oocyte activation-inducing ability of mouse sonicated (killed) spermatozoa with increasing time after sonication and storage in THF medium

Time after sonication (exp.)	No. of oocytes injected	No. of oocytes surviving (%)[a]	No. of oocytes arrested at MII (%)[b]	No. of oocytes activated (%)[b]	No. of 2PN + 2PB eggs (%)[b]	No. of other eggs (%)[b]
<1 h (6)	84	60 (71)	0 (0)	60 (100)	60 (100)	0 (0)
5 h (2)	51	36 (71)	1 (2.7)	35 (97)	35 (97)	0 (0)
10 h (2)	60	38 (63)	1 (2.6)	37 (97)	34 (90)	3 (7.9)
20 h (2)	35	26 (74)	5 (19)	18 (69)	18 (69)	3 (12)
30 h (2)	27	23 (85)	6 (26)	15 (65)	14 (61)	3 (13)
48 h (2)	27	24 (89)	12 (50)	11 (46)	11 (46)	1 (4.2)
72 h (2)	28	21 (75)	12 (57)	3 (14)	3 (14)	6 (29)

[a]Oocyte as percentage of injected oocytes.
[b]Oocytes as percentage of surviving oocytes.

Table 2 The Ca oscillation-inducing ability of mouse sonicated (killed) spermatozoa with increasing time after sonication and storage in THF medium

Time after sonication (exp.)	No. of oocytes injected	Normal oscillation pattern (A) eggs (%)	Transient pattern (B) eggs (%)	No response pattern (C) eggs	Atypical pattern eggs
<1 h (4)	14	14 (100)	0 (0)	0	0
1.5 h (1)	8	6 (75)	2 (25)	0	0
3 h (2)	15	5 (33)	10 (67)	0	0
5 h (2)	13	4 (31)	9 (69)	0	0
10 h (4)	35	4 (11)	28 (78)	0	3
20 h (2)	8	0 (0)	6 (100)	0	0
30 h (1)	6	0 (0)	6 (100)	0	0
48 h	19	0 (0)	18 (95)	1	0
72 h	9	0 (0)	7 (78)	2	0

patterns had irregular increases in $[Ca^{2+}]$i that did not fit into any of the above three patterns.

Results

Oocyte activation and $[Ca^{2+}]$i responses following the injection of mouse sonicated spermatozoa

Tables 1 and 2 summarize the activation rates and $[Ca^{2+}]$i responses of mouse oocytes injected with sonicated (dead) mouse spermatozoa, which had various maintenance intervals between sonication and injection. More than 90% of sonicated mouse sperm-injected oocytes were normally activated (2PN) with an approx. 10 h maintenance interval between sonication and ICSI. The rate of normal activations gradually declined when the maintenance interval was extended to longer than 20 h. When the maintenance interval was prolonged to 72 h, the rate of normal activations became extremely poor (14%).

The $[Ca^{2+}]$i responses of the sperm-injected oocytes were more sensitive to incubation period than activation rate. The rates of sonicated mouse sperm-injected oocytes with normal oscillation patterns remained relatively high with maintenance intervals up to 1.5 h, but the rates of injected oocytes with normal oscillation patterns began to decline with intervals longer than 3 h.

Embryo development following injection of mouse sonicated spermatozoa

When mouse spermatozoa were injected into oocytes immediately after sonication and cultured continuously for up to 96 h, 46% of the fertilized oocytes developed into blastocysts. The rate of blastocyst development was 3–35% when the maintenance interval between sonication and ICSI was between 3 to 5 h. However, the rates declined substantially when the maintenance interval was prolonged to longer than 10 h (Table 3).

Injection of human sonicated sperm into mouse oocytes

Tables 4 and 5 summarize the activation and $[Ca^{2+}]$i responses of mouse oocytes injected with sonicated (dead) human spermatozoa after various maintenance intervals between sonication and injection. Ninety-seven per cent of the injected oocytes were normally

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Table 3 Embryo development of mouse eggs injected with mouse sonicated spermatozoa with increasing time after sonication and storage in THF medium

Time after sonication (exp.)	No. of oocytes injected	No. of oocytes surviving (%)[a]	No. of eggs fertilized (%)[b]	No. of eggs developed to (%)[c]					
				2-cell	4-cell	8-cell	Morula	Blastocyst	Fragment/Degraded
<1 h (6)	149	105 (70)	102 (97)	2	3	16	32	47 (46)	0
3 h (4)	110	80 (73)	77 (96)	2	7	11	29	29 (35)	2
5 h (3)	109	75 (69)	74 (97)	0	13	8	29	23 (31)	1
10h (3)	99	67 (68)	61 (91)	2	8	16	27	8 (13)	1
20 h (2)	53	39 (74)	28 (72)	1	13	7	8	1 (3.6)	2

[a]Oocytes as percentage of injected oocytes.
[b]Oocytes as percentage of surviving oocytes.
[c]Oocytes as percentage of fertilized oocytes.
Injected oocytes were cultured in THF medium for 96 h and evaluated for their stage of development.

Table 4 The oocyte activation-inducing ability of human sonicated (killed) spermatozoa with increasing time after sonication and storage in THF medium

Time after sonication (exp.)	No. of oocytes injected	No. of oocytes surviving (%)[a]	No. of oocytes arrested at MII (%)[b]	No. of oocytes activated (%)[b]	No. of 2PN + 2PB eggs (%)[b]	No. of other eggs (%)[b]
<1 h (3)	39	30 (77)	1 (3.3)	29 (97)	29 (97)	0 (0)
1.5 h (1)	30	21 (70)	2 (9.5)	19 (90)	17 (81)	0 (0)
3 h (3)	80	69 (86)	13 (19)	53 (77)	50 (72)	1 (1.4)
6 h (2)	32	27 (84)	12 (44)	15 (56)	15 (56)	0 (0)
12 h (1)	10	7 (70)	4 (57)	3 (43)	3 (43)	0 (0)
20 h (2)	46	43 (93)	33 (77)	8 (24)	8 (24)	2 (4.7)
30 h (2)	42	33 (74)	31 (94)	0 (0)	0 (0)	2 (6.1)

[a]Oocyte as percentage of injected oocytes.
[b]Oocytes as percentage of surviving oocytes.

Table 5 The Ca-oscillation-inducing ability of human sonicated (killed) spermatozoa with increasing time after sonication and storage in THF medium

Time after sonication (exp.)	No. of oocytes examined	Normal oscillation pattern (a) eggs (%)	Transient pattern (b) eggs (%)	No response pattern (c) eggs	Atypical pattern eggs
<1 h (4)	10	9 (90)	0 (0)	1	0
3 h (3)	22	6 (27)	12 (55)	1	1
6 h (3)	21	2 (9.5)	16 (76)	1	2
20 h (2)	16	0 (0)	5 (31)	11	0
30 h (2)	14	0 (0)	4 (29)	10	0

activated (2PN) when the human spermatozoa were injected immediately after sonication. However, the rate of normal activations gradually declined when the maintenance intervals were prolonged to over 1.5 h. Moreover, no oocytes were activated when the maintenance interval was longer than 30 h.

Ninety per cent of the injected oocytes had normal $[Ca^{2+}]$i oscillation patterns when injected with human spermatozoa immediately after sonication. However, when the maintenance interval was increased to 3 h, the rate of oocytes with normal oscillation pattern declined to 27%. No oocytes exhibited a normal $[Ca^{2+}]$i oscillation pattern when the maintenance interval was prolonged to 20 h.

Discussion

In previous reports, we had examined whether the spermatids from some experimental rodents and humans possessed oocyte-activating and Ca^{2+} oscillation-inducing abilities by using a mouse oocyte activation assay method (Yazawa *et al*., 2000, 2001, 2007). These abilities of ROSs and ELSs differed between species. For example, hamster, rabbit and human ROSs could activate mouse oocytes, while mouse and rat ROSs could not. ELS from all of the above species could activate mouse oocytes, but normal $[Ca^{2+}]$i oscillation was induced only by injection of hamster, rabbit and human ELSs. Additionally, we

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confirmed that the timing of the acquisition oocyte activation-inducing and Ca^{2+} oscillation-inducing abilities in spermatogenic cells was dissociated. For example, mouse ELSs could activate oocytes but could not induce Ca^{2+} oscillation. Similar results were obtained from hamster and rabbit ROSs. Based on these findings, we estimated that the sperm-borne oocyte-activating and Ca^{2+} oscillation-inducing factor (SOA-COIF, which is presumably identical to the so-called SOAF or sperm factor) appears (or becomes active) gradually when the spermatogenic cells mature. When these factors (SOAF or sperm factor) become fully active, injected oocytes can be activated and exhibit Ca^{2+} oscillation, while no activation occurs when spermatogenic cells that lack SOA-COIF are injected. When the SOA-COIF becomes intermediately active during spermiogenesis, the injected oocytes are activated without normal Ca^{2+} oscillation (only several transient $[Ca^{2+}]$i rises are observed).

In our experiments, we analysed the activation rates and $[Ca^{2+}]$i responses of mouse oocytes that were injected with sonicated mature spermatozoa to evaluate the biological SOA-COIF activities of dead spermatozoa, after various lengths of time between their death (by sonication) and injection into oocytes.

In the ICSI procedures, it is well known that sperm immobilization (killing) prior to injection is very important for obtaining successful results. Immobilization is commonly achieved by scoring the sperm tail with an injection pipette on the bottom of the dish, applying a Piezo pulse to sperm tail, or by sonication. These procedures disintegrate the sperm plasma membrane and facilitate the immediate mixing of the intracellular components of the sperm (such as the nucleus and the sperm factor) with the oocyte cytoplasm, leading to effective oocyte activation and embryo development (Yanagimachi, 2005). The more extensively the sperm plasma membrane is damaged, the faster oocyte activation is induced (Kasai *et al*., 1999). In clinical ICSI treatments, immotile spermatozoa must be used in the cases of severe asthenozoospermia or necrozoospermia (Dozortsev *et al*., 1995; Nagy *et al*., 1995). Most of the ejaculated immotile spermatozoa were found to be dead (Eliasson, 1977), but it is unclear when the spermatozoa died, i.e. before or after ejaculation. Moreover, pregnancies have not been reported using dead ejaculated spermatozoa. Another important part of ICSI procedure is the timing of the immobilization (Yanagimachi, 2005). Once the sperm plasma membrane is disrupted, irreversible damage to the sperm nucleus occurs within a certain interval. Disintegrations of the chromosomes of the dead spermatozoa (Rybouchkin *et al*., 1997) and the spermatozoa killed by sonication and storage (Tateno *et al*., 2000) was demonstrated by their injection into mouse oocytes. This may be due to the influx of Na^{+}-rich extracellular medium, which is extremely different from the intracellular environment, because the incidence of structural chromosomal aberrations was decreased (delayed) when sonicated spermatozoa were stored in K^{+}-rich NIM medium prior to injection (Tateno *et al*., 2000). Additionally, improved embryo development was seen when dead (killed) sperm or spermatids were stored in NIM medium for certain periods prior to injection (Suzuki *et al*., 1998; Mizuno *et al*., 2002).

Figure 4 shows the activation rates and $[Ca^{2+}]$i responses of mouse oocytes injected with mouse sonicated mature spermatozoa and mouse immature spermatogenic cells (ROSs or ELSs), examined in this study and in our previous studies (Yazawa *et al*., 2000, 2001, 2007). We confirmed in this study that the SOAF (sperm factor) activities of dead spermatozoa gradually disappeared, in accordance with the maintenance interval between sonication and injection. As the incubation time after sonication was increased, normal Ca^{2+} oscillations disappeared first (in the earliest interval assayed, approx. 3 h) and then blastocyst development (approx. 10 h) was gradually obstructed at the earlier interval assayed. In contrast, the oocyte-activating ability was maintained for a relatively long period, but declined after the spermatozoa were incubated for 20 h. Interestingly, when the incubation period for the killed spermatozoa was prolonged from 3 to 10 h, most of the injected oocytes were normally activated (>90%) without normal Ca^{2+} oscillations. This same phenomenon was observed in the maturation process of immature spermatogenic cells (in the case of mouse ELSs) and occurs when the SOAF activity is sufficient for the induction of oocyte activation, but is insufficient to induce Ca^{2+} oscillations. The transient Ca^{2+} response patterns were least affected by increasing the incubation time intervals after sonication, as 78% of the oocytes that were injected with 72 h-incubated sonicated spermatozoa had transient responses, even though their activation rate was decreased to 14%. These results indicated that Ca^{2+} oscillation was the most sensitive indicator for evaluating the SOAF activities of injected spermatozoa or spermatogenic cells. Moreover, although ELS-injected oocytes had only transient Ca^{2+} response patterns with high rates of activation, they maintained a reasonable rate of blastocyst development when compared with controls. However, oocytes injected with 10–20 h-incubated sonicated spermatozoa (that had the same pattern of Ca^{2+} response and activation rate) had disappointing rates of blastocyst development.

Figure 5 shows the activation rates and $[Ca^{2+}]$i responses of mouse oocytes injected with human sonicated mature spermatozoa and human immature spermatogenic cells (ROSs or ELSs). Compared with

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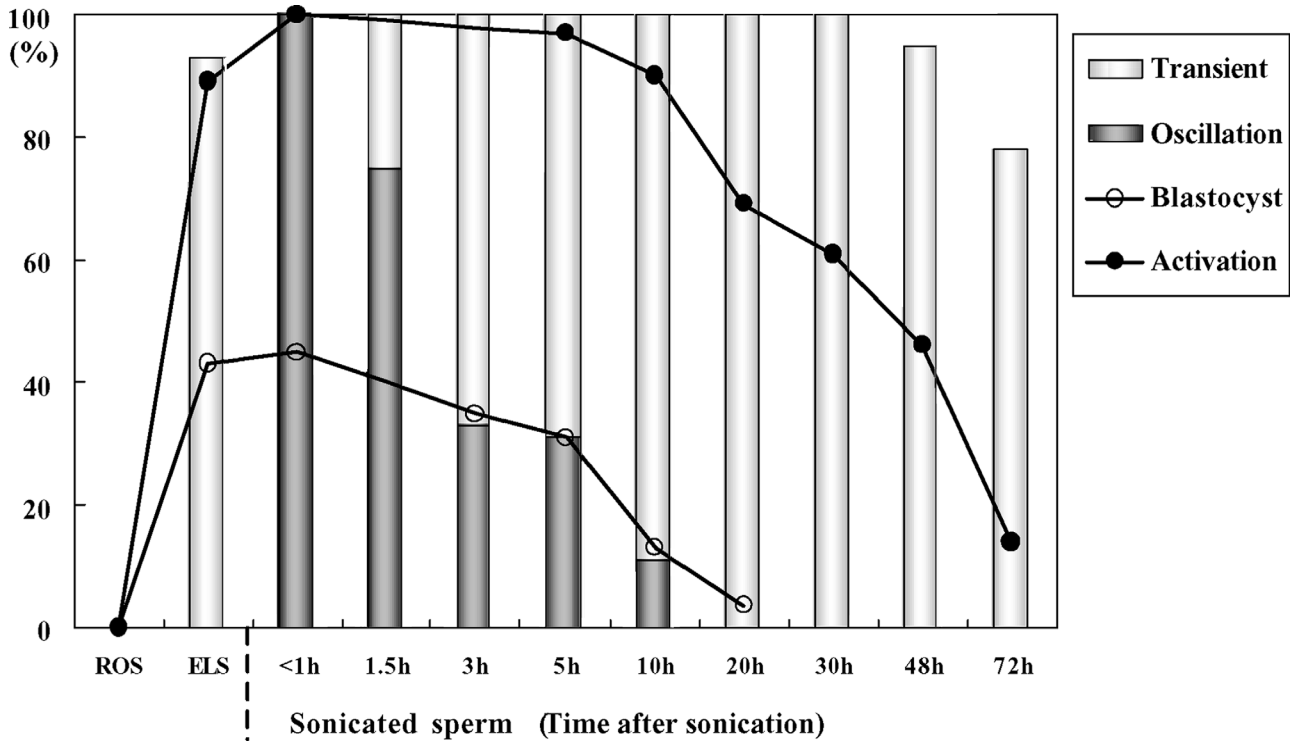

Figure 4 The activation rates and Ca^{2+} responses of mouse oocytes injected with mouse immature spermatogenic cell (ROS, ELS) and mouse sonicated spermatozoa.

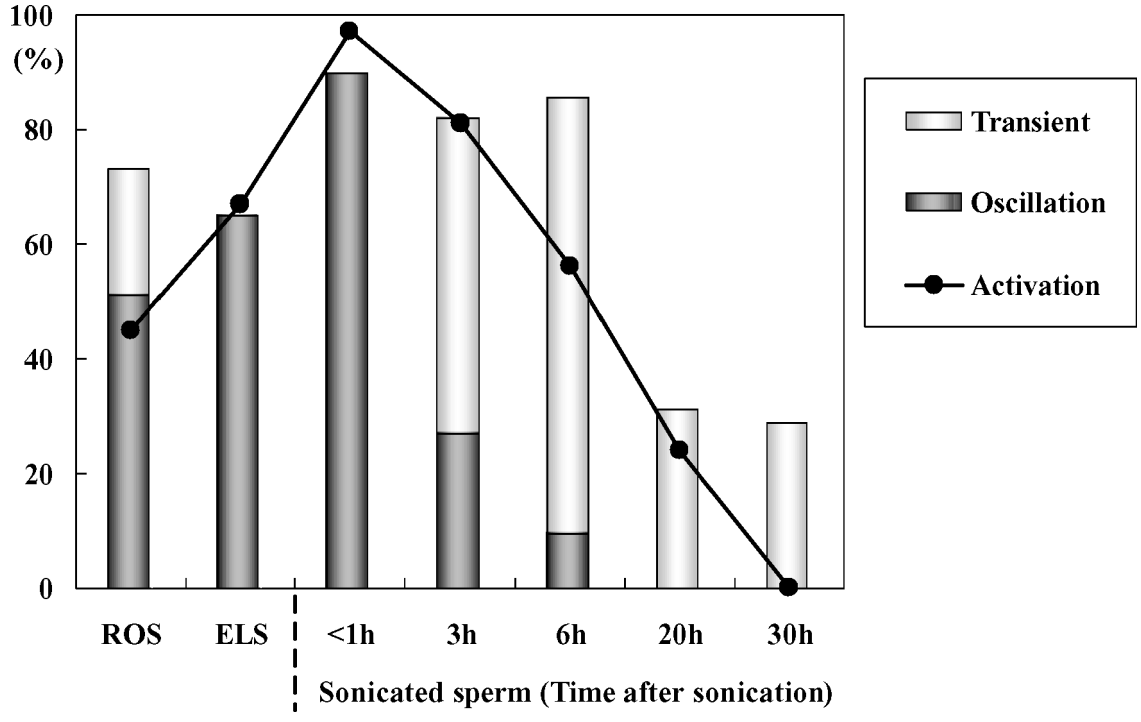

Figure 5 The activation rates and Ca^{2+} responses of mouse oocytes injected with human immature spermatogenic cell (ROS, ELS) and human sonicated spermatozoa.

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mouse sperm or spermatogenic cells, SOAF activity began to appear in the more immature stages of the spermatogenic cells, but began to disappear after shorter incubation intervals prior to sperm injection

In the mouse, although ROSs could not activate oocytes, normal offspring were obtained by ROSI or by using electrofusion, with the induction of oocyte activation by an electric pulse (Ogura *et al*., 1994; Kimura & Yanagimachi, 1995b). Mouse ELS-injected oocytes, which did not exhibit normal [Ca^{2+}]i oscillation patterns, also produced normal offspring (Yazawa *et al*., 2001). Despite the fact that immature spermatogenic cells lack SOAF activity, injection and supplementation of SOAF activities (with electric pulse or Ca^{2+} ionophore) can produce normal development to term, because these cells possess genetically normal nuclei, similar to that for mature spermatozoa. On the other hand, irreversible damage to the sperm nucleus occurs during prolonged incubation of dead spermatozoa and supplementation of SOAF activities by any method may not be enough to rescue their function.

Recently, a sperm-specific zeta isoform of phospholipase C (PLCζ) in mouse and human has been identified and demonstrated as a powerful candidate to be a sperm factor (Cox *et al*., 2002; Saunders *et al*., 2002). Microinjection of PLCζ cRNA triggered Ca^{2+} oscillation in mouse and human oocytes, similar to that observed during fertilization, and evoked embryo development to blastocysts (Saunders *et al*., 2002; Roger *et al*., 2004). Similarly, mouse oocytes injected with anti-PLCζ antibody-treated sperm extract did not exhibit Ca^{2+} responses. The activity of PLCζ is conserved across mammalian and non-mammalian vertebrates (Cox *et al*., 2002; Coward *et al*., 2005). In spermatozoa, PLCζ is located mainly at perinuclear matrix of post-acrosome lesion, where the acrosome-reacted spermatozoa first fuse with the oocyte plasma membrane and enter the oocyte at fertilization (Fujimoto *et al*., 2004). In the activated egg, exogenously expressed PLCζ is distributed throughout the cytoplasm before pronucleus formation and then accumulates in pronuclei after their formation (Larman *et al*., 2004; Yoda *et al*., 2004; Sone *et al*., 2005; Oda, 2006) and sperm factor is distributed in a similar manner (Kono *et al*., 1995; Ogonuki *et al*., 2001). Together, these data indicate that PLCζ is the sperm factor that triggers egg activation and Ca^{2+} oscillations during fertilization. Recently, Yu *et al*., clearly demonstrated the relationship between injected PLCζ levels, the pattern of Ca^{2+} oscillation and embryo development (Yu *et al*., 2007). The more PLCζ protein expressed, the earlier the first Ca^{2+} oscillation occurred and the higher the frequency of transient Ca^{2+} spikes. However, higher levels of PLCζ resulted in the earlier cessation of the Ca^{2+} responses (shorter duration of Ca^{2+} oscillation), arrest of most embryos around the 2-cell stage and failure to develop to blastocysts. This confirmed that, despite the fact that oocyte activation and Ca^{2+} oscillations occurs over a wide range of PLCζ levels, successful development of an embryo into blastocyst could only be achieved by a relatively narrow and appropriate level of PLCζ expression. Additionally, low levels of PLCζ induced high rates of oocyte activation (i.e. induced relatively low frequent Ca^{2+} oscillations), but this did not lead to effective development into blastocysts.

Once the sperm plasma membrane is disrupted with immobilization procedure, irreversible damage of SOAF activity and the sperm nucleus occurs during a certain interval. In these experiments, we confirmed that the normal oscillation pattern disappeared within 3 h of incubation and the Ca^{2+} oscillation is the most sensitive indicator of the SOAF activity of dead spermatozoa. We also reconfirmed that using live spermatozoa and immobilization immediately prior to injection is important for obtaining successful results from the ICSI procedure.

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