Pre-treatment of sperm reduces success of ICSI in the pig

Michiko Nakai, Junya Ito¹, Ken-ichi Sato², Junko Noguchi, Hiroyuki Kaneko, Naomi Kashiwazaki¹ and Kazuhiro Kikuchi

Reproductive Biology Research Unit, Division of Animal Sciences, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan, ¹Laboratory of Animal Reproduction, School of Veterinary Medicine, Azabu University, Sagami-hara, Kanagawa 252-5201, Japan and ²Department of Molecular Biosciences, Faculty of Life Sciences, Kyoto Sangyo University, Kyoto, Kyoto 603-8555, Japan

Correspondence should be addressed to M Nakai; Email: nakai3@affrc.go.jp

Abstract

In pigs, although ICSI is a feasible fertilization technique, its efficiency is low. In general, injected pig sperm are insufficient to induce oocyte activation and embryonic development. Pretreatments for disrupting sperm membranes have been applied to improve the fertility of ICSI oocytes; however, we hypothesize that such pretreatment(s) may reduce the ability of the sperm to induce oocyte activation. We first evaluated the effects of sperm pretreatments (sonication (SO) to isolate the sperm heads from the tails, Triton X-100 (TX), and three cycles of repeated freezing/thawing (3×-FT) for disrupting sperm membranes) on the rate of pronucleus (PN) formation after ICSI. We found that oocytes injected with control (whole) sperm had higher rates of PN formation than those obtained after subjecting the sperm to SO, TX, and 3×-FT. The amounts of phospholipase C ζ (PLC ζ), which is thought to be the oocyte-activating factor in mammalian sperm, in sperm treated by each method was significantly lower than that in whole untreated sperm. Furthermore, using immunofluorescence, it was found that in pig sperm, PLC ζ was localized to both the post-acrosomal region and the tail area. Thus we demonstrated for the first time that sperm pretreatment leads to a reduction of oocyte-activating capacity. Our data also show that in addition to its expected localization to the sperm head, PLC ζ is also localized in the tail of pig sperm, thus raising the possibility that injection of whole sperm may be required to attain successful activation in pigs.

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Introduction

Ovulated mammalian oocytes remain arrested until fertilization at the metaphase of the second meiosis (metaphase-II). In mammals, repetitive rises in the intracellular level of free Ca²⁺, also known as Ca²⁺ oscillations, are induced immediately after sperm–oocyte fusion, which is a prerequisite event for oocyte activation (Miyazaki & Ito 2006). Research has indicated that phospholipase C ζ (PLC ζ) might be the sperm factor that triggers Ca²⁺ responses during fertilization in fish (Ito *et al.* 2008), rodents (Saunders *et al.* 2002, Ito *et al.* 2008), birds (Coward *et al.* 2005, Mizushima *et al.* 2009), primates (Cox *et al.* 2002), and also domestic species (cattle (Ross *et al.* 2008) and pigs (Yoneda *et al.* 2006)). The amplitude and number of rises of Ca²⁺ are suggested to play an important role in the completion of the events of oocyte activation, including the regulation of maternally stored mRNA in the early zygote (Ducibella *et al.* 2002), which might affect gene expression at later stages of preimplantation embryo development (Ozil *et al.* 2006) as well as cell composition in blastocysts (Bos-Mikich *et al.* 1997).

ICSI has been used to generate offspring in infertile human couples and also in experimental and domestic animals (Yanagimachi 2005). In studies using rodent sperm, injection of whole sperm (mouse (Kimura & Yanagimachi 1995, Kuretake *et al.* 1996)) or of a sperm head (mouse (Kuretake *et al.* 1996), rat (Hirabayashi *et al.* 2002, Nakai *et al.* 2005), and hamster (Yamauchi *et al.* 2002)) is sufficient to generate full-term development. These results suggest that in these species, a large part of the activating stimulus resides in the sperm head.

In large animals, the birth of live offspring after ICSI has been reported (pigs (Kolbe & Holtz 2000, Martin 2000, Nakai *et al.* 2003), cattle (Goto *et al.* 1990); sheep (Catt *et al.* 1996), and horses (Cochran *et al.* 1998)), although it is well known that developmental success in these species remains remarkably low after this technique. In natural fertilization, oocytes penetrated by spermatozoa are activated normally (as evidenced by extrusion of the second polar body and formation of the male and female pronuclei), but the efficiency of oocyte activation after ICSI is quite low unless the oocytes further receive artificial stimulation (Fujinami *et al.* 2004,

Table 1 The effects of sperm treatments on pronuclear formation after ICSI.

Sperm treatments ^a	Number of the injected oocytes (N)	Number of pronuclear formed oocytes (% from N)		
		Total	Normal fertilization ^b	Others ^c
Whole	79	43 (54.4±4.5)*	33 (41.8±7.4)*	10 (12.6±6.5)
SO	59	12 (20.3±3.6) [†]	8 (13.5±0.8) [†]	4 (6.8±3.2)
TX	74	7 (9.5±5.8) [†]	4 (5.4±3.8) [†]	3 (4.1±3.9)
3×-FT	64	14 (21.9±5.0) [†]	9 (14.1±5.3) [†]	5 (7.8±3.0)
Sham	69	6 (8.7±2.7) [†]	0 (0) [†]	6 (8.7±2.7)
Handling	82	1 (1.2±0.9) [†]	0 (0) [†]	1 (1.2±0.9)

Data are presented as mean±S.E.M. of more than three separate replicates. Different superscripts within each column (*, †) indicate values that are significantly different ($P<0.05$).

^aFrozen-thawed spermatozoa (whole) were treated with sonication (SO), 0.1% Triton X-100 (TX), or freezing/thawing without cryoprotectant three times (3×-FT). As negative controls, sham and handling groups were also prepared. ^bNormal fertilization is defined with two polar bodies and two pronuclei. ^cOthers are categorized other than normal fertilization.

Nakai *et al.* 2006). Therefore, it is suspected that delivery of the oocyte activation signal by the sperm in large domestic species may be compromised.

Various artificial stimulation protocols, such as electrical stimulation (Probst & Rath 2003, Nakai *et al.* 2006), application of calcium ionophore (Kolbe & Holtz 1999, Probst & Rath 2003) or ionomycin (Galli *et al.* 2003), and combined treatment with ionomycin and 6-dimethylaminopurine (Gou *et al.* 2002, Jimenez-Macedo *et al.* 2005), ethanol (Fujinami *et al.* 2004), or CaCl_2 (Probst & Rath 2003), have been used to improve ICSI in domestic species. Although these protocols improve pronucleus (PN) formation and embryonic development, the subsequent developmental rates do not reach those observed after IVF, at least in pigs (Kikuchi *et al.* 2002, Nakai *et al.* 2003, 2006).

In pig oocytes, failure of male PN formation frequently occurs after ICSI, even though female PN formation seems to occur normally (Kren *et al.* 2003, Lee *et al.* 2003). To improve male PN formation, various treatments of sperm have been applied to sperm such as sonication (SO; Kim *et al.* 1999), incubation with Triton-X 100 (TX; Lee & Yang 2004), exposure to progesterone (Katayama *et al.* 2002), or repetitive freezing/thawing (FT) without cryoprotectants (Katayama *et al.* 2002). However, our studies using some of these procedures have shown that the rates of male PN formation are still remarkably low (Nakai *et al.* 2006). Therefore, we have hypothesized that such treatments may reduce the ability of pig sperm to induce oocyte activation. In this study,

we investigated the effects of several sperm treatments on the concentration and localization of PLC ζ in pig sperm and on the ability of these sperm to induce oocyte activation.

Results

Treatment of sperm before ICSI affects PN formation

The ability of pretreated sperm to induce PN formation after ICSI was examined (Table 1). The percentage of PN formation in the SO (20.3±3.6%), TX (9.5±5.8%), and three cycles of repeated freezing/thawing (3×-FT; 21.9±5.0%) treatment groups was lower ($P<0.05$) than that for oocytes injected with whole sperm (54.4±4.5%) and in fact was as low as that after sham injection (8.7±2.7%) or dummy handling (1.2±0.9%). In addition, the proportion of normally fertilized zygotes among oocytes injected with whole sperm (41.8±7.4%) was significantly ($P<0.05$) higher than those in the other groups (SO (13.5±0.8%), TX (5.4±3.8%), and 3×-FT (14.1±5.3%)).

We next examined whether injection of multiple sonicated sperm heads would be able to induce higher rates of PN formation. When one sperm head was injected, more than one PN formation rate was low (20.4±3.6%; Table 2). However, as the number of sperm heads increased, the rates of oocyte with more than one PN also increased, reaching 78.0±7.5% when three sperm heads were injected.

Table 2 Improvement of oocyte activation by injection of multiple sperm heads.

Group ^a	Number of oocytes	Oocytes with first polar body				Oocytes with two polar bodies				1PN < (%)
		1PN (%)	2PN (%)	3PN (%)	4PN (%)	1PN (%)	2PN (%)	3PN (%)	4PN (%)	
Head-1	59	1 (1.7±1.9)	1 (1.7±1.9)	0 (0)	0 (0)	2 (3.4±1.5)	8 (13.6±0.7)	0 (0)	0 (0)	12 (20.3±3.6)*
Head-2	52	0 (0)	0 (0)	2 (3.8±2.4)	1 (1.9±3.3)	1 (1.9±1.3)	4 (7.7±4)	10 (19.2±3.9)	1 (1.9±2)	19 (36.5±5.2)*
Head-3	50	1 (2.0±3.3)	0 (0)	1 (2.0±3.3)	1 (2.0±3.3)	5 (10.0±5.2)	6 (12.0±3.8)	12 (48±5.8)	13 (26.0±8.4)	39 (78.0±7.5) [†]

Data are presented as mean±S.E.M. of more than three separate experiments. Different superscripts (*, †) indicate values that are significantly different ($P<0.05$).

^aOne (head-1), two (head-2), or three (head-3) sonicated sperm head(s) were injected into oocytes.

Figure 1 Decrease in the quantity of PLC ζ in pig spermatozoa after demembrane treatments. Frozen-thawed spermatozoa were treated with sonication (SO), 0.1% Triton X-100 (TX), or freezing/thawing without cryoprotectant three times (3 $\times$ -FT). Western blot analysis is shown in the upper panels and the mean values of band intensities in the lower panel. Blotting with anti- β actin antibody is also shown as a protein loading control. The concentration of sperm was 1×10^5 cells/lane. *Data are expressed in terms of the fold strength of the PLC ζ level in the whole group and are presented as mean $\pm$ S.E.M. for three separate experiments. Different letters (a, b) indicate values that are significantly different ($P < 0.05$).

PLC ζ concentration and distribution are altered after sperm treatments

We next examined how the aforementioned treatments affected the concentration of PLC ζ . The concentrations of PLC ζ in the SO, TX, and 3 $\times$ -FT groups were markedly reduced in comparison with that in the whole group ($P < 0.05$; Fig. 1), although all the treatments induced the same degree of PLC ζ loss (Fig. 1).

Furthermore, we investigated the localization of PLC ζ in boar sperm before and after each sperm treatment (Fig. 2). A strong immunoreactive signal was observed in the acrosomal and post-acrosomal regions of whole sperm, which were used as positive controls (Fig. 2A, A'). In addition, the tail region, especially around the neck, also showed a faint fluorescent signal (Fig. 2A, A'). To confirm whether the signals were specific to PLC ζ , samples were pretreated with an antigenic peptide. As a result, the signal in the post-acrosomal and tail regions disappeared, but the signal localized to the acrosomal

area did not (Fig. 2B, B'). In the absence of primary antibody, the fluorescence for PLC ζ was not detectable (Fig. 2C, C'). Importantly, all of the aforementioned treatments caused the immunoreactive area around the post-acrosomal region to disappear (TX: Fig. 2D, D', 3 $\times$ -FT: Fig. 2E, E', and SO: Fig. 2F, F').

PLC ζ detection in supernatant after treatment of pig sperm

Given the reduced presence of PLC ζ after the treatments, we speculated that PLC ζ might be released into the surrounding medium. Figure 3 shows that this was indeed the case, as western blot analysis detected a much greater amount of immunoreactive PLC ζ in the supernatant from the SO, TX, and 3 $\times$ -FT groups than in that from whole untreated sperm.

Sperm tail also participates in induction of oocyte activation

Given the presence of PLC ζ in the sperm tail, suggested by our immunofluorescence studies, we evaluated

Figure 2 Immunofluorescence analysis of PLC ζ localization in pig spermatozoa treated with 0.1% TritonX-100 (D, D'), repetitive free-freezing/thawing without cryoprotectant three times (E, E') and sonication (F, F'), or without each treatment respectively (A, A', B, B', C, C'). Labeling of pig sperm with antibody against PLC ζ (green: A, B, C, D, E, F) and combined with PI (red: A', B', C', D', E', F'). Negative controls were incubated in the absence of primary antibody (C, C') or with antigenic peptide (10 ng/ μ l; B, B'). Magnified views of sperm (identified as circles in A, A', B, B') are also shown (a, a', b, b'). Arrows and arrowheads indicate the equatorial and acrosomal regions, respectively. Scale bars: 5 μ m.

Figure 3 Detection of PLC ζ in supernatants of sperm cells subjected to different treatments. The amount of PLC ζ loaded was the PLC ζ released into the surrounding medium from 5×10^5 cells immediately after each treatment. Frozen-thawed spermatozoa (whole) were treated with sonication (SO), 0.1% Triton X-100 (TX) or F/T without cryoprotectant three times (3 $\times$ -FT). *Data are expressed in terms of the fold strength of the PLC ζ level in supernatants from whole sperm and are presented as mean $\pm$ S.E.M. for three separate experiments.

whether injection of sperm tails would be able to induce oocyte activation events (Fig. 4). The following injection groups were prepared: 1) one sperm head (head group), 2) one sperm tail (1-tail group), 3) three sperm tail (3-tail group), 4) one sperm head and one tail (head+tail group), and 5) sham injection (sham group). The rates of metaphase-II-arrested oocytes in the head ($64.4 \pm 7.9\%$), 1-tail ($64.4 \pm 2.7\%$), 3-tail ($51.3 \pm 7.2\%$), and head+tail ($29.6 \pm 5.8\%$) groups were lower than those in the sham group ($89.9 \pm 2.8\%$; $P < 0.05$). In addition, the rate of PN formation in oocytes injected with head+tail ($40.7 \pm 6.2\%$) was higher than that in the other groups ($P < 0.05$). There were no significant differences in the proportions of oocytes showing PN formation among the head ($20.3 \pm 3.6\%$), 1-tail ($3.4 \pm 3.6\%$), 3-tail ($20.5 \pm 2.7\%$), and sham ($8.7 \pm 2.7\%$) groups. However, there found to be a tendency of difference ($P = 0.085$) between 1-tail and 3-tail groups after the analysis used in this study. The proportions of oocytes in the transitional stage in the 1-tail ($32.2 \pm 4.3\%$), 3-tail ($25.6 \pm 5.7\%$) and head+tail ($29.6 \pm 8.2\%$) groups were significantly higher than those in the sham group ($1.4 \pm 1.7\%$; $P < 0.05$). Furthermore, we confirmed the presence of PLC ζ in sperm tails using western blotting (Fig. 5).

Discussion

In all domestic animals tested to date, including pigs, the developmental competence of zygotes generated by the ICSI technique has been low in comparison to those obtained using the same procedure in rodents (Kimura & Yanagimachi 1995, Kuretake *et al.* 1996, Garcia-Rosello *et al.* 2009). We hypothesized that the problem may lie in an insufficient oocyte activation stimulus generated by the injected sperm. Initially, therefore, we investigated the membrane-damaging effects of certain treatments on the ability of sperm to induce PN formation after ICSI; some of these treatments are applied to assist decondensation of the sperm head (Lee & Yang 2004). We then

also noted that the rates of PN formation in oocytes injected with treated sperm were lower than those obtained after injection of whole sperm. Similar results have already been reported following treatment of sperm by FT without cryoprotectants or dithiothreitol, although the underlying mechanisms were not investigated (Yong *et al.* 2005). In addition, we found that injection of multiple sperm heads improved the rates of PN formation (Table 2). Together, we considered these results to indicate that the PLC ζ concentration/activity in sperm may be decreased by sperm treatments that are commonly used prior to ICSI in large domestic species.

To explore the possibility that PLC ζ had been lost from the sperm, we performed western blotting on supernatants of the sperm preparations and immunoreactive PLC ζ was indeed detected in these samples (Fig. 3). This was not unexpected, as characterizations of the sperm factor have been reported previously using samples of supernatants obtained from sonicated pig sperm (Kuretake *et al.* 1996). Furthermore, we observed a reduction of immunofluorescent signals for PLC ζ in sperm that had undergone the aforementioned treatments (Fig. 2). These findings demonstrated that treatment of pig sperm leads to a reduced ability to induce oocyte activation and that this should be taken into consideration when preparing samples for ICSI (Table 1). Remarkably, in rats, the presence of PLC ζ in the head region has been shown to be maintained even after SO and demembration treatments (Seita *et al.* 2009). Consistent with these findings, the ability of rat sperm to induce oocyte activation and full-term development following SO and demembration was not diminished by some of the aforementioned

Figure 4 Participation of sperm tails in the induction of oocyte activation. Oocytes were injected with one sonicated sperm head (head), one sonicated sperm tail (1-tail), three sonicated sperm tail (3-tail), or a head and a tail (head+tail). Sham-injected oocytes were prepared as a negative control. At 10 h, oocytes were classified into the following three categories: 1) M-II, metaphase-II stage; 2) transitional period: total of anaphase-II, telophase-II, and metaphase-III stages; and 3) 1PN $\leq$: formation of more than one pronucleus. Data are presented as mean $\pm$ S.E.M. for more than three separate experiments. Different superscripts (a, b, c) within each category indicate values that are significantly different ($P < 0.05$).

Figure 5 Sperm tails also contain PLC ζ . A band of ~ 72 kDa was detected in the sperm tail sample. The concentration of sperm tails was 4×10^6 cells/lane.

treatments (Seita *et al.* 2009). Similarly, sonicated mouse sperm heads still seem to retain their ability to induce oocyte activation (Yazawa *et al.* 2009). Therefore it seems that the sperm of various mammalian species differ in their PLC ζ content and/or storage site.

It has been reported that PLC ζ is detectable in the post-acrosomal region (Yoon & Fissore 2007) and in the acrosomal and post-acrosomal regions (Young *et al.* 2009) of mouse sperm, in the acrosomal region of rat sperm (Seita *et al.* 2009), in the equatorial (Yoon *et al.* 2008), or equatorial and post-acrosomal regions (Heytens *et al.* 2009) of human sperm, and in the equatorial region of bull sperm (Yoon & Fissore 2007). In mice, it is known that the components of the tail are not required for induction of oocyte activation (Kuretake *et al.* 1996). However, subsequent to the submission of this manuscript, the existence of PLC ζ in equine sperm tail was suggested (Bedford-Guaus *et al.* 2011). In this study, we also suggest that PLC ζ was detectable in both the post-acrosomal region and the tail region of pig sperm (Figs 2 and 5), suggesting that pig sperm tails may be capable of triggering oocyte activation and embryo development. Our results showed that a higher proportion of oocytes injected with sperm tails resumed the second meiosis than sham-injected oocytes, although most of them failed to reach the PN stage. These results suggest that pig sperm tails possess PLC ζ activity but that its concentration and/or activity is insufficient for PN formation. Indeed, combined injection of a head and a tail greatly improved the rates of metaphase-II exit and PN formation (Fig. 4). Interestingly, despite the presence

of PLC ζ in both the head and the tail, injection of whole sperm induced normal fertilization in only 40% of the oocytes (Table 1) and similar results have been published by others (Katayama *et al.* 2005). In contrast, following IVF, nearly 100% of sperm-penetrated oocytes display activation events resulting in PN formation (Kikuchi *et al.* 1999). This suggests that ICSI in these species does not quite replicate the events of fertilization. There are two possible explanations for why this might be the case. First, it is well documented that high rates of polyspermy occur following IVF in the pig (Nagai 1994), which might explain the higher activation rates following this procedure. Alternatively, as we found that an appreciable proportion of pig sperm stained for PLC ζ did not show any immunoreactivity (data not shown), some of the sperm used for ICSI may lack the enzyme and therefore fail to robustly activate oocytes.

Taken together, our present findings show that treatments used to aid sperm head decondensation before ICSI in the pig also cause loss of PLC ζ , resulting in a weakened sperm activation signal. We have also showed for the first time that PLC ζ is localized in the post-acrosomal and tail regions of pig sperm and that sperm tails contribute to oocyte activation, although PLC ζ in the tail region does not represent the main activation stimulus. It is hoped that these findings, and a better understanding of the early stages of oocyte activation, will improve the efficiency of ICSI in the pig and other large domestic species.

Materials and Methods

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Japan.

Sperm collection, treatment, and preparation

Ejaculated semen was collected from boars of the Landrace breed, and cryopreservation of the sperm was carried out as described previously (Kikuchi *et al.* 1998, Ikeda *et al.* 2002). Cryopreserved spermatozoa were thawed in TCM 199 (with Earl's salts; Gibco Life Technologies) adjusted to pH 7.8 and centrifuged for 2 min at 600 *g*. The sperm pellet was resuspended in pig-fertilization medium (Pig-FM; Suzuki *et al.* 2002) supplemented with 5 mg/ml BSA (Fraction V; Sigma–Aldrich Corp.). Sperm (whole group) was then subjected to the following three treatments: 1) SO at room temperature for 10 s using an ultrasonic cleaner (UT-305; Sharp Manufacturing Systems Corporation, Osaka, Japan) to isolate the sperm heads from the tails (SO group), 2) repetitive FT without cryoprotectant three times at -196°C and 37°C respectively ($3 \times$ -FT group), and 3) incubation in Pig-FM supplemented with 0.1% TX (Sigma) at 37°C for 1 min (TX group). The treated sperm were centrifuged for 2 min at 600 *g* and the sperm pellets were resuspended in Pig-FM for injection. Injection was conducted within 30 min after each sperm treatment. For

western blotting analysis, the treated spermatozoa were centrifuged for 5 min at 1400 *g* in PBS (Nissui Pharmaceutical Co., Tokyo, Japan) three times. The pellets were then diluted in Laemmli sample buffer (Bio-Rad Laboratories) and stored at -20°C .

Preparation of supernatants from treated sperm

For collection of supernatants, all sperm treatments were conducted in PBS containing protease inhibitors such as 0.2 mM phenylmethylsulfonylfluoride (Sigma), 10 $\mu\text{g}/\text{ml}$ pepstatin A (Wako Pure Chemical Industries, Osaka, Japan), 10 $\mu\text{g}/\text{ml}$ leupeptin (Wako), 1 mM ethylenediamine-*N,N,N',N'*-tetraacetic acid (Dojindo Laboratories, Kumamoto, Japan), 10 mM β -glycerophosphate (Sigma), and 1 mM dithiothreitol (Wako). Immediately after each treatment, the sperm suspension was centrifuged (5 min, 1400 *g*) and cells and other components (supernatant) were separated. The supernatant was diluted in sample buffer and stored at -20°C until western blotting analysis.

Collection of sperm tails

Sperm tails were collected by density-gradient centrifugation as follows. Spermatozoa were washed three times in PBS. The pellet was then resuspended in PBS including protease inhibitors and sonicated for 10 s. The sonicated sample was layered on a 150 μl discontinuous gradient (consisting of 500 μl layers of 40, 60, and 80% solutions respectively) of Percoll (GE Healthcare, Buckinghamshire, UK) in PBS supplemented with protease inhibitors, then subjected to centrifugation at 3200 *g* for 20 min at 4°C . After centrifugation, sperm tails had gathered in the 60% solution layer. To purify the sperm tails, the collected 60% solution was layered on the same type of Percoll gradient and centrifuged again. The tail fraction collected from the 60% solution layer after the second centrifugation was mixed with PBS supplemented with protease inhibitors and centrifuged at 9600 *g* for 10 min at 4°C . The pellets were finally resuspended in sample buffer and stored at -20°C until western blotting analysis.

Oocyte collection and in vitro maturation

Ovaries were obtained from prepubertal cross-bred gilts (Landrace, Large White, and Duroc breeds) at a local slaughterhouse and transported to the laboratory at 35°C . Cumulus-oocyte complexes (COCs) were collected from follicles 2–6 mm in diameter in TCM 199 (with Hanks' salts; Sigma) supplemented with 10% (v/v) fetal bovine serum (Gibco, Invitrogen), 20 mM Hepes (Dojindo Laboratories), 100 IU/ml penicillin G potassium (Sigma), and 0.1 mg/ml streptomycin sulfate (Sigma). Maturation culture was performed as reported previously (Kikuchi *et al.* 2002). In brief, about 40 COCs were cultured in 4-well dishes (Nunc Multidishes; Thermo Fisher Scientific, Inc., Roskilde, Denmark) for 20–22 h in 500 μl maturation medium, a modified North Carolina State University (NCSU)-37 solution (Petters & Wells 1993) containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine (Sigma), 50 μM β -mercaptoethanol (Sigma), 1 mM

dibutyl cAMP (dbcAMP; Sigma), 10 IU/ml eCG (PMS 1000 Tani NZ; Nihon Zenyaku Kogyo, Koriyama, Japan), and 10 IU/ml hCG (Puberogen 1500 U; Sankyo, Tokyo, Japan). They were subsequently cultured for 24 h in maturation medium without dbcAMP and hormones. Maturation culture was carried out at 39°C in an atmosphere of CO_2 , O_2 , and N_2 adjusted to 5, 5, and 90% respectively. After maturation culture, cumulus cells were removed from the oocytes by treatment with 150 IU/ml hyaluronidase (Sigma) and gentle pipetting. Denuded oocytes with the first polar body were harvested under a stereomicroscope and used as *in vitro* matured oocytes.

Procedure of sperm injection

In total, two solutions were prepared for ICSI: 1) for oocytes, a modified NCSU-37 solution without glucose but supplemented with 0.17 mM sodium pyruvate, 2.73 mM sodium lactate, 4 mg/ml BSA, 50 μM β -mercaptoethanol (IVC-PyrLac; Kikuchi *et al.* 2002), and 20 mM Hepes, with the osmolality adjusted to 285 mOsm/kg (IVC-PyrLac-Hepes; Nakai *et al.* 2003), and 2) for sperm, IVC-PyrLac-Hepes supplemented with 4% (w/v) polyvinyl pyrrolidone (molecular weight 3 60 000; Sigma; IVC-PyrLac-Hepes-PVP). Spermatozoa were injected as described previously (Nakai *et al.* 2003). About 20 oocytes were transferred to a 20 μl drop of IVC-PyrLac-Hepes. The solution containing the mature oocytes was placed on the cover of a plastic dish (Falcon 35-1005; Becton Dickinson and Company, Franklin Lakes, NJ, USA). A small volume (0.5 μl) of the sperm suspension was transferred to a 2 μl drop of IVC-PyrLac-Hepes-PVP, which was prepared close to the drops used for the oocytes. All drops were covered with paraffin oil (Paraffin Liquid; Nakarai Tesque, Inc., Kyoto, Japan). The spermatozoa were injected into the ooplasm by using a Piezo-actuated micromanipulator (PMAS-CT150; Prime Tech Ltd, Tsuchiura, Japan). Sperm-injected oocytes were then cultured in IVC-PyrLac at 38.5°C under 5% O_2 .

Assessment of oocyte activation

Injected oocytes were cultured for 10 h and then mounted on glass slides. The mounted oocytes were fixed in 25% (v/v) acetic acid in ethanol and stained with 1% (w/v) orcein in 45% (v/v) acetic acid. The nuclear status of the oocytes was observed using a phase-contrast microscope. We classified the states of the oocytes as follows: 1) 'normal fertilization' characterized by the presence of two PN's (male and female) and two polar bodies; 2) 'other PN', including oocytes that had formed PN other than normal fertilized oocytes; 3) 'PN formation', including all oocytes with one or more PN(s), i.e. the total in categories 1 and 2; 4) 'transitional stage', in which oocytes had resumed meiosis but before any PN formation, i.e. total in anaphase-II, telophase-II, and metaphase-III (an abnormal stage in which chromosomes remain condensed even after telophase-II due to insufficient ooplasmic activation); and 5) 'metaphase-II', in which oocytes were arrested at the metaphase-II stage. We considered categories (1–4) to be oocytes exhibiting activation phenomena. The assay was replicated at least three times using more than 50 oocytes per each injection group.

Western blotting

Western blotting was carried out as described previously (Ito *et al.* 2010) with some modifications. Porcine PLC ζ (pPLC ζ) was detected using anti-PLC ζ rabbit serum generated against a 19-mer sequence (MENKWFLSMVRDDFKGGKI) at the N-terminus of pPLC ζ (accession no. BAC78817) as described previously (Kurokawa *et al.* 2005). After denaturation by boiling at 99.5 °C for 3 min, samples were separated by SDS-PAGE on 12.5% polyacrylamide gel and then transferred onto a PVDF membrane (GE Healthcare). The membrane was blocked using blocking buffer (5% (w/v) BSA in PBS) supplemented with 0.1% Tween 20 (T-PBS), then incubated with anti-PLC ζ antibody (1:5000) overnight at 4 °C in T-PBS. After three washes in T-PBS, the membranes were treated with HRP-labeled anti-rabbit IgG (1:5000, Cell Signaling Technology, Danvers, MA, USA) in blocking buffer for 1.5 h at room temperature. After one wash for 15 min and five washes for 5 min each with T-PBS, peroxidase activity was visualized using the ECL Plus western blotting detection system (GE Healthcare) in accordance with the manufacturer's instructions. The intensity of the bands was analyzed using ImageJ software (Ver. 1.41, National Institutes of Health, Bethesda, MD, USA).

Immunofluorescence

Immunofluorescence was performed as reported previously (Seita *et al.* 2009). Immediately after each treatment, sperm was centrifuged (600 g, 2 min) and the pellet was resuspended and incubated with 3.7% paraformaldehyde in PBS for 30 min at 4 °C. For permeabilization, 0.1% (v/v) TX-PBS was added to the samples and incubated for 10 min at room temperature. The sperm suspension was then spotted as 20 μ l drops onto glass slides and allowed to attach to the slides for 20 min at 37 °C. The slides were blocked in 5% normal goat serum (NGS, Cedarlane Laboratories Ltd, Hornby, ON, Canada) in PBS for 3 h at 4 °C and then incubated overnight at 4 °C with anti-pPLC ζ (1:200) in 5% NGS. Washes were performed with T-PBS, followed by 1 h incubation at room temperature with Alexa Fluor 488-labeled goat anti-rabbit antibody (1:200; Molecular Probes, Inc., Eugene, OR, USA) as the secondary antibody. After several washings in T-PBS, samples were counterstained with propidium iodide (Molecular Probes). Fluorescence images were obtained using a fluorescence microscope (IX-71; Olympus, Tokyo, Japan). As a negative control, sperm were immunostained using the protocol described above, but without the primary antibody. We also confirmed the specificity of pig PLC ζ using an antigenic peptide for PLC ζ 10 ng/ μ l.

Statistical analysis

Statistical analysis of the data from more than three replicates was carried out by ANOVA and Tukey's multiple range tests (Statistical Analysis System (SAS, ver. 9.2; SAS Institute, Inc., Cary, NC, USA). All percentage data were subjected to arcsine transformation (Snedecor & Cochran 1989) before statistical analysis. Differences were considered significant at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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