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—Original Article—

Lack of calcium oscillation causes failure of oocyte activation after intracytoplasmic sperm injection in pigs

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Abstract. In pigs, the efficiency of embryo production after intracytoplasmic sperm injection (ICSI) is still low because of frequent failure of normal fertilization, which involves formation of two polar bodies and two pronuclei. To clarify the reasons for this, we hypothesized that ICSI does not properly trigger sperm-induced fertilization events, especially intracellular Ca^{2+} signaling, also known as Ca^{2+} oscillation. We also suspected that the use of *in vitro*-matured oocytes might negatively affect fertilization events and embryonic development of sperm-injected oocytes. Therefore, we compared the patterns of Ca^{2+} oscillation, the efficiency of oocyte activation and normal fertilization, and embryo development to the blastocyst stage among *in vivo*- or *in vitro*-matured oocytes after ICSI or *in vitro* fertilization (IVF). Unexpectedly, we found that the pattern of Ca^{2+} oscillation, such as the frequency and amplitude of Ca^{2+} rises, in oocytes after ICSI was similar to that in oocytes after IVF, irrespective of the oocyte source. However, half of the oocytes failed to become activated after ICSI and showed no Ca^{2+} oscillation. Moreover, the embryonic development of normal fertilized oocytes was reduced when *in vitro*-matured oocytes were used, irrespective of the fertilization method employed. These findings suggest that low embryo production efficiency after ICSI is attributable mainly to poor developmental ability of *in vitro*-matured oocytes and a lack of Ca^{2+} oscillation, rather than the pattern of oscillation.

Key words: Ca^{2+} oscillation, Fertilization, Intracytoplasmic sperm injection, Phospholipase C-ζ, Pig

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Ovulated mammalian oocytes remain arrested at metaphase of the second meiosis (M-II) until fertilization. Fertilizing sperm evoke repetitive rises in the intracellular level of free Ca^{2+}, known as Ca^{2+} oscillation, immediately after sperm-oocyte fusion [1]. Previous studies have indicated that sperm-specific phospholipase C-ζ (PLCζ) is delivered from the fertilizing sperm into the ooplasm triggering Ca^{2+} oscillation in mouse [2, 3], rat [3], human [3, 4], cynomolgus monkey [4], bovine [5, 6], pig [7, 8], equine [9, 10], medaka (fish) [11], chicken [12], and quail [13]. PLCζ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into 1,4,5-trisphosphate (IP_3) and diacylglycerol. Binding of IP_3 to its receptors (IP_3Rs) on the endoplasmic reticulum (ER) membrane elicits the release of Ca^{2+} from ER, resulting in Ca^{2+} oscillation [14].

Intracytoplasmic sperm injection (ICSI) is a useful tool for producing live offspring from immotile sperm, and for preventing polyspermy, which frequently occurs with *in vitro* fertilization (IVF) in pigs. However, the efficiency of *in vitro* embryo production by ICSI and the quality of the embryos are inferior compared to IVF. It has also been reported that the pattern of Ca^{2+} oscillation in human oocytes after ICSI differs from that in oocytes penetrated with sperm [15]. The pattern of Ca^{2+} oscillation is suggested to play an important role in the resumption of meiosis, recruitment of maternal mRNAs, formation of pronucleus (PN), expression of genes, and development to term [16–18].

Despite the use of *in vitro*-matured pig oocytes in a wide range of studies, their developmental capacity is lower than that of *in vivo*-matured oocytes [19]. Furthermore, *in vitro*-matured oocytes from humans are deficient in Ca^{2+} release in response to IP_3 due to their inability to synthesize the IP_3 receptor protein [20] compared to *in vivo*-matured oocytes.

Therefore, we have hypothesized that the patterns of Ca^{2+} oscillation in *in vitro*-matured oocytes and sperm-injected oocytes are different from that in *in vivo*-matured oocytes after IVF. Such different patterns bring about failure of oocyte activation and normal fertilization, which involves the formation of two polar bodies and two PNs, and a reduction of embryonic developmental ability. To our knowledge, no previously reported study has compared the pattern of Ca^{2+} oscillation in sperm-penetrated oocytes with that in oocytes after

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ICSI in pigs. Herein, we investigated the pattern of Ca^{2+} oscillation, such as the number, amplitude, and interval of Ca^{2+} rises, and *in vitro* developmental capacity after ICSI or IVF using *in vivo*- or *in vitro*-matured pig oocytes.

Materials and Methods

Protocols for the use of animals were approved by the Animal Care Committee of the Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Japan. All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Preparation of in vivo-matured oocytes

Prepubertal (< 6 months of age) crossbred gilts (Landrace, Large White, and Duroc breeds) received 1500 IU of equine chorionic gonadotropin (eCG: PMS A for Animal; ZENOAQ, Fukushima, Japan) and, 72 h later, 750 IU of human chorionic gonadotropin (hCG: Puberogen; ZENOAQ). At 44 h after administration of hCG, the gilts were euthanatized and their ovaries, oviducts, and uteri were collected. Cumulus-oocyte complexes (COCs) were collected by oviduct perfusion and washed with phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mg/ml bovine serum albumin (BSA; Fraction V) and 150 IU/ml hyaluronidase. Denuded oocytes with the first polar body were harvested under a stereomicroscope and used as *in vivo*-matured oocytes.

Preparation of in vitro-matured oocytes

Ovaries from prepubertal crossbred gilts (Landrace, Large White, and Duroc breeds) were obtained at a local slaughterhouse and transported to the laboratory at 35°C. COCs were collected from follicles 2–6 mm in diameter in glucose-free, HEPES-buffered Tyrode medium [21]. Maturation culture was performed as reported previously [22]. In brief, COCs were cultured in six-well dishes (Research Institute for Functional Peptides, Yamagata, Japan) for 20–22 h in 100 µl of maturation medium, a modified North Carolina State University (NCSU)-37 solution [23] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 µM β-mercaptoethanol, 1 mM dibutyl cAMP (dbcAMP), 10 IU/ml eCG, and 10 IU/ml hCG. 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 (5% CO_2 and 5% O_2). After maturation culture, cumulus cells were removed from the oocytes by treatment with 150 IU/ml hyaluronidase and gentle pipetting. Denuded oocytes with a first polar body were harvested under a stereomicroscope and used as *in vitro*-matured oocytes.

Preparation of sperm

Epididymal spermatozoa were collected from a Landrace boar and cryopreserved [24, 25]. The spermatozoa were thawed in Medium 199 (with Earle's salts; Thermo Fisher Scientific, Waltham, MA, USA) adjusted to pH 7.8 and centrifuged at 600 × *g* for 2 min. For IVF, the sperm pellet was resuspended in Medium 199 (pH 7.8), preincubated at 38°C for 15 min in Medium199 (pH 7.8), and used for IVF. For ICSI, the sperm pellet was resuspended in PBS supplemented with 5 mg/ml BSA (PBS-BSA) and maintained at room temperature (25°C) until ICSI.

Sperm injection procedure

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 (Kanto Chemical, Tokyo, Japan), 4 mg/ml BSA, 50 µM β-mercaptoethanol (IVC-PyrLac [22]) and 20 mM HEPES (Dojindo, Kumamoto, Japan), with the osmolality adjusted to 285 mOsm/kg (IVC-PyrLac-HEPES [26]); (2) for sperm: IVC-PyrLac-HEPES supplemented with 4% (w/v) polyvinyl pyrrolidone (PVP360) (IVC-PyrLac-HEPES-PVP). Spermatozoa were injected as described previously [26]. 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 then transferred to a 2-µl drop of IVC-PyrLac-HEPES-PVP, which had been placed close to the drops used for the oocytes. All drops were covered with paraffin oil (Paraffin Liquid; Nacalai Tesque, Kyoto, Japan). The spermatozoa were immobilized and injected into the ooplasm using a piezo-actuated micromanipulator (PMAS-CT150; Prime Tech, Tsuchiura, Japan). Sperm-injected oocytes were then cultured in IVC-PyrLac at 38.5°C, 5% CO_2, and 5% O_2.

In vitro fertilization (IVF) procedure

IVF was carried out according to the method described by Kikuchi *et al*. [22]. The oocytes were washed three times in pig fertilization medium (Pig-FM [27]) and then placed in individual 80-µl drops of the same medium that had been covered with warm paraffin oil. Next, 10 µl of preincubation medium containing sperm was added to each fertilization drop to give a final concentration of 1×10^5 sperm/ml and then co-incubated for 3 h at 39°C under 5% CO_2 and 5% O_2.

In vitro culture (IVC)

At 10 h after ICSI or insemination, oocytes were placed in 700 µl of IVC-PyrLac-HEPES and centrifuged at 10,000 × *g* at 37°C for 20 min in a microcentrifuge [28]. The centrifuged oocytes were examined for their content of PN and polar bodies under an inverted microscope. Normal fertilized oocytes that had two polar bodies and two PNs were cultured for 6 days.

Two types of IVC medium were prepared [22]. The first was IVC-PyrLac. The second contained 5.55 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), as originally reported in the NCSU-37 medium, and supplemented with 4 mg/ml BSA and 50 mM β-mercaptoethanol (IVC-Glu). For the first 2 days, IVC-PyrLac was used. The medium was changed once, to IVC-Glu, on the second day and this medium was used for subsequent culture for 4 days. The IVC was carried out at 38.5°C, 5% CO_2, and 5% O_2.

Assessment of oocyte activation, normal fertilization, and embryonic development

The oocytes and cultured embryos were mounted on glass slides and fixed in 25% (v/v) acetic acid in ethanol, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and observed under a phase-contrast microscope. The nuclear status of the oocytes was observed at 10 h

after injection or insemination and was categorized into three states: (1) metaphase-II: M-II; (2) transitional period, in which oocytes had resumed meiosis but before any PN formation, i.e., all in anaphase-II, telophase-II, or metaphase-III; and (3) formation of more than 1 PN (1 PN ≤) [29]. We defined normal fertilization as a zygote with two polar bodies and two PNs. The rate of blastocyst formation and the mean number of cells per blastocyst were also examined on Day 6 (the day of injection or insemination was defined as Day 0).

Measurement of intracellular calcium

After sperm injection or insemination, each oocyte was loaded with 50 μg Fura-PE3 (Santa Cruz Biotechnology, Dallas, Texas, USA) supplemented with 0.02% Pluronic F-127 (Thermo Fisher Scientific) at 38°C for 30 min. The Fura-PE3 prelabeled oocytes were monitored in 50-μl drops of PyrLac-HEPES without BSA on a thin glass coverslip (Electron Microscopy Sciences, Hatfield, PA, USA) fitted into a stainless steel well, covered with paraffin oil. The Ca^{2+} imaging was performed using an inverted microscope and AQUACOSMOS (Hamamatsu Photonics, Hamamatsu, Japan). Measurements were taken every minute and are reported as the ratios of 340/380 nm fluorescence. The amplitude of Ca^{2+} rise was calculated by subtracting the fluorescence ratio before Ca^{2+} rise from that in the peak of Ca^{2+} rise. After measurement, PN formation in each oocyte was observed individually by aceto-orcein staining, and the Ca^{2+} response in normal fertilized oocytes was determined.

Statistical analysis

All percentage data were subjected to arcsine transformation [30] before statistical analysis. The data for activated oocytes, blastocyst formation, and number of cells per blastocyst were analyzed by two-way analysis of variance (ANOVA) using the Statcel 2 program (OMS Publishing, Saitama, Japan). Furthermore, the data for calcium measurement were analyzed by ANOVA and Tukey's multiple range test. Differences were considered significant at $P < 0.05$. All data were expressed as mean ± SEM. Experiments were repeated more than three times.

Results

Failure of oocyte activation after ICSI

The nuclear status of *in vivo*- and *in vitro*-matured oocytes after IVF and ICSI (vivo-ICSI, vitro-ICSI, vivo-IVF, and vitro-IVF, respectively) was investigated (Fig. 1, Table 1). Regardless of their source, more than half of the oocytes after ICSI remained at the M-II stage (vivo-ICSI and vitro-ICSI, 51.7 ± 3.8% and 56.4 ± 5.2%, respectively). In contrast, all oocytes in the IVF groups resumed the second meiosis. Statistical analysis revealed that the method of fertilization significantly affected the resumption of second meiosis ($P < 0.001$). The proportions of oocytes showing formation of more than 1 PN after IVF (vivo-IVF and vitro-IVF, 100% and 88.2 ± 6.4%, respectively) were higher than those after ICSI (vivo-ICSI and vitro-ICSI, 44.8 ± 6.5% and 28.2 ± 5.1%, respectively). The differences in the two effects, oocyte source and method of fertilization, were also statistically significant ($P < 0.01$); the F-value for the fertilization method was particularly high, meaning that it had a more important effect on PN formation. In addition, *in vivo*-matured oocytes supported PN formation to a greater extent than *in vitro*-matured oocytes. There were no significant inter-group differences in the proportions of oocytes in the transitional period.

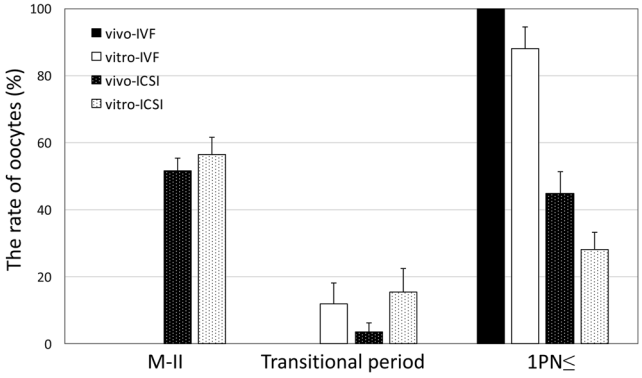

Fig. 1. The induction of oocyte activation in *in vivo*- or *in vitro*-matured oocytes after IVF or ICSI. At 10 h, oocytes were classified into the following three categories: (1) M-II, metaphase-II; (2) transitional period: total of anaphase-II, telophase-II, and metaphase-III stages; and (3) 1PN ≤ : formation of more than one pronucleus. Data are presented as mean ± SEM for more than three separate experiments.

Table 1. Two-way ANOVA of the status of *in vivo*- or *in vitro*- matured oocyte nuclei after IVF or ICSI

Source	DF [a]	F-value		
		M-II [d]	Transitional period [e]	PN [f]
Source of oocytes [b]	1	4.12337	3.85167	16.4186 **
Fertilization methods [c]	1	113.701 ***	0.63273	110.623 ***

[a] degree of freedom. [b] *in vivo*- or *in vitro*-matured oocytes. [c] IVF or ICSI. [d] M-II: metaphase-II. [e] transitional period: total of anaphase-II, telophase-II, and metaphase-III stages. [f] PN: formation of more than one pronucleus. ** $P < 0.01$, *** $P < 0.001$.

Similarity of Ca^{2+} oscillation patterns in oocytes after ICSI and IVF

The frequency, amplitude and interval of Ca^{2+} oscillations in normal fertilized oocytes after IVF or ICSI were investigated (Table 2). The typical patterns of Ca^{2+} oscillation in each treated oocyte are shown in Fig. 2. We consider that the pattern of Ca^{2+} oscillation in *in vivo*-matured oocytes, penetrated and activated by sperm, is closest to that in the *in vivo* "physiologically fertilized" oocytes. Therefore, the pattern of Ca^{2+} oscillation such as number, interval, and amplitude of Ca^{2+} rises in the vivo-IVF group was set as the basis for comparison among the patterns of Ca^{2+} oscillation in each group. The pattern from the vivo-IVF group was characterized as a low frequency of oscillation (1–2 times/4 h), a long interval between each Ca^{2+} rise (156 ± 19.7 min), and a rise in Ca^{2+} level of 0.97 ± 0.07. It seems that this Ca^{2+} oscillation pattern is typical in pigs. The vitro-IVF and vivo-ICSI groups also showed similar frequencies, amplitudes, and intervals of Ca^{2+} oscillation compared with the

Table 2. Ca^{2+} responses in normal fertilized* pig oocytes after IVF or ICSI

Source of oocytes	Fertilization methods	Total no. of oocytes	No. of 2PB2PN	No. of oocytes with Ca^{2+} signal	Ca^{2+} rise number	No. of oocytes (%) **	Amplitude of Ca^{2+} rise **	Ca^{2+} rise interval (min) **
in vivo	ICSI	41	17	9	1	5 (55.6 ± 17.4)		
					2	3 (33.3 ± 18.1)	0.78 ± 0.07	120 ± 23.7 [a]
					3 <	1 (11.1 ± 8.3)		
in vitro	ICSI	97	32	18	1	11 (61.1 ± 13.7)		
					2	4 (22.2 ± 11.3)	0.92 ± 0.06	37.1 ± 6.6 [b]
					3 <	3 (16.7 ± 7.9)		
in vivo	IVF	56	17	7	1	4 (57.1 ± 22.4)		
					2	3 (42.9 ± 22.4)	0.97 ± 0.07	156 ± 19.7 [a]
					3 <	0 (0)		
in vitro	IVF	117	30	15	1	11 (73.3 ± 12.4)		
					2	3 (20.0 ± 7)	0.9 ± 0.06	106.5 ± 21.4 [a]
					3 <	1 (6.7 ± 6.3)		

* Oocytes formed two polar bodies and two pronuclei. ** Mean ± SEM for oocytes that exhibited Ca^{2+} oscillations. [a–b] Values with different superscripts within same column are significantly different ($P < 0.01$).

vivo-IVF group because there was no significant difference among those categories. The vitro-ICSI also showed a low frequency of oscillation (1–4 times/4 h) and a rise in Ca^{2+} level of 0.92 ± 0.06; however, the interval between each Ca^{2+} rise in *in vitro*-matured oocytes after ICSI was shorter (37.1 ± 6.6 min) than that in the other groups. Nevertheless, more than half of the oocytes with Ca^{2+} oscillation in the vitro-ICSI group showed a single Ca^{2+} rise like the other groups. These data indicated that the sperm-injected oocytes and *in vitro*-matured oocytes are able to develop a Ca^{2+} oscillation pattern which is similar to that of *in vivo*-matured oocytes after IVF. Yet, most of the oocytes that failed to undergo PN formation after ICSI showed no Ca^{2+} signal. Lastly, in a few oocytes that remained at the M-II stage, a low amplitude Ca^{2+} oscillation was observed (Fig. 3, Table 3).

Developmental ability of normal fertilized oocytes after ICSI and IVF

We compared the ability of embryos to develop into blastocysts, and the mean number of cells per blastocyst, after ICSI or IVF (Fig. 4, Table 4). The proportions of embryos undergoing blastocyst formation in the vivo-IVF, vivo-ICSI, vitro-IVF, and vitro-ICSI groups were 80.8 ± 7.1%, 85.7 ± 6.3%, 71.9 ± 6.2%, and 65.0 ± 10.9%, respectively. Statistical analysis revealed that blastocyst formation was affected by the oocyte source, and not by the fertilization method. The mean numbers of cells per blastocyst in the vivo-IVF, vivo-ICSI, vitro-IVF, and vitro-ICSI groups were 91.8 ± 7.0, 65.6± 6.7, 67.1 ± 5.6, and 54.3 ± 7.0, respectively. Statistical analysis revealed that the differences between the two effects were significant ($P < 0.05$). These data suggested that *in vitro*-matured oocytes were inferior to *in vivo*-matured oocytes in terms of developmental ability and embryo quality. Fertilization by ICSI also had a negative effect on embryo quality.

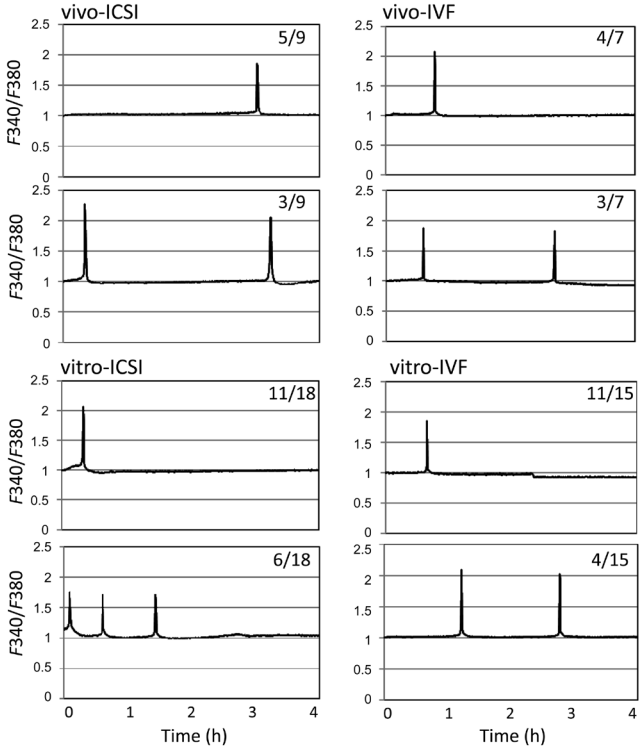

Fig. 2. The typical pattern of intracellular Ca^{2+} responses in 2PB2PN formed pig oocytes, matured *in vivo* or *in vitro* after IVF and ICSI. vivo- or vitro-ICSI: *in vivo*- or *in vitro*-matured oocytes injected with sperm. vivo- or vitro-IVF: *in vivo*- or *in vitro*-matured oocytes inseminated. Data show the ratiometric value of 340/380 nm fluorescence over time.

Discussion

The low efficiency of *in vitro* embryo production by ICSI in pigs has been an unresolved problem. We hypothesized that *in vitro*-matured oocytes or sperm-injected oocytes were unable to generate the pattern of Ca^{2+} oscillation in "physiological fertilization", and this might

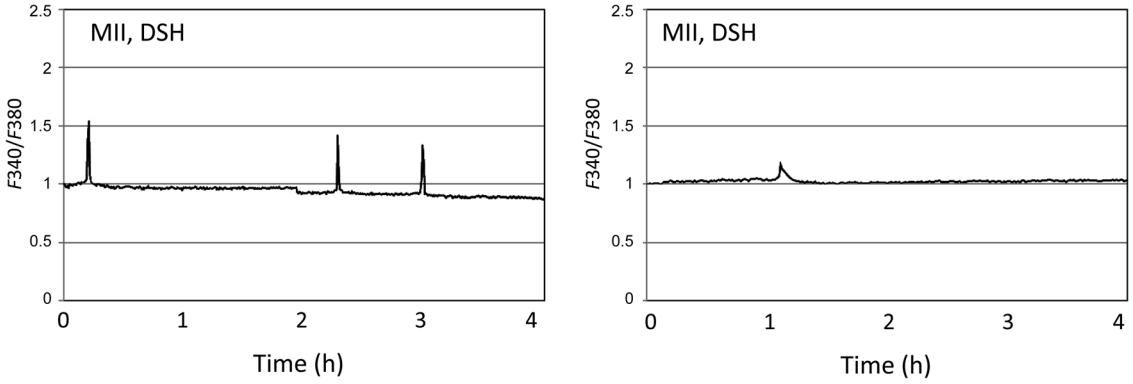

Fig. 3. The pattern of intracellular Ca^{2+} responses from *in vivo*-matured oocytes that failed to form PN after ICSI. Both oocytes remained in the MII stage and contained a decondensed sperm head (DSH). Data show the ratiometric value of 340/380 nm fluorescence over time.

Table 3. Ca^{2+} responses in pig oocytes that failed in PN formation after ICSI

Source of oocytes	No. of oocytes failed in PN formation	No. of oocytes with Ca^{2+} signal	Ca^{2+} rise number	No. of oocytes	Amplitude of Ca^{2+} rise *	Ca^{2+} rise interval (min)*
in vivo	22	3	1	3		
			2	0	0.49 ± 0.16	—
			3 <	0		
in vitro	50	5	1	3		
			2	0	0.51 ± 0.06	49.6 ± 12.06
			3 <	2		

* Mean ± SEM for oocytes that exhibited Ca^{2+} oscillations.

lead to failure of oocyte activation, fertilization, and embryonic development. The pattern of Ca^{2+} oscillation has been suggested to play an important role in the completion of oocyte activation events, fertilization, and embryonic development [17, 18, 31]. Contrary to our expectations, the pattern of Ca^{2+} oscillation was not affected by two factors (ICSI and *in vitro* matured oocyte) because oocytes in the vivo-ICSI and vitro-IVF groups showed a similar pattern of Ca^{2+} oscillation compared with that of the vivo-IVF group (Fig. 2, Table 2). Some of the *in vitro*-matured oocytes after ICSI showed a short interval of Ca^{2+} rise (Table 2). Considering the fact that the pattern was observed in normal fertilized *in vitro*-matured oocytes after ICSI, the short interval of Ca^{2+} rise might not have caused the failure of oocyte activation. However, in the present study, whether the short interval of Ca^{2+} rise shows negative effects on embryonic development is not yet clear.

Half of the *in vivo*- and *in vitro*-matured oocytes injected with sperm remained at the M-II stage (Fig. 1) and most of them exhibited no Ca^{2+} signals (Fig. 3, Table 3). The membranes of pig sperm can be damaged during the freezing and thawing processes associated with cryopreservation [32], and such damage can lead to leakage of intracellular PLCζ [29]. Indeed, immediately after thawing, more than half of the pig sperm were found to have lost PLCζ immunoreactivity, leading to failure of oocyte activation after ICSI [33]. Use of sperm with appropriate levels of PLCζ for ICSI actually increases the efficiency of oocyte activation and normal fertilization [33]. Our findings suggest that the primary reason for failure of oocyte activation after ICSI may be a deficit, rather than a difference, in the pattern of Ca^{2+} oscillation. Some oocytes that failed to become activated showed small-amplitude Ca^{2+} oscillation (Fig. 3). Sperm containing an insufficient quantity of PLCζ probably induce this kind of small-amplitude Ca^{2+} oscillation. Even if the level of intracellular Ca^{2+} increases, Ca^{2+} signals below a minimum threshold may not be able to induce oocyte activation.

The frequency of Ca^{2+} oscillation induced by one sperm in a single oocyte is much lower in pig than in hamster (20–30 times/h [34]) and mouse (5–30 times/h [35–37]). Considering that injection of pig sperm into mouse oocytes triggers an extremely high frequency of Ca^{2+} oscillation [7], the activity of PLCζ in pig may be higher than that in mouse. Thus, it appears that the sensitivity of the IP_3Rs to IP_3 or the speed of Ca^{2+} refilling of ER varies among species.

Use of *in vitro*-matured oocytes led to a low efficiency of blastocyst formation and a decline in the number of cells per blastocyst (Fig. 4, Table 4). It has been reported that the level of glutathione (GSH) in *in vitro*-matured oocytes is lower than that in *in vivo*-matured oocytes [19]. GSH is one of the most important antioxidants, and GSH levels affect oocyte maturation, fertilization, embryonic development, and quality [38–40]. Therefore, increasing the level of GSH in *in vitro*-matured oocytes may improve the efficiency of embryo production. Moreover, fertilization by ICSI caused no decline in developmental ability (Table 4). Thus, if oocytes show normal

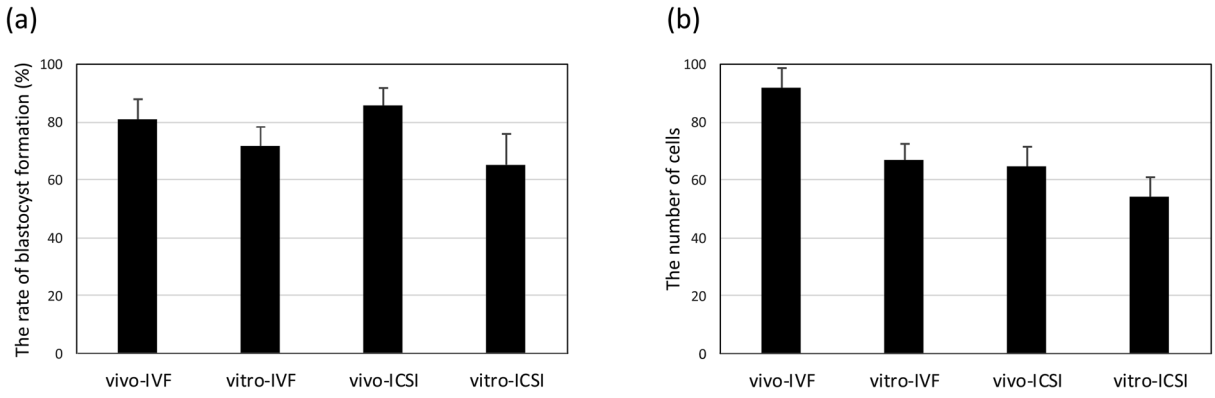

Fig. 4. Effects of the source of oocytes (*in vivo*- or *in vitro*-matured) and the fertilization method (IVF or ICSI) on blastocyst formation (a) and number of cells per blastocyst (b). Data are presented as mean ± SEM for more than three separate experiments.

Table 4. Two-way ANOVA of the rate of blastocyst formation and number of cells per blastocyst

Source	DF[a]	F-value	
		Blastocyst formation	No. of cells per blastocyst
Source of oocyte [b]	1	8.62204 *	5.61664 *
Fertilization method [c]	1	0.73464	6.80907 *

[a] degree of freedom. [b] *in vivo*- or *in vitro*-matured oocytes. [c] IVF or ICSI. * $P < 0.05$.

fertilization after ICSI, they appear to develop better. However, the number of cells per blastocyst after ICSI was lower than that after IVF (Table 4). It has been reported that mouse embryos activated without Ca^{2+} oscillation have a smaller number of inner mass cells and a higher proportion of apoptotic cells than embryos with Ca^{2+} oscillation [41]. Therefore, we considered that the small number of cells per blastocyst in ICSI-derived pig embryos might relate to a lack of Ca^{2+} oscillation. However, in this study, most of the normal fertilized oocytes after ICSI exhibited a pattern of Ca^{2+} oscillation similar to that after IVF. Thus, the number of cells per blastocyst appears to be influenced by other factors, such as mitochondrial function and expression of genes associated with apoptosis [42, 43]. Further studies will be needed to determine the reason for the small number of cells in ICSI-derived embryos.

In conclusion, *in vitro*- and *in vivo*-matured oocytes after ICSI were able to mount a Ca^{2+} oscillation similar to that of *in vivo*- and *in vitro*- matured oocytes after IVF. The main cause of oocyte activation failure in some oocytes after ICSI appeared to be a lack of Ca^{2+} oscillation, rather than a difference in the pattern of Ca^{2+} oscillation. Furthermore, normal fertilized oocytes after ICSI showed *in vitro* developmental ability equivalent to those after IVF.

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