Activation and Development of Pig Oocytes after Microinjection of Crude Sperm Extract

Konosuke Okada[1]*, Takashi Miyano[2] and Masashi Miyake[1]

[1]*Department of Life Science, Graduate School of Science and Technology, and*
[2]*Laboratory of Reproductive Biology and Biotechnology, Faculty of Agriculture,*
Kobe University, Kobe 657-8501, Japan

Abstract: *This work was undertaken to investigate the activation of pig oocytes after microinjection of crude sperm extract prepared from ejaculated boar spermatozoa. The results were compared with those of electro-stimulated oocytes. When in vitro-matured pig oocytes were microinjected with sperm extract and others were electro-stimulated, 100% and 92%, respectively, of the oocytes were released from arrest at metaphase II (MII) and formed female pronuclei. To test their developmental ability, the injected oocytes were treated with cytochalasin B and then cultured in NCSU23 medium. After 168 h, 30% and 44% of the oocytes that had been microinjected with sperm extract and electro-stimulated, respectively, developed to the blastocyst stage. At 6–8 h after the microinjection of sperm extract or electro-stimulation, cortical granules were released from the oocytes. In addition, Cdc2 kinase activity declined to a low level in the treated oocytes. These results indicate that microinjection of crude sperm extract induces the release of in vitro-matured pig oocytes from MII-arrest and leads them into a series of events related to oocyte activation.*
Key words: *Blastocyst, Electro-stimulation, Oocyte activation, Pig, Sperm extract*

Introduction

Parthenogenetic activation of mammalian oocytes is artificially induced by chemical agents including calcium ionophore [1], diluted ethanol [2], strontium [3], and cycloheximide [4], but these agents do not effectively activate pig oocytes. In the pig, an electro-stimulation technique [5] has been used for oocyte activation [6–8]. In addition, pig oocytes are activated by the microinjection of calcium chloride [9, 10] and soluble sperm extract [11]. In many species studied so far, such as the hamster [12], mouse [13], human [14], cow [15] and pig [11], injection of sperm extract activates oocytes with repetitive increases in intracellular Ca^{2+} concentration (Ca^{2+} oscillations), a result which is similar to what occurs in those of fertilization. On the other hand, most of the parthenogenetic treatments including electro-stimulation cause a monotonic increase in the intracellular Ca^{2+} concentration. Moreover, it has been reported that oocytes injected with sperm extract develop to blastocysts in the mouse [16], cow [16] and pig [11].

The objective of the present study was to investigate a series of events during oocyte activation including cortical granule exocytosis, meiotic resumption, Cdc2 kinase inactivation, pronuclear formation and the subsequent development of *in vitro*-matured pig oocytes after microinjection of crude sperm extract. The results were compared with those for electro-stimulated oocytes.

Materials and Methods

Oocyte maturation

Pig ovaries were obtained from prepubertal gilts at a local slaughterhouse. After three washes in Dulbecco's phosphate-buffered saline containing 0.1% polyvinyl alcohol (PBS-PVA), intact healthy antral follicles 4.0–6.0 mm in diameter were dissected in PBS-PVA from ovaries with the technique described by Moor and Trounson [17]. Follicles were opened in HEPES-

buffered medium 199 (Nissui Pharmaceutical, Tokyo, Japan), and oocyte-cumulus-granulosa cell complexes (OCGCs) were isolated from the follicles. After two washes, OCGCs were cultured in 2 ml of bicarbonate-buffered medium 199 supplemented with 10% fetal bovine serum (FBS; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, 0.1 IU/ml human menopausal gonadotropin (hMG; Pergonal, Teikokuzoki, Tokyo, Japan), and two everted theca shells with gentle agitation. The cultures of OCGCs were carried out in an atmosphere of 5% CO_2 in humidified air at 38.5°C for 48 h. After the culture, OCGCs were recovered and treated with 0.01% hyaluronidase (Sigma Chemical Co., St Louis, MO, USA) to remove cumulus cells. Oocytes were then denuded completely by pipetting with a small-bore pipette. Oocytes emitting the first polar body were selected to be used as mature oocytes (MII-oocytes) in the following experiments.

Preparation and microinjection of crude sperm extract

Crude sperm extract was prepared from boar spermatozoa by the method described by Swann [12] and Wu *et al.* [18], with some modifications. The sperm-rich fraction of an ejaculate of a fertile boar was collected and the spermatozoa were washed twice in HEPES-buffered Tyrode's T6 medium [19] and then resuspended in extraction buffer (intracellular-like medium [12]). The buffer consisted of 120 mM KCl, 20 mM HEPES, 100 μM ethyleneglycol-bis-(β-aminoethylether)*N,N,N',N'*-tetraacetic acid (EGTA), 10 mM Na-glycerophosphate, 200 μM *p*-amidinophenylmethylsulfonyl fluoride (*p*-APMSF) and 1 mM dithiothreitol (DTT), and pH was adjusted to 7.5. The sperm suspension, containing approximately 5 × 10^8 cells/ml, was sonicated for 15 min at 4°C (VC-501, Sonic and Material Inc., CT, USA). Homogenates were then centrifuged twice at 10,000 g for 15 min at 4°C. The resulting supernatant was centrifuged at 100,000 g for 45 min at 4°C, and the clear supernatant was collected as the crude sperm extract. The extract was washed with HEPES buffered solution (75 mM KCl, 20 mM HEPES, pH 7.2) and concentrated to 25 mg/ml of protein with ultrafiltration membranes (Centriplus-100, Amicon Inc., Berverly, MA, USA). Aliquots of the sperm extract were stored at –80°C before use.

Microinjection was performed in Ca^{2+} and Mg^{2+}-free HEPES-TL-PVA[10]. MII-oocytes were microinjected with 8.2–14.1 pl (0.9–1.6% of the oocyte volume) of sperm extract with a micro-injector (IM-30 or IM-300; Narishige Inc., Tokyo, Japan). The injection volume

was estimated by measuring the volume of a sphere formed after releasing the solution (sperm extract or HEPES buffered solution) under paraffin oil before the injection. As a control, HEPES buffered solution was injected into MII-oocytes in the same manner.

Electro-stimulation

Electro-stimulation was performed according to the method of Kure-bayashi *et al.* [20]. The field solution for electro-stimulation consisted of 0.3 M mannitol, 0.05 mM $CaCl_2$ and 0.1 mM $MgSO_4$ [5]. MII-oocytes were washed 3 times with the solution and were transferred into a chamber (FTC-03; Shimadzu Co. Ltd., Kyoto, Japan), which was filled with 0.1 ml field solution. Oocytes were manually aligned between the two electrodes at a distance of 2 mm, and subjected to a single square pulse of direct current of 100 μsec duration at 1,500 V/cm from an electric cell-fuser (EFC-2001; Riko Chemical Co., Kyoto, Japan or ECM-200; BTX Inc., San Diego, CA, USA).

Examination of meiotic resumption and development of oocytes

After microinjection of sperm extract or electro-stimulation, oocytes were cultured in TL-PVA [10] for 6 h, then, mounted on slides, fixed in an acetic acid-ethanol (1:3, v/v) solution, and stained with 1% aceto-orcein. Meiotic resumption of oocytes and their pronuclear formation were examined under a Nomarsky interference microscope.

Development of activated oocytes was examined in the manner described by Okada *et al.* [10]. Briefly, oocytes after microinjection of sperm extract or electro-stimulation were cultured for 4 h in NCSU23 medium [21] containing 5 μg/ml cytochalasin B (Sigma) and 4 mg/ml bovine serum albumin (BSA, Serologicals Co., Norcross, GA, USA), and further cultured in NCSU23 medium containing 4 mg/ml BSA. Cleavage of oocytes was examined after 48 h, and development to the blastocyst stage was evaluated after 168 h. Blastocysts were stained with Hoechst 33342 (Polysciences Inc., Warrington, PA, USA), and the number of nuclei was counted under an epifluorescence microscope.

Staining of cortical granules

Cortical granules of oocytes were labeled as described previously[22]. MII-oocytes after electro-stimulation (n=15) and microinjection of sperm extract (n=22) were cultured for 6–8 h in TL-PVA, and then fixed in 3% paraformaldehyde in PBS-PVA for 30 min. After being rinsed in PBS containing 10 mg/ml BSA

Table 1. Activation of pig oocytes after microinjection of sperm extract

Treatment	No. of oocytes examined	No. (%) of oocytes	
		resuming meiosis	having a pronucleus
Sperm extract	39	39 (100)	39 (100)
Electro-stimulation	48	44 (92)[a]	44 (92)
Control[1]	71	1 (1)[b]	0 (0)

[1]HEPES buffered 75 mM KCl-solution. [a,b]Values within a column having different superscripts are significantly different (P<0.05).

(PBS-BSA), the oocytes were kept in PBS-BSA at least overnight. They were then treated with Triton X-100 (Sigma) in PBS-PVA for 5 min and rinsed in PBS-BSA. The oocytes were incubated in 20 μg/ml fluorescein isothiocyanate (FITC)-labeled peanut agglutinin (Sigma) in PBS-PVA for 30 min. After rinsing in PBS-BSA, DNA was counterstained with 400 μg/ml propidium iodide (Sigma) for 15–20 min. After rinsing, the oocytes were mounted in an anti-fade medium (Vector Laboratories Inc., Burlingame, CA, USA) and observed under a confocal laser-scanning microscope (MRC 1024 system; Bio-Rad, Hercules, CA, USA). As the control, non-treated MII-oocytes (n=6) and oocytes injected with HEPES buffered solution (n=13) were treated and observed in the same manner.

Cdc2 kinase assay

Cdc2 kinase activity was measured with histone H1 as a substrate [23]. Groups of two oocytes were recovered 0.5, 1 or 6 h after electro-stimulation or microinjection of sperm extract. The oocytes were transferred into tubes with PBS-PVA and extraction buffer [24], and stored at –80°C before the kinase assay. After thawing, the oocytes were centrifuged at 13,000 g for 2 min, then kinase buffer and histone H1 solution were added and the oocytes were incubated for 20 min. The kinase buffer was composed of 75 mM β-glycerophosphate, 75 mM HEPES (pH 7.2), 0.1 mM EGTA, 75 mM MgCl$_2$, 6 mM DTT, 10 mM ATP, 15 μM cAMP-dependent protein kinase inhibitor peptide (Sigma) and 0.3 μCi/μl [γ-^{32}P]ATP (250 μCi/25 μl, Amersham Pharmacia Biotech, UK). The reaction was terminated by the addition of 4-times-concentrated SDS sample buffer [25]. The samples were boiled for 5 min and then loaded onto 13% SDS-polyacrylamide gels to separate the labeled histone H1. After being run, the gels were dried and autoradiographed. As the control, oocytes at the germinal vesicle stage, MII-oocytes, and oocytes injected with HEPES buffered solution were used. The experiment was repeated three times.

Statistical analysis

The numbers of oocytes in the different treatment groups were analyzed by means of the chi-square test. The nuclear numbers of blastocysts were analyzed by Student's *t*-test. A *P*-value less than 0.05 was considered statistically significant.

Results

Oocyte activation and development after microinjection of sperm extract

When pig MII-oocytes were injected with sperm extract, all oocytes were released from MII-arrest and formed female pronuclei (Table 1). On the other hand, 92% of electro-stimulated oocytes resumed meiosis and formed female pronuclei. The control oocytes injected with HEPES buffered solution resumed meiosis at a significantly lower rate (P<0.05). Three oocytes (6%) degenerated after electro-stimulation, although no oocyte degenerated in the other groups.

After microinjection of sperm extract and electro-stimulation, 75% and 60% of the oocytes cleaved, respectively (Table 2). These percentages were significantly higher than that in the control group (P<0.05). After 168 h, 30% and 44%, respectively, of the oocytes injected with sperm extract and those that were electro-stimulated, developed to the blastocyst stage, while other oocytes in these groups and all of the control oocytes stopped their development before the blastocyst stage or degenerated. The mean cell numbers of blastocysts that developed from the oocytes injected with sperm extract and electro-stimulated were 21 ± 8 and 28 ± 12, respectively (P>0.05).

Exocytosis of cortical granules after microinjection of sperm extract

In MII-oocytes (6/6), cortical granules were localized just under the plasma membrane as a bright continuous ring (Fig. 1a). In control oocytes injected with HEPES buffered solution (12/13), the cortical granules showed a similar distribution to MII-oocytes after 6–8 h (data not

Table 2. Development of pig oocytes after microinjection of sperm extract

Treatment	No. of oocytes examined	No. (%) of		Nuclear no. of blastocyst[3]
		cleaved oocytes[2]	blastocysts[2]	
Sperm extract	40	30 (75)[a]	12 (30)	21 ± 8
Electro-stimulation	43	26 (60)[a]	19 (44)	28 ± 12
Control[1]	23	4 (17)[b]	0 (0)	–

[1]HEPES buffered 75 mM KCl-solution. [2]Cleavage of oocytes was examined after 48 h, and development to the blastocyst stage was examined after 168 h. [3]Mean ± SD. [a,b]Values within a column having different superscripts are significantly different (P<0.05).
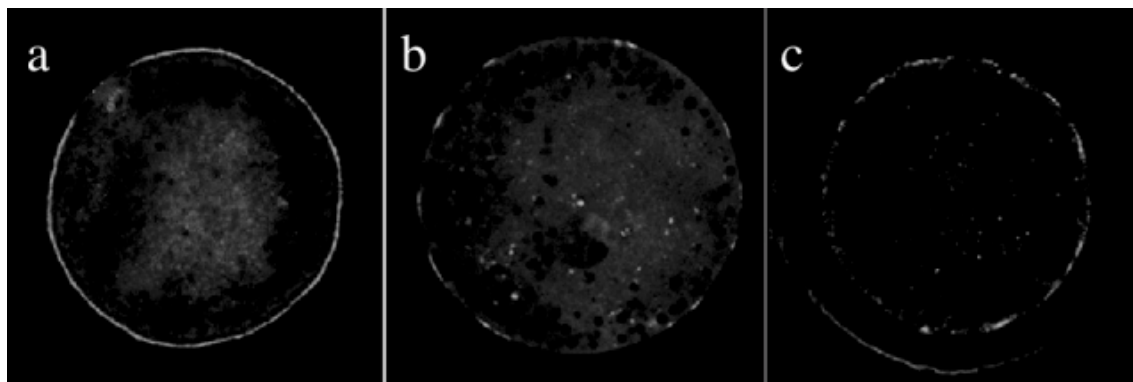

Fig. 1. Confocal images of cortical granules of pig oocytes stained with FITC-labeled peanut agglutinin. Pig oocytes cultured for 48 h (a) were electro-stimulated (b) or injected with 8.2–14.1 pl of crude sperm extract (c), and were further cultured in TL-PVA medium for 6–8 h. After the culture, the oocytes were fixed, stained and examined for cortical granule exocytosis. Control oocytes were immediately fixed after maturation-culture and stained in the same manner (a).
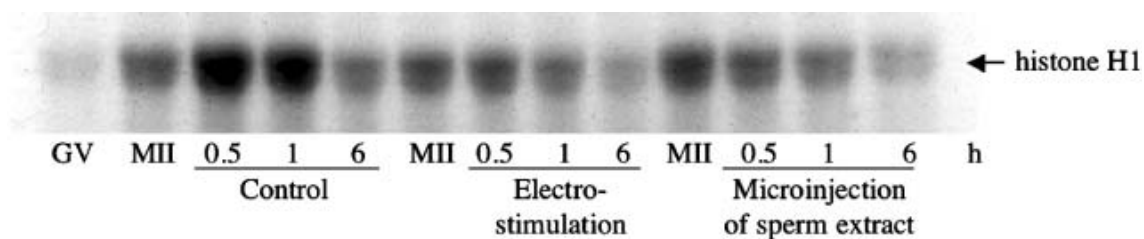

Fig. 2. Changes in the activities of Cdc2 kinase at 0.5, 1, or 6 h after treatments. Pig oocytes cultured for 48 h were electro-stimulated or injected with 8.2–14.1 pl of sperm extract, and further cultured in TL-PVA medium for 0.5, 1, or 6 h. Cdc2 kinase activities of the oocytes were detected by phosphorylation of histone H1. GV: germinal vesicle, MII: metaphase II, Control: HEPES buffered 75 mM KCl-solution.

shown). On the other hand, in electro-stimulated oocytes (12/15), the ring of cortical granules became discontinuous, and patches of cortical granule materials were observed on the oocyte surface (Fig. 1b). After oocytes were injected with sperm extract, the exudation of cortical granules was observed in 18 of 22 injected oocytes (Fig. 1c), and cortical granules were completely lost in 8 oocytes.

Inactivation of Cdc2 kinase after microinjection of sperm extract

Activity of Cdc2 kinase was low in the oocytes at the germinal vesicle stage, and high in MII-oocytes (Fig. 2). After 1 h, the activity slightly declined in both the sperm extract-injected and electro-stimulated oocytes. After 6 h, the activity further declined in both experimental groups. In the control oocytes, the activity declined at 6

h post-treatment, although the activity was higher than in the experimental groups.

Discussion

The results of the present study suggest that pig MII-oocytes are activated by microinjection of crude sperm extract of boar spermatozoa to a degree similar to their activation by electro-stimulation. Microinjection of sperm extract not only induced the resumption and completion of meiosis of pig MII-oocytes but also induced a series of activation events including cortical granule exocytosis, the formation of a female pronucleus, and development to the blastocyst stage in a manner analogous to those observed in the previous study by Macháty *et al.* [11].

In many mammals studied so far (hamster [12], mouse [26], rabbit [27], bovine [16] and pig [11]), sperm extract induces oocyte activation. In the present study, oocytes injected with sperm extract were effectively activated. In general, the activation of oocytes and the related events have been thought to be triggered by a transient in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and subsequent Ca^{2+} oscillations after sperm penetration [28]. There are two hypotheses, which are named the receptor-mediated hypothesis [29, 30] and the sperm-factor hypothesis [12], regarding how the spermatozoon induces the release of Ca^{2+} from intracellular stores of the oocyte. The former holds that the activating signal originates in a receptor localized on the surface of the oocytes and travels through the plasma membrane into the cytoplasm through the polyphosphoinositide messenger system. As a result, inositol 1,4,5-trisphosphate induces Ca^{2+} release from stores via the activation of phospholipase C (PLC). The latter hypothesis, on the other hand, proposes that after membrane fusion spermatozoa introduce a factor into the oocyte cytoplasm that has Ca^{2+}-releasing ability. In recent studies, a novel sperm-specific isoform of PLC, named PLCζ, has been identified as the factor, and it has been reported that microinjection of complementary RNA for PLCζ from mouse, hamster, human and monkey spermatozoa elicits Ca^{2+} oscillations in mouse oocytes equivalent to those seen during fertilization in mice [31, 32]. Our results may support the sperm-factor hypothesis.

When mouse and bovine oocytes were injected with sperm extract, 45% and 26% of them developed to the blastocyst stage, respectively [16]. Macháty *et al.* [11] reported that 2% of pig oocytes injected with sperm extract developed to blastocysts in NCSU23 medium.

In the present study, a relatively high percentage (30%) of pig oocytes injected with sperm extract developed to blastocysts. The reason for this is thought to be that the oocytes were treated with cytochalasin B to prevent the extrusion of the second polar body. The treatment of cytochalasin B produces diploid embryos that have a better chance to develop to the blastocyst stage than haploid embryos have [33, 34], but the rate of development of the oocytes microinjected with sperm extract was lower than that of the electro-stimulated oocytes, although there was no significant difference between the two treatments. The sperm extract prepared in this study may contain not only the essential components for oocyte activation but also unnecessary components, such as acrosin and hyaluronidase, which are thought to be naturally lost before sperm-egg fusion. Such components may affect the development of the oocytes injected with sperm extract.

After *in vitro* fertilization, pig oocytes show cortical granule exocytosis within 6 h [35]. The release of cortical granules is induced by the increased $[Ca^{2+}]_i$ caused by the spermatozoon. It is also induced by different treatments, such as electro-stimulation [7] and microinjection of Ca^{2+} [9, 10] in the pig, calcium ionophore in the hamster [1], and exposure to the medium containing Sr^{2+} in the mouse [36]. In the present study, cortical granules were released within 6–8 h after electro-stimulation or microinjection of sperm extract, and the result was consistent with the previous report [11]. The exocytotic patterns were similar to those of the oocytes injected with divalent cations such as Ca^{2+}, Sr^{2+} and Ba^{2+} [10]. Cortical granules were completely released from some oocytes. It is possible that repetitive increases in $[Ca^{2+}]_i$ may accelerate or promote the complete release of cortical granules, since sperm extract induces the Ca^{2+} oscillation in injected pig oocytes [11] (our personal data).

Both electro-stimulation and microinjection of sperm extract induced the inactivation of the Cdc2 kinase of the oocytes. The activity of Cdc2 kinase is an indicator of maturation-promoting factor (MPF) activity. Fertilization or parthenogenetic activation induces a transient increase in $[Ca^{2+}]_i$ that leads to cyclin B degradation to inactivate Cdc2 kinase. The inactivation of Cdc2 kinase allows oocytes to release MII-arrest. In our experiment, Cdc2 kinase activity slightly decreased at 1 h and further decreased at 6 h after both treatments in a similar pattern. In the control oocytes, the activity also declined at 6 h after the injection of HEPES buffered solution, although these oocytes did not form the pronucleus (Table 1). It is possible that this decline

in the activity may be spontaneous and then insufficient for meiotic resumption and pronuclear formation.

In conclusion, the present study proves that microinjection of sperm extract induces pig oocytes to be released from MII-arrest and leads to a series of events, including cortical granule exocytosis, pronuclear formation, and Cdc2 kinase inactivation, that are similar to those observed in electro-stimulated oocytes.

Acknowledgements

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