Effects of Miniature Pig Sperm Extracts and Their Treatments on Porcine or Bovine Oocyte Activation

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Abstract: *This study investigated the activation of oocytes after microinjection of sperm extracts (SE) from miniature pig spermatozoa. SE was prepared from miniature pig sperm by sonication for 35 min and centrifugation 100,000 g for 1 h. Injection of SE 0.3 mg/ml into porcine oocytes produced the highest activation rate (41.8%). SE injection into bovine oocytes induced similar activation (43.3%). The porcine oocyte activation rate (63.4%) was significantly (p<0.05) improved when SE was purified with a 2-D clean-up kit. When SE was heat treated at 60°C for 30 min, it failed in activation. However, SE succeeded in activation when stored at 4°C for 48 h or was freeze-thawed 5 times using liquid nitrogen. The activation rates were 42.1% and 31.4%, respectively. The results indicate that SE from miniature pig sperm might have an oocyte activation factor, and that the factor might have stability at low temperatures, though it appears to be inactivated at high temperatures.*
Key words: *Porcine oocyte, Oocyte activation, Miniature pig spermatozoa, Sperm extracts, Thermal stability*

Introduction

It is well known that a spermatozoon releases cytosolic substances, called sperm factor (SF), into an oocyte at fertilization, and that the SF induces $[Ca^{2+}]i$ oscillations [1–3]. Some studies have attempted to specify the SF. Phospholipase C ζ (PLC ζ) and Tr-kit have been advocated as the two main candidates for SF. Saunders *et al.* [4] demonstrated that the molecular

weight of PLC ζ was approximate 74 kDa, and that PLC ζ triggered $[Ca^{2+}]i$ oscillations in mouse oocytes. Sette *et al.* [5] showed that Tr-kit, a 23 kDa protein, was a truncated product of the c-kit gene [6], and that it caused meiotic resumption through activation of phospholipase Cγ1 (PLC γ1) and $[Ca^{2+}]i$ oscillations in mouse oocytes. Fissore *et al.* [7] reported that injection of sperm extracts from boar spermatozoa induced high rates of activation and cleavage in bovine and mouse oocytes. Injection of the sperm extracts into mouse and bovine oocytes triggered $[Ca^{2+}]i$ rises that resembled spontaneous oscillation [3]. Kimura *et al.* [8] designated sperm extracts as sperm-borne oocyte-activating factor.

As mentioned above, it is clear that oocyte activation and development of cleavage is induced by the injection of sperm extracts. There has, however, been little research elucidating their characteristics. The aim of this study was to investigate whether extracts from miniature pig sperm can induce porcine and bovine oocyte activation, and to examine whether the extracts have thermal stability against heat or low temperatures.

Materials and Methods

Sperm preparation

Whole semen from Gottingen miniature pigs (2–3 years old) was collected once weekly by a glove-hand technique and filtered through cotton gauze to remove the gel particles. Ten or more ejaculated semen samples from two boars were used in this study. The semen was centrifuged at 400 × g for 10 min and the sediments were removed. Then, the supernatants were transferred into a new tube and centrifuged at 700 × g for 5 min. The sperm pellets were suspended and washed twice in TALP-Hepes (TL-Hepes) medium [9] at 700 × g for 5 min. Washed spermatozoa were

resuspended in cell lysis buffer (75 mM KCl, 20 mM Hepes, 1 mM EDTA, 10 mM glycerophosphate, 1 mM DTT, 200 μM PMSF, 10 μg/ml pepatatin, 10 μg/ml leupeptin, pH7.0), and then the sperm concentration was adjusted to 1×10^9/ml.

Method of adjusting SE

The protocol modified by Wu *et al.* [10] was adapted for the method of adjusting SE. Briefly, sperm suspensions were sonicated (UD-200, TOMY, Tokyo, Japan) for 35 min at 4°C in dry ice alcohol. The lysate was centrifuged twice at 10,000 × g, and further ultaracentrifuged at 100,000 × g (TL-100, Beckman, USA) for 1 h at 4°C. The clear supernatant was filtered through a 3,000 molecular weight cut-off membrane, (Microcon YM-3; Millipore Corp, Bedford, MA). The substances on the ultrafiltration membranes were washed twice with washing buffer solution consisting of 75 mM KCl and 20 mM Hepes (pH 7.0). Then the protein concentration of the diluted substances was adjusted to 1.0 mg/ml with the same buffer. The adjusted substances were stored at –80°C until use.

Treatment of the cytosolic fraction with 2-D clean-up kit

The aim of treatment with the 2-D clean-up kit (Amersham Biosciences Corp. San Francisco, CA, USA) was to remove detergents, salt, lipids, phenol and nucleic acids from SE. The purification with the kit was performed according to the manufacturer's protocol. Briefly, 300 μl precipitant was added to 100 μl SE and left on ice for 15 min. It was centrifuged for 5 min at 12,000 × g to remove the supernatant. Then 25 μl of distilled water was added to the pellet, and it was vortexed to disperse it. After addition of 1 ml "chilled wash buffer" and 5 μl "wash additive", the precipitate was collected by centrifugation for 5 min at 15,000 × g, resuspended with washing buffer, and washed two times. The sample was dissolved in the injection buffer. Finally, the protein concentration of the sample was measured by DC Protein Assay Kits (Bio-Rad), and adjusted to 6.0 mg/ml. The adjusted sample was stored at –80°C until use.

Porcine oocyte maturation

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported within 2 h to the laboratory in 0.9% (w/v) saline solution containing 100 mg/ml kanamycin sulfate (Meiji Seika, Ltd, Tokyo, Japan) at 30°C. The follicular contents were recovered by cutting them from visible small antral follicles (about 2–5 min in diameter) with a razor, and by scraping the inner surface of the follicle walls with a disposable surgical blade. The cumulus oocyte complexes (COCs) with a uniform ooplasm and a compact cumulus cell mass were placed in phosphate-buffered saline (PBS) containing 0.01% (w/v) polyvinylpyrrolidone (PVP) (Sigma, St. Louis, MO, USA). They were washed two times with the maturation medium (BSA-free NCSU37 [11] supplemented with 0.6 mM cysteine (Sigma), 10% FCS (Gibco BRL, Grand Island, NY, USA), 0.6 μg/ml porcine-FSH (Sigma), and 1.3 μg/ml equine-LH (Sigma). Twenty COCs were cultured for 48 h in a NUNC 48-well multidish (NUNC, Roskilde, Denmark) containing 300 μl of maturation medium.

Bovine oocyte maturation

Bovine ovaries were collected from a local slaughterhouse and transported to the laboratory in 0.9% saline solution at 30°C. Using a 10-ml syringe with a 21-gauge needle (TERUMO), COCs were aspirated from the antral follicles (2–8 mm in diameter). Only oocytes with intact cytoplasm and compact cumulus oophorus were used. The COCs were washed with Dulbecco's phosphate buffered saline containing 0.05% PVP, and then washed twice with TCM-199 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 15% bovine follicular fluid, 0.6 μg/ml p-FSH (Sigma), 1.3 μg/ml e-LH (Sigma) 0.2 mM sodium-pyruvate and 50 μg/ml gentamicin (Sigma). Fifteen to twenty COCs were cultured per well with 300 μl maturation medium for 20 h at 38°C in a humidified atmosphere of 5% CO_2 in air.

Microinjection of SE into Oocytes

After cultivation of porcine and bovine COCs, the cumulus cells were removed by pipetting. For injection of SE, a Cell-Injector (Microinjector, CIJ-1, SHIMADZU, Inc, Koto, Japan) and micromanipulator (System PMM-120, PRIMA, Japan) equipped with a piezo-micropipette driving unit (Model PMM-01, Prima Meat Packers, Tsuchiura-City, Ibaraki-ken, Japan) were used. The injection procedure was based on the protocol of Kimura and Yanagimachi [12]. The amount injected was 5 pl, and the injection was performed on a heating stage at 30°C. The injected oocytes were immediately transferred into the culture medium (TCM-199 containing 10% FCS) and incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Both nuclear formation and cleavage were evaluated after cultivation for 48 h.

Observation of cortical granule exocytosis

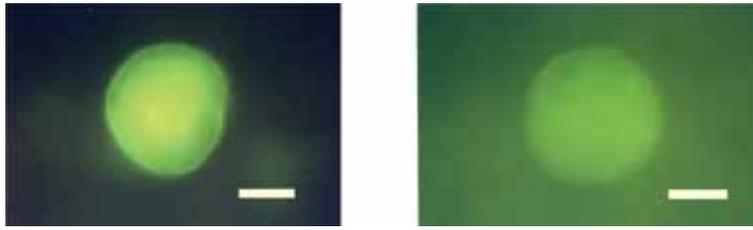The oocytes injected with SE were cultured for 4 h in

Fig. 1. Distributional patterns of CGs in porcine oocytes Type I: CGs localize just under the plasma membrane as a bright continuous ring. Type II: Patches of CGs are observed on the oocyte surface. Scale bars represent 50 μm.

TCM-199 containing 10% FCS. After fixation with 3% paraformaldehyde (Sigma) in PBS for 30 min at room temperature, the oocytes were washed twice in PBS and then placed in PBS containing 0.5% (w/v) pronase (Sigma) for 5 min to dissolve the zona pellucida. They were further treated with 0.1% (w/v) TritonX-100 (Sigma) in PBS for 5 min and then stained in PBS supplemented with 3 mg/ml BSA (Sigma) and 40 μg/ml FITC-PNA (Sigma) for 20 min. The stained oocytes were washed three times in PBS containing 3 mg/ml BSA and put between a coverslip and a glass slide supported by 4 columns of a mixture of Vaseline and paraffin (9:1). Cortical granule (CG) exocytosis was examined using a fluorescence microscope (Nikon, 515 nm wavelength). The distributional pattern of CGs was classified into the following two types: Type I, CGs localized just under the plasma membrane as a bright continuous ring; Type II, patches of CG materials observed on the oocyte surface (Fig. 1).

Evaluation of nuclear status

After culturing for 48 h, the oocytes were mounted on slides, compressed with coverslips and fixed with ethanol : acetic acid (3:1) for more than 48 h at room temperature. The fixed oocytes were stained with 1% lacmoid in 45% acetic acid. Nuclear status was examined by phase-contrast microscopy ($\times$ 400).

Experimental design

In Experiment 1, the effect of injection of SE on parthenogenetic porcine oocyte activation was examined. The protein concentrations in SE extracted from miniature pig spermatozoa were adjusted to 0.03, 0.08, 0.3 and 0.8 mg/ml. Various concentrations of the protein were injected into the matured oocytes. The rates of pronuclear formation and cleavage were examined after culturing for 48 h. In Experiment 2, the effect of SE injection on bovine oocyte activation was examined. In Experiment 3, cortical granule (CG) exocytosis was examined after the injection of SE. In Experiment 4, the effectiveness of purification treatment of SE with 2-D clean-up kit was tested. In Experiment 5, three treatments were done to characterize SE. The treatments were as follows: incubation for 30 min at 60°C, incubation for 48 h at 4°C, and repeated freeze-thawing (5 times) by dipping into nitrogen liquid. The treated SE were injected into porcine oocytes.

Statistical analysis

Statistical comparison of the data was carried out using one-way analysis of variance (ANOVA). All percentage data were subjected to arc sine transformation before statistical analysis. All data were compared using the Tukey-Kramer honesty significant difference test. All experiments were repeated at least three times. Statistical comparisons were performed using JMP IN software (SAS Institute Inc., Cary, NC). Differences were considered to be significant at P<0.05.

Results

Experiment 1

Table 1 shows both the rates of pronucleus formation and cleavage after injection of SE at different protein concentrations. In the control group no activation occurred, however oocytes in the treated groups injected with SE were activated. The highest activation was induced by injection of 0.3 mg/ml SE.

Experiment 2

Table 2 shows the results of bovine oocytes injected with 0.3 mg/ml SE. Although no activation occurred in the control group, SE injection induced oocyte activation. The rate (43.3%) was similar to the rate of porcine oocyte activation (41.8%).

Table 1. Porcine oocyte activation after injection of different concentrations of sperm extracts (SE) from miniature pig sperm

Concentration of SE	No. of oocytes	No. of oocytes with pronucleus (%)	No. of cleaved oocytes (%)		Activation rate (%)
			≥2-cell	≥8-cell	
0 (buffer)	92	0 (0.0 ± 0.0)[a]	0 (0.0 ± 0.0)[a]	0 (0.0 ± 0.0)[a]	0 (0.0 ± 0.0)[a]
0.03 mg/ml	56	1 (1.8 ± 1.0)[ab]	0 (0.0 ± 0.0)[a]	0 (0.0 ± 0.0)[a]	1 (1.8 ± 1.0)[a]
0.08 mg/ml	51	4 (11.8 ± 1.3)[c]	5 (9.3 ± 8.5)[b]	2 (3.7 ± 3.2)[b]	11 (20.4 ± 8.5)[b]
0.3 mg/ml	67	13 (19.4 ± 1.2)[d]	10 (15.1 ± 2.2)[b]	5 (7.3 ± 1.4)[c]	28 (41.8 ± 1.5)[c]
0.8 mg/ml	61	15 (24.6 ± 1.2)[e]	5 (8.2 ± 2.8)[b]	1 (1.6 ± 2.8)[bc]	21 (34.4 ± 4.1)[c]

Data are expressed as mean ± SEM. Values within the same column with different letters (a–e) differ significantly (P<0.05).

Table 2. Bovine oocyte activation after injection of sperm extracts (SE) from miniature pig sperm

Treatment	No. of oocytes	No. of oocytes with pronucleus (%)	No. of cleaved oocytes (%)		Activation rate (%)
			≥2-cell	≥8-cell	
Injection of buffer	28	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)	0 (0.0 ± 0.0)
Injection of SE (0.3 mg/ml)	30	11 (36.7 ± 0.6)*	0 (0.0 ± 0.0)	2 (6.7 ± 0.6)	13 (43.3 ± 10.0)*

Data are expressed as mean ± SEM. Values within the same column with asterisk (*) differ significantly (P<0.05).

Table 3. Effect of injection of sperm extracts (SE) on exocytosis of cortical granules (CGs) in porcine oocytes

Treatment	No. of oocytes	Distributional patterns of CGs (%)	
		I[a]	II[b]
Non-injection	27	27 (100)	0 (0.0)
Injection of buffer	14	13 (92.9)	1 (7.1)
Injection of 0.3 mg/ml SE	24	2 (8.3)	22 (91.7)

Type I: CGs localized just under the plasma membrane as a bright continuous ring.
Type II: Patches of CGs observed on the oocyte surface.

Experiment 3

As shown in Table 3, almost all of the uninjected oocytes and the oocytes injected with buffer contained intact cortical granules (Type I: localized under the plasma membrane). In contrast, oocytes injected with SE exhibited CG exocytosis (Type II: patches of CG materials on the oocyte surface) judged by FITC-PNA staining (Fig. 1).

Experiment 4

Purification with the 2-D clean-up kit, resulted in the percentage of oocyte activation being significantly (P<0.05) improved (Fig. 2).

Experiment 5

As shown in Fig. 3, the activation rate of the control group was 41.6%. Activation was absent (0%) after heat treatment of SE at 60° C for 30 min, whereas after the other treatments (storage at 4° C for 48 h and freeze-
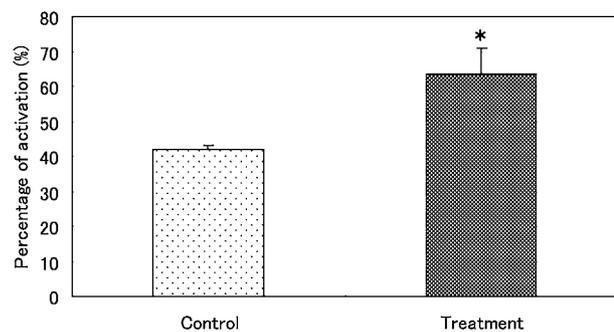

Fig. 2. Porcine oocyte activation after injection of untreated SE (control) and SE treated for protein purification kit (treatment). Values are mean ± SEM of three replications. *Significant difference (P<0.05).

thawing 5 times) SE successfully activated oocytes at rates of 42.1% and 31.4%, respectively. These rates did not differ significantly (P>0.05) from the control.
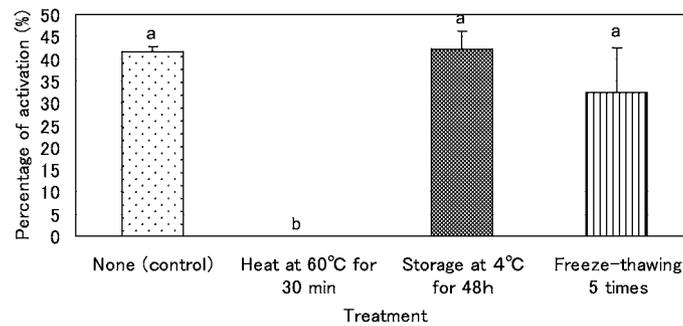
Fig. 3. Effects of different thermal treatments of SE from miniature pig sperm on porcine oocyte activation. Values are mean ± SEM of three replicates. [a, b]Different letters show significant difference (P<0.05).

Discussion

The present study examined whether SE extracted from miniature pig sperm could elicit oocyte activation. The porcine and bovine oocytes injected with SE were activated (Experiments 1 and 2), and the porcine oocytes injected with SE exhibited CG exocytosis (Experiment 3). From these results, it was considered that injection of SE into the oocyte was an effective method of activating an oocyte. Fissore *et al.* [7] reported that microinjection of 10 mg/ml porcine sperm crude extracts into bovine oocytes induced a high rate of activation. Their report and the results of the present study indicate that injection of SE could be used to promote activation in intracytoplasmic sperm injection (ICSI). Palermo *et al.* [13] demonstrated that human oocytes previously unfertilized by ICSI formed two pronuclei or a mosaic diploid after injection of human sperm cytosolic factor.

When SE was purified by 2-D clean-up kit, the percentage of oocyte activation was significantly (P<0.05) improved (Experiment 4), though the reason why the activation rate was improved is not certain. It may have been due to the removal of salts, lipids and nucleic acids from SE, because the treatment of 2-D clean-up kit removes these substances.

In Experiment 5, the effect of heat treatment of SE on porcine oocyte activation was investigated. SE failed to activate oocytes after heat treatment at 60°C for 30 min. This result means that SE contains an oocyte activating factor, and that the factor seems to be a protein degenerated by heat treatment. Wu *et al.* [10] also showed that pig sperm factor treated at 60°C for 30 min failed to activate. Furthermore it was reported that the activation ability of demembranated mouse sperm heads was denatured after heating at 44°C for 30 min [14].

For SE to be used in ICSI effectively, SE needs to be preserved, e.g. by refrigeration at 4°C for several days or by freezing and storage freezer at –20 – –80°C for longer periods. Furthermore preservation by freeze-thawing needs to be repeatable. Therefore, the effect of low temperatures on SE was examined. Oocytes injected with SE preserved at 4°C for 48 h and freeze-thawed 5 times were successfully activated at rates of 42.1% and 31.4%, respectively, and these values were not significantly different from the control (the activation rate of the oocytes injected with untreated SE). From these results, we consider that SE has thermal stability at low temperatures, and that SE could be used in ICSI effectively.

Although the present study demonstrated characteristics of SE extracted from miniature pig sperm for oocyte activation, it is unclear whether or not these characteristics are common to the extracts from boar or other mammalian sperm. Further work is required to investigate these characteristics.

In summary, the present study showed that SE extracted from miniature pig sperm induced comparatively high rates of oocyte activation. Though SE was denatured by heating at 60°C, it was tolerant of low temperatures.

Acknowledgements

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