

—Technology Report—

Efficient pig ICSI using Percoll-selected spermatozoa; evidence for the essential role of phospholipase C- ζ in ICSI success

Michiko NAKAI¹⁾, Shun-ichi SUZUKI¹⁾, Junya ITO²⁾, Dai-ichiro FUCHIMOTO¹⁾, Shoichiro SEMBON¹⁾, Junko NOGUCHI¹⁾, Akira ONISHI³⁾, Naomi KASHIWAZAKI²⁾ and Kazuhiro KIKUCHI¹⁾

¹⁾Division of Animal Sciences, Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Ibaraki 305-8602, Japan

²⁾Laboratory of Animal Reproduction, School of Veterinary Medicine, Azabu University, Kanagawa 252-5201, Japan

³⁾College of Bioresource Sciences, Nihon University, Kanagawa 252-0880, Japan

Abstract. In pigs, the damaged sperm membrane leads to leakage of phospholipase C- ζ (PLC ζ), which has been identified as a sperm factor, and a reduction of oocyte-activating ability. In this study, we investigated whether sperm selected by Percoll gradient centrifugation (Percoll) have sufficient PLC ζ , and whether the efficiency of fertilization and blastocyst formation after intracytoplasmic sperm injection (ICSI) using Percoll-selected sperm can be improved. Percoll-selected sperm (Percoll group) or sperm without Percoll selection (Control group) were used. A proportion of the oocytes injected with control sperm were subjected to electrical stimulation at 1 h after ICSI (Cont + ES group). It was found that the Percoll group showed a large amount of PLC ζ in comparison with the Control group. Furthermore, application of Percoll-selected sperm for ICSI increased the efficiency of fertilization and embryo development. Thus, these results indicate the Percoll-selected sperm have sufficient PLC ζ and high oocyte-activating ability after ICSI in pigs.

Key words: Fertilization, Intracytoplasmic sperm injection, Oocyte activation, Pig

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In mammals, repetitive rise in the intracellular level of free Ca²⁺, also known as Ca²⁺ oscillations, are induced by sperm-specific phospholipase C- ζ (PLC ζ), which has been identified as the most probable candidate sperm factor [1], during fertilization. This triggers a series of oocyte activation events, such as the destruction of cytostatic factor, degradation of maturation promoting factor, resumption of meiosis, extrusion of cortical granules, and transformation of sperm and oocyte nuclei into male and female pronuclei (PNs), respectively, which in turn leads to embryonic development [2].

Unlike the situation in rodents, the efficiency of PN formation after intracytoplasmic sperm injection (ICSI) in pigs is quite low if the ICSI-oocytes do not receive additional artificial stimulation [3, 4]. Therefore, artificial oocyte stimulation, by electrical stimulation [3–6] or calcium ionophore application [6] is considered to be essential for a successful ICSI. We have previously demonstrated that treatments for disrupting sperm membranes before ICSI, such as sonication to isolate the sperm heads from the tails or treatment with detergent and repeated freezing/thawing, led to leakage of PLC ζ and reduction in oocyte-activating ability in pigs [7]. The membranes of a majority of boar sperm were damaged and the sperm

were immobilized after freezing and thawing procedure. However, we used all the freeze-thawed sperm samples for ICSI irrespective of being live or immobilized because it was difficult to select only live sperm with an intact membrane owing to the fact that sperm motility is lost soon after its transfer into the injection medium during ICSI. Given that the oocytes penetrated by sperm undergo PN formation, it would be expected that such sperm, which have a high ability to fertilize, have sufficient PLC ζ . Therefore, we hypothesized that utilization of sperm containing a large amount of PLC ζ might considerably improve the efficiency of fertilization and embryonic development after ICSI without any artificial stimulation. Superior boar sperm with high motility and penetrability and intact membranes were selected by Percoll gradient centrifugation (Percoll) [8–10]. The cell density of dead sperm decreases because the extracellular fluid enters into the cytoplasm via passive transport [11]. Therefore, Percoll can separate live and dead sperm by the differences in cell densities. When boar sperm harvested after Percoll were examined by immunofluorescence assay, PLC ζ was observed to be localized in the post-acrosomal and tail regions, as described previously [7] (Fig. 1-a, b). The signals from the acrosomal region are considered non-specific [7]. The proportion of sperm containing PLC ζ in the Percoll-selected sperm (Percoll group: 74.2 \pm 7.8%) was significantly higher than that in the frozen-thawed sperm without Percoll selection (Control group: 45.7 \pm 5.7%) (Fig. 1-c, -d and Fig. 2). Furthermore, a dense band for PLC ζ in the sperm selected by Percoll was detected in the western blotting assay (Fig. 3). As expected, sperm with intact membranes contain sufficient PLC ζ because it is membrane damage that causes PLC ζ loss [7].

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Correspondence: M Nakai (e-mail: nakai3@affrc.go.jp)

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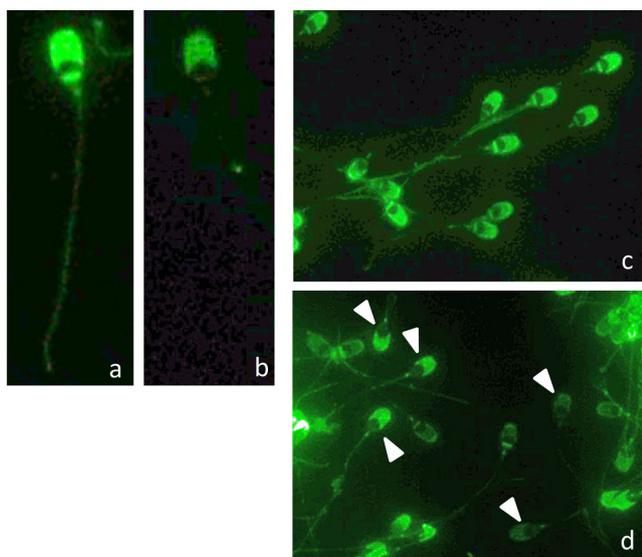


Fig. 1. (a) Labeling of pig sperm with antibody against PLC ζ (green). PLC ζ is localized in the post-acrosomal tail regions and sperm tails in pig sperm. (b) Sperm, which loses PLC ζ , shows no signal. Arrow heads show sperm with weak- or non-PLC ζ signal. Immunofluorescence analysis of localization of phospholipase C- ζ (PLC ζ) in pig spermatozoa treated with (c) or without (d) Percoll gradient centrifugation.

We evaluated the oocyte-activating ability of Percoll-selected sperm after ICSI (Table 1). The rate of normal fertilization (2 polar bodies and 2 PN, 2PB2PN) in the Percoll group was significantly higher than that in the Control group ($P < 0.05$) and equals to that in the electric stimulated oocytes after ICSI (Cont + ES group). In the Control group, more than half of the injected oocytes were arrested at the metaphase-II (M-II) stage but most of the injected sperm nuclei underwent decondensation spontaneously triggered by ooplasmic glutathione [12]. A rise in the intracellular level of free Ca^{2+} is not necessary for the decondensation [13], whereas transition of the decondensed sperm nucleus to the male PN does require this either [4]. These results suggest that a decrease of oocyte-activating ability of the sperm, possibly attributable to the quantity of PLC ζ present in the sperm, also leads to failure of male PN formation. However, in cattle, it has been also suggested that no rise in intracellular Ca^{2+} levels may be caused by the low activity of PLC ζ or presence of PLC ζ inside the sperm [14]. Further studies will be needed to clarify the effect of PLC ζ activity in the injected sperm on induction of oocyte activation after ICSI.

In addition, we investigated the *in vitro* developmental ability of oocytes injected with Percoll-selected sperm (Table 2). The rate of blastocyst formation in the Percoll and Cont + ES groups was significantly higher than that in the Control group ($P < 0.05$). In contrast, the mean number of cells per blastocyst, which is an important indicator of embryo quality [13], after electrical stimulation was lower in both, the Percoll and Control groups (Table 2). It has been reported that the pattern of Ca^{2+} oscillation induced by electrical stimulation differs from that observed in the sperm-penetrated oocytes [15]. It is probable that inappropriate Ca^{2+} oscillation can

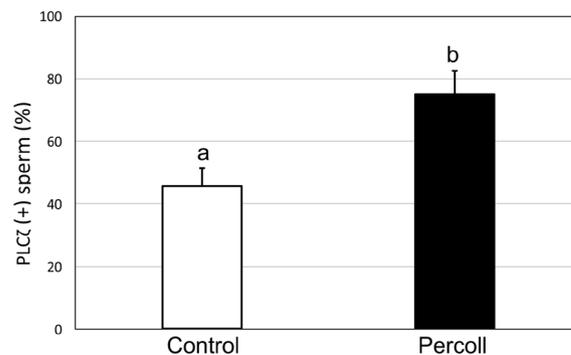


Fig. 2. Selection of sperm with phospholipase C- ζ (PLC ζ) by Percoll gradient centrifugation. Sperm with PLC ζ signal, as observed via immunofluorescence analysis, were counted. Data are presented as mean \pm SEM for more than three separate experiments. Different superscripts (a vs. b) indicate values that are significantly different ($P < 0.05$).

act as a signal for induction of apoptosis [16, 17]. Considering the fact that most blastocysts with low cell numbers are categorized as “partial blastocysts”, in which a proportion of blastomeres have died or degenerated, it may be suggested that electrical stimulation was insufficient to induce optimal activation of oocytes injected with sperm in this study. However, the high mean number of cells per blastocyst in the Percoll and Control groups may have been caused by induction of an appropriate Ca^{2+} oscillation via PLC ζ released from the injected sperm.

In conclusion, use of sperm containing an appropriate amount of PLC ζ for ICSI increases the efficiency of fertilization and embryo development without the need for any artificial stimulation. In addition, physiological oocyte activation via sperm may improve embryo quality.

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 (NARO), Tsukuba, Japan. All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Oocyte collection and *in vitro* maturation

Ovaries were obtained from pre-pubertal 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 glucose-free, HEPES-buffered Tyrode medium [18]. In brief, COCs were cultured in six-well dishes (Research Institute for the Functional Peptides, Yamagata, Japan) for 20–22 h in 100 μ l of maturation medium, a modified North Carolina State University (NCSU)-37 solution [19] containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μ M β -mercaptoethanol, 1 mM dibutyl cAMP (dbcAMP), 10 IU/ml equine chorionic gonadotropin (PMS A for Animal; ZENOAQ, Fukushima, Japan), and 10 IU/ml human chorionic gonadotropin

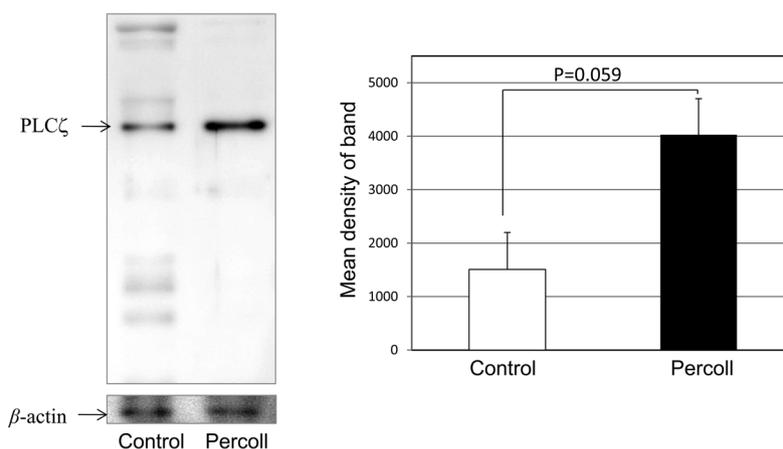


Fig 3. The sperm selected by Percoll contains comparatively more phospholipase C- ζ (PLC ζ). Western blot analysis is shown in the left panels, and the mean values of band intensities are shown in the right panel. Blotting with antibody against β -actin is also shown as a protein loading control. The concentration of sperm was 5×10^4 cells/lane. Data are presented as mean \pm SEM for three separate experiments.

Table 1. The effects of Percoll-selected sperm derived after gradient centrifugation on oocyte activation

Treatment	No. of injected oocytes	M-II (%)	Transitional Period (%)	Oocytes with IPN<	
				2PB2PN (%)	Others (%)
Control	60	33 (55.0 \pm 6.2 ^a)	6 (10.0 \pm 3.3)	19 (31.7 \pm 3.4 ^a)	2 (3.3 \pm 1.4 ^a)
Percoll	73	12 (16.4 \pm 5.4 ^b)	10 (13.7 \pm 4.9)	48 (65.8 \pm 3.0 ^b)	3 (4.1 \pm 3.0 ^a)
Cont + ES	59	2 (3.4 \pm 1.7 ^b)	13 (22.0 \pm 7.1)	33 (55.9 \pm 4.4 ^b)	11 (18.6 \pm 4.4 ^b)

The injected sperm is selected with or without Percoll gradient centrifugation (Percoll group and Control group). A part of oocytes injected with control sperm were activated by electrical stimulation (Cont + ES group). At 10 h after ICSI, oocytes were classified into following four categories: 1) M-II: metaphase-II stage, 2) transitional period: total of anaphase-II, telophase-II, metaphase-III stages, 3) 2PB2PN: oocytes with two polar bodies and two pronuclei, 4) others: more than one pronucleus formation except 2PB2PN. Different superscripts (a, b) within same column indicate values that are significantly different ($P < 0.05$). Data are presented as mean \pm SE of more than three separate experiments.

Table 2. The effects of Percoll-selected sperm derived after gradient centrifugation on *in vitro* embryonic development

Treatment	No. of injected oocytes	Blastocyst formation (%)	Mean No. of cells per blastocyst
Control	41	8 (19.5 \pm 4.1 ^a)	53.5 \pm 14.6 ^{ab}
Percoll	47	17 (36.2 \pm 6.7 ^b)	52.2 \pm 5.2 ^a
Cont + ES	44	16 (36.4 \pm 1.4 ^b)	31.1 \pm 3.9 ^b

The injected sperm is selected with or without Percoll gradient centrifugation (Percoll group and Control group). A part of oocytes injected with control sperm were activated by electrical stimulation (Cont + ES group). Different superscripts (a, b) within same column indicate values that are significantly different ($P < 0.05$). Data are presented as mean \pm SE of more than three separate experiments.

(Puberogen; ZENOAQ). 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 CO₂, O₂, and N₂ adjusted to 5%, 5%, and 90%, respectively (5% CO₂ and 5%

O₂). After the 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 [20, 21]. The spermatozoa were thawed in phosphate-buffered saline (PBS; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 5 mg/ml bovine serum albumin (BSA; Fraction V) (PBS-BSA), and centrifuged for 2 min at $700 \times g$. The sperm pellet was resuspended in PBS-BSA (Control group).

Percoll gradient centrifugation was performed as described by Noguchi *et al.* [10]. The 50% and 80% (v/v) solution of Percoll (GE Healthcare UK, Buckinghamshire, England) was prepared by adding five-strength modified Modena solution. Thawed semen (0.5 ml) was mixed with 2 ml of 50% Percoll and then slowly layered over 2 ml of 80% Percoll in a 15-ml tube. After centrifugation at $700 \times g$ for 20 min, the pellet at the bottom of the tube was recovered and washed in PBS-BSA by centrifugation at $700 \times g$ for 2 min to remove the Percoll (Percoll group). Each batch of treated sperm was maintained at room temperature (25°C) until use.

Procedure for sperm injection

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 μ M β -mercaptoethanol (IVC-PyrLac) [22], and 2) for sperm, IVC-PyrLac-HEPES supplemented with 4% (w/v) polyvinyl pyrrolidone (MW 360,000) (IVC-PyrLac-HEPES-PVP). Spermatozoa were injected as described previously [23]. About 20 oocytes were transferred to a 20- μ l drop of IVC-PyrLac-HEPES. The solution containing the mature oocytes was placed on the cover of a plastic dish (Falcon 35-1005; Thermo Fisher Scientific, Waltham, MA, USA). A small volume (0.5 μ l) of the sperm suspension was transferred to a 2- μ l 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; Nacalai Tesque, Kyoto, Japan). The spermatozoa were subjected with a piezo-pulse on their neck region and then injected into the ooplasm using a piezo-actuated micromanipulator (PMAS-CT150; Prime Tech, Ibaraki, Japan). The sperm-injected oocytes were then cultured in IVC-PyrLac at 38.5°C under 5% CO₂ and 5% O₂.

Electrical stimulation (ES)

One h after the injection, several oocytes injected with control sperm were transferred to stimulation solution consisting of 0.28 M *d*-mannitol, 0.05 mM CaCl₂ (Katayama Chemical, Osaka, Japan), 0.1 mM MgSO₄, and 0.1 mg/ml BSA and washed three times. They were then stimulated with a direct current pulse of 1.5 kV/cm for 20 μ sec, using a somatic hybridizer (SSH-2 Shimadzu, Kyoto, Japan). After stimulation, the oocytes were washed and cultured in IVC-PyrLac.

In vitro culture (IVC)

Two types of IVC medium were prepared [22]. The first was IVC-PyrLac and the second contained 5.55 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), as used in the originally reported NCSU-37 medium, and was supplemented with 4 mg/ml BSA and 50 mM β -mercaptoethanol (IVC-Glu). For the first two

days, IVC-PyrLac was used. The medium was changed once, to IVC-Glu, on the second day of IVC, and this medium was used for subsequent culture for four days. The IVC was carried out at 38.5°C under 5% CO₂ and 5% O₂.

Assessment of fertilization and embryonic development

The sperm-injected 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 proportion of fertilized oocytes was examined at 10 h after injection. We defined fertilization as the presence of two polar bodies and two PNs (2PB2PN). The rate of blastocyst formation and the mean number of cells per blastocyst were also examined at Day 6 (the day of injection and/or electrical stimulation was defined as Day 0).

Western blotting

Western blotting was carried out as described previously [24] with some modifications. Porcine PLC ζ (pPLC ζ) was detected using anti-PLC ζ rabbit serum generated against a 19-mer sequence (MENKWFSLMVRDDFKGGKI) at the N-terminus of pPLC ζ (accession no. BAC78817) [25]. Each sample was mixed with $2 \times$ Laemmli Sample Buffer (Bio Rad, Hercules, CA, USA) and was stored at -80°C until use. After denaturing by boiling at 99.5°C for 3 min, samples were separated by SDS-PAGE using 10% polyacrylamide gel and then transferred to PVDF membranes (Millipore, Darmstadt, Germany). Each membrane was blocked with blocking buffer [5% (w/v) skimmed milk in PBS] supplemented with 0.1% Tween 20 (T-PBS), followed by incubation 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 horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG; 1:5000, Cell Signaling Technology, Danvers, MA, USA) in T-PBS for 1 h at 4°C . After one wash at 15 min and five washes at 5 min each with T-PBS, peroxidase activity was visualized using the ECL Plus Western blotting detection system (GE Healthcare Japan, Tokyo, Japan), according to the manufacturer's instructions. The intensity of the bands was analyzed using ImageJ software (Ver. 1.41, National Institutes of Health, Bethesda, Maryland, USA).

Immunofluorescence assay

Immunofluorescence assay was performed as reported previously [26]. Immediately after each treatment, sperm sample was centrifuged ($600 \times g$, 2 min) and the pellet was resuspended and incubated with 3.7% paraformaldehyde in PBS for 30 min at 4°C . After centrifugation, the pellet was resuspended in PBS containing 0.1% (v/v) Triton X-100 and then 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 slide for 20 min at 37°C . The slides were blocked in 5% normal goat serum (NGS, Cedarlane Laboratories, 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, Eugene, OR, USA) as the secondary antibody. After several washings in T-PBS, the

presence of PLC ζ was observed using a standard inverse microscope equipped with appropriate standard fluorescence facilities for green fluorescent protein (GFP) dye at a magnification of $\times 200$. For each group, the fluorescence of three independent samples of more than 100 cells each was evaluated.

Statistical analysis

Percentage data were subjected to arcsine transformation [27] before statistical analysis. All data were subjected to analysis of variance (ANOVA) and Tukey's multiple range test using the Statcel 2 program (OMS Publishing, Saitama, Japan). Differences were considered significant at $P < 0.05$. All data were expressed as mean \pm SEM, and experiments were repeated more than three times.

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