Enhanced Oocyte Activation by Intracytoplasmic Injection of Porcine Spermatozoa Pre-treated with Dithiothreitol

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Abstract: The aim of this study was to confirm the effect of dithiothreitol (DTT)-treated spermatozoa on oocyte activation following intracytoplasmic sperm injection (ICSI). Boar spermatozoa with or without DTT treatment (5 mM, 30 min) were injected into in vitro matured porcine oocytes, and the nuclear phase in presumptive zygotes was observed at 3 h intervals up to 12 h after ICSI. Furthermore, developmental competence of embryos produced by DTT-treated or non-treated spermatozoa was monitored after cultivation in vitro for 144 h. Male and female pronuclear formation rates in the oocytes injected with DTT-treated spermatozoa were significantly ($P < 0.05$) higher than those in the oocytes injected with non-treated spermatozoa. Additionally, we observed that female pronuclear formation was linked to male pronuclear formation. Sperm treatment with DTT improved ($P < 0.05$) subsequent development up to the blastocyst stage. These findings confirm the efficiency of DTT in in vitro porcine embryo production mediated by ICSI. We conclude that DTT treatment improves the formation of not only male pronuclei but also female pronuclei in porcine ICSI.

Key words: Assisted reproductive technology, Dithiothreitol, Intracytoplasmic sperm injection, Oocyte activation

Received: December 8, 2008
Accepted: February 16, 2009
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Introduction

During spermatogenesis, the nuclei of spermatozoa are highly packed by the disulfide bonds between protamines, sperm-specific proteins. The paternal genetic resources are transferred into oocytes in a stable form. A spermatozoon which has penetrated an oocyte releases a 'sperm factor', which triggers the resumption of the second meiosis. Thus, early embryonic development is achieved by interaction between a spermatozoon and an oocyte. However, oocyte activation occasionally fails in *in vitro* production of embryos mediated by intracytoplasmic sperm injection (ICSI). Therefore, various activation regimes have been attempted following ICSI for cows [1–3] and pigs [4]. Moreover, it has been reported that microinjection of sperm extract into oocytes induces successful oocyte activation [5, 6].

In embryo production by ICSI, many workers have performed sperm pre-treatments with various chemicals for enhancement of male pronucleus (MPN) formation. In particular, dithiothreitol (DTT), a disulfide bond reducing agent, has been frequently used for sperm pre-treatment, and it results in adjustment of the timing of MPN formation [7]. Sperm pre-treatment with DTT also improved the rates of MPN formation [8] and normal fertilization [1, 8] in bovine ICSI. In porcine, we reported that DTT treatment of spermatozoa for 30 min prior to ICSI was effective for improvement of fertilizability and subsequent development [9]. In that study [9], DTT

treatment induced a higher normal fertilization rate rather than MPN formation only. In other words, DTT affected not only MPN but also female pronucleus (FPN) formation. However, in that study we could not demonstrate whether DTT-treated spermatozoa induced effective oocyte activation or not. To the best of our knowledge, there is no report on oocyte activation by DTT-treated spermatozoa. Therefore, detailed observations of the fertilization process of oocytes injected with DTT-treated spermatozoa are warranted to further embryo productivity and understanding of fertilization mechanisms.

We report here that boar spermatozoa treated by DTT induced improvement of FPN formation as well as MPN formation in porcine oocytes.

Materials and Methods

Oocyte collection and in vitro maturation (IVM)

Porcine ovaries were obtained at a local slaughterhouse and brought to the laboratory in sterilized saline (0.9% NaCl) solution at 37°C within 3 h. After the ovaries were washed twice in sterilized saline solution, cumulus-oocyte complexes (COCs) were aspirated from the ovaries using an 18-gauge needle attached to a 5 ml disposable syringe. Those COCs, having three or more layers of intact cumulus cells, and with uniform cytoplasm, were rinsed three times in Hepes buffered Tyrode's medium [10] containing 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, Inc., St. Louis, MO, USA: TLH-PVA), and cultured for IVM.

For IVM of oocytes, medium 199 (with Earle's salts, L-Glutamine, and 2,200 mg/l sodium bicarbonate; Sigma) supplemented with 10% (v/v) newborn calf serum (Gibco-BRL, Grand Island, NY, USA), 3.05 mM glucose (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.91 mM Na-pyruvate (Wako), 100 mM cysteamine (Sigma), 10 ng/ml epidermal growth factor (Sigma) and 75 mg/l kanamycin (Sigma) was used (IVM medium). After the selected COCs were washed three times in IVM medium, 10–15 COCs were cultured at 39°C in 5% CO_2 in air in a 100 μl droplet of the IVM medium covered with mineral oil (Sigma) with hormonal supplements (0–22 h), 10 IU/ml PMSG (Teikoku-Zouki Co., Tokyo, Japan) and 10 IU/ml hCG (Teikoku-Zouki Co.), without hormonal supplements (22–44 h).

Oocyte, sperm preparation and procedure of intracytoplasmic sperm injection (ICSI)

After IVM culture for 44 h, the oocytes were denuded of their cumulus cells by gentle pipetting. Denuded oocytes were kept in fresh IVM medium until ICSI.

Pelleted frozen boar semen [11] was thawed by adding pre-warmed (39°C) Dulbecco's phosphate buffered saline (PBS; Gibco-BRL) containing 0.1% PVA (PBS-PVA). A 100-μl aliquot of diluted semen was placed at the bottom of a 1.7-ml centrifuge tube (Multi SafeSeal™; Sorenson BioScience, Inc., UT, USA) containing 1 ml of PBS-PVA for swim-up, for 30 min at 39°C. Motile spermatozoa were recovered from the top of the tube and used for ICSI in the non-treated (control) group. In the DTT-treated group, motile spermatozoa were kept in PBS-PVA supplemented with 5 mM DTT (Sigma) at 39°C for 30 min. The DTT-treated spermatozoa were washed twice by centrifugation at 600 × g for 5 min in PBS-PVA, and used immediately for ICSI.

Sperm injection was performed using a previously reported method [12] with slight modifications. Briefly, manipulation was conducted with the aid of a pair of micromanipulators (Leitz, Wetzlar, Germany) under an inverted microscope. Seven-microliter drops of PBS-PVA containing spermatozoa and TLH-PVA containing oocytes were placed on the lid of a 50 × 9 mm Petri dish (Falcon 1006; Becton Dickinson Labware, Franklin Lakes, NJ, USA) and covered with mineral oil. A spermatozoon was aspirated into the injection pipette tail-first without immobilizing treatment such as tail-scoring/cutting, and transferred to the drop containing oocytes. After an oocyte was fixed in a position in which the first polar body was positioned at 6 or 12 o'clock, the aspirated single spermatozoon was injected into the oocyte cytoplasm and mixed with cytoplasmic components thoroughly by open tubing regulated by mouth.

In vitro culture (IVC)

Injected oocytes were immediately transferred into a North Carolina State University (NCSU)-23 medium containing 0.4% (w/v) bovine serum albumin (Sigma) and 0.5% (v/v) Eagle's minimum essential medium essential amino acids (Gibco-BRL) and washed. Oocytes were cultured (under 5% CO_2, 5%O_2 and 90% N_2 at 39°C) in 30 μl droplets of the same medium covered with mineral oil for 12 and 144 h.

Observation of pronuclear formation, cleavage and blastocyst cell number

At 3, 6, 9 and 12 h after ICSI, oocytes were fixed in 25% (v/v) acetic acid (Wako) in ethanol (Wako) for 24 h, and stained with 1% (w/v) orcein (Sigma) in 45% acetic acid solution for observation of MPN, FPN, two polar

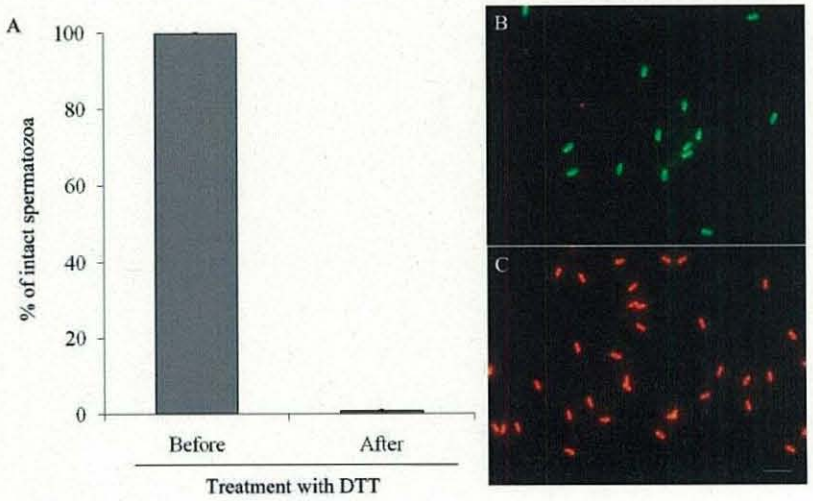

Fig. 1. Visualization of the effect of DTT on disulfide bonds in boar sperm.
DTT treatment (5 mM, 30 min) effectively reduced disulfide bonds in the sperm head (A). Percentages of disulfide bond cross-linked sperm were calculated before (B) and after (C) DTT treatment. Bar = 20 μm.

bodies and sperm tail. In this study, oocytes having 1 MPN, 1 FPN, two polar bodies and sperm tail were considered as normally fertilized.

The cleavage rate was determined at 48 h from the onset of culture. After culture for 144 h, produced blastocysts were treated with 0.5% protease (Actinase E; Kaken Pharmaceuticals, Tokyo, Japam) in PBS to digest zona pellucida. Then, they were kept in a hypotonic solution consisting of equal volumes of 1% (w/v) sodium citrate and 30% (v/v) fetal calf serum (Gibco-BRL). The samples were prepared by the gradual-fixation/air drying method [13] using ethanol in place of methanol. The slides were conventionally stained with 2% (v/v) Giemsa (Merck Japan, Tokyo, Japan) in buffered saline (pH 6.8) for 10 min. The cell number of produced blastocysts was determined by a phase-contrast microscope.

Detection of disulfide bonding pattern in sperm head

The disulfide bond integrity in sperm nuclei was detected by a previously reported method [7]. Briefly, DTT- and non-treated spermatozoa were smeared on glass slides and fixed in fixation (methanol : acetic acid = 3 : 1). After fixation, the slides were stained with 0.2% acridine orange (Sigma) for 5 min, and examined using a fluorescence microscope with a 450–490 nm excitation filter. Under these conditions, sperm nuclei with rich and reduced disulfide bonds fluoresce green and red, respectively, and sperm nuclei of intermediate status fluoresce yellow. In the present study, sperm nuclei with green and yellow colors were classified as disulfide bond intact, and red colored sperm nuclei were classified as disulfide bond reduced.

Statistical analysis

All data were analyzed by the chi-square test. Differences were considered significant when the *P* value was less than 0.05.

Results

Disulfide bond of boar spermatozoa before and after DTT treatment

A total 711 and 751 spermatozoa before and after DTT treatment, respectively, were examined for cross-linkage of the disulfide bond in the sperm head (Fig. 1). Before DTT treatment, almost all boar spermatozoa (709 out of 711: 99.7%) had a tightly-linked disulfide bond in the sperm head. However, the linkage was effectively reduced by DTT treatment for 30 min, with the percentage of intact spermatozoa decreasing to a level of less than 1% (5 out of 751: 0.7%).

Fertilizability and developmental competence of embryos derived from DTT-treated spermatozoa

A total 593 matured oocytes were injected with boar spermatozoa with or without DTT treatment. Some of these oocytes were fixed and observed for the paternal

Table 1. Effects of culture duration and DTT treatment on pronuclear formation in porcine ICSI

Culture time (h)	DTT	No. oocytes observed	No. (%) of female nuclei			No. (%) of male nuclei		No. (%) of normal fertilization
			MII	AII/TII	FPN	DSH	MPN	
3	–	45	9 (20.0)	36 (80.0)	0 (0)	16 (35.6)[a]	0 (0)	0 (0)
	+	47	3 (6.4)	43 (91.5)	1 (2.1)	32 (68.1)[b]	1 (2.1)	0 (0)
6	–	43	5 (11.6)	21 (48.8)	17 (39.5)	17 (39.5)	7 (16.3)[a]	6 (14.0)[a]
	+	47	3 (6.4)	23 (48.9)	21 (44.7)	23 (48.9)	18 (38.3)[b]	16 (34.0)[b]
9	–	42	3 (7.0)	23 (53.5)[a]	16 (37.2)[a]	16 (38.1)	8 (19.0)[a]	7 (16.7)[a]
	+	49	2 (4.1)	14 (28.6)[b]	33 (67.3)[b]	18 (36.7)	25 (51.0)[b]	24 (49.0)[b]
12	–	44	1 (2.3)	25 (56.8)[a]	18 (40.9)[a]	21 (47.7)	12 (27.3)[a]	12 (27.3)[a]
	+	48	2 (4.2)	14 (29.2)[b]	32 (66.7)[b]	13 (27.1)	26 (54.2)[b]	24 (50.0)[b]

Cumulative number of observations from six replicates. a–b: Values with different letters are different ($P < 0.05$) between the pairs. DTT: dithiothreitol, MII: Metaphase II, AII/TII: Anaphase II or Telophase II, FPN: female pronucleus, DSH: decondensed sperm head, MPN: male pronucleus, normal fertilization: both FPN and MPN formation. There was a significant interrelation between the formation rates of MPN and FPN ($r = 0.8011$, $P < 0.0001$).

Table 2. Developmental competence of porcine oocytes injected with DTT-treated boar spermatozoa

DTT	No. injected oocytes	No. (% ± SEM) cleaved oocytes on Day 2	No. (% ± SEM) developed blastocysts on Day 6	No. cells/blastocyst (Mean ± SEM)
–	121	93 (77.0 ± 3.6)	53 (42.6 ± 9.3)[a]	49.9 ± 3.6
+	107	90 (82.8 ± 7.2)	62 (56.8 ± 7.4)[b]	53.0 ± 3.0

Cumulative number of observations from six replicates. DTT: dithiothreitol. a–b: Values with different letters are different ($P < 0.05$) between the pairs.

and maternal nuclei at 3 h intervals up to 12 h after sperm injection (Table 1). At 3 h after ICSI, sperm head swelling was accelerated ($P < 0.05$) by DTT treatment and this was followed by improvement ($P < 0.05$) of MPN formation at a later period (6–12 h after ICSI). In maternal nuclei, DTT treatment decreased ($P < 0.05$) the number of AII/TII oocytes, and frequent FPN formation was concurrently induced ($P < 0.05$) at 9 h after ICSI. Fertilized zygotes with both MPN and FPN were observed at 6 h after ICSI or later. Furthermore, DTT treatment showed a higher ($P < 0.05$) fertilization rate than the non-treated group. Interestingly, as shown in the footnote of Table 1, there was a significant ($P < 0.05$) interrelation between the rates of MPN and FPN formation ($r = 0.8011$, $P < 0.0001$).

The remainder, 228 oocytes injected with spermatozoa, were cultured for 144 h for determination of their developmental competence. As shown in Table 2, 62 out of 107 oocytes (56.8 ± 7.4%) injected with DTT-treated spermatozoa developed successfully up to the blastocyst stage, which is a higher ($P < 0.05$) rate than the non-treated group. The produced blastocysts were morphologically normal (Fig. 2), and had a consistent cell number with blastocysts derived from non-treated spermatozoa.

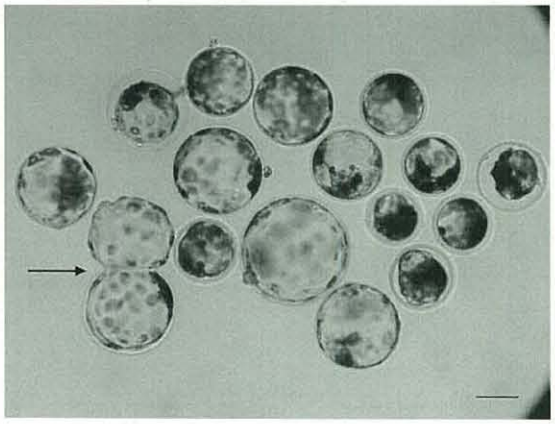

Fig. 2. Blastocysts produced by injection of DTT-treated sperm.

All blastocysts were morphologically normal. One blastocyst was hatching (arrow). Bar = 100 μm.

Discussion

In *in vitro* porcine embryo production mediated by ICSI, a major reason of fertilization failure is a low rate of formation of MPN [14]. We previously reported that

the acceleration of sperm head swelling by *in vitro* reduction of the disulfide bond using DTT successfully improved the fertilization rate in pigs [9]. In that stady [9], we also noted that DTT-treated spermatozoa apparently enhanced FPN formation. Therefore, in the present study, we investigated the effects of sperm pre-treatment with DTT on oocyte activation following ICSI. To the best of our knowledge, this is the first report of porcine sperm pre-treated with DTT improving FPN formation following ICSI.

To date, sperm pre-treatment with DTT has been the most popular and appropriate method for the improvement of fertilizability [7, 8]. The present results of sperm staining (Fig. 1) and oocyte fixation (Table 1) demonstrate that sperm pre-treatment with DTT (5 mM for 30 min) reduced the disulfide bond in the sperm head effectively. As a result of accelerated reduction of the disulfide bond, sperm head swelling and MPN formation improved at 3 and 6–12 h after ICSI, respectively. In parallel, DTT treatment led to frequent FPN formation, although the timing (9–12 h) of FPN formation was delayed compared with MPN formation (6 h or later). The improvement in FPN formation was improbably induced by the chemical effect of DTT *per se*, because the treated sperm were thoroughly flushed twice by centrifugation. It has been reported that sperm washing by centrifugation is unlikely to influence the efficiency of *in vitro* fertilization [15]. Therefore, the accelerated FPN formation observed in the present study might have been induced by physiological change of spermatozoa treated with DTT. In fact, DTT treatment destroyed sperm plasma membrane in a time-dependent manner in bovine [2]. Disrupted sperm plasma membrane may allow contact of sperm perinuclear material, that sheltered sperm factor [16, 17], with ooplasm. However, it should be noted that the rates of arrested oocytes at the MII stage after ICSI were similar between the DTT- and non-treated groups. This means that the present sperm injection procedure triggered sufficient oocyte activation. In turn, injection of DTT-treated spermatozoa resulted in later events in oocyte activation: transformation into FPN from AII/TII stage was increased. Furthermore, we observed that the formation of both MPN and FPN was frequently concomitant, leading to significant interrelation between MPN and FPN formation (Table 1). In the case of heterologous zygotes between human spermatozoa and porcine oocytes, similar outcomes were reported [18]. It might be possible to interpret this as MPN formation stimulated by DTT assisting FPN formation. However, the detailed mechanism is still unknown. Further detailed observation is required to clarify the mechanism of oocyte activation induced by injecting DTT-treated spermatozoa.

In the present study, we obtained a very high rate of blastocyst formation (56.8 ± 7.4%). To the best of our knowledge, the developmental competence observed in the present study was the highest in porcine embryo production by ICSI without artificial oocyte activation treatment. However, the rates of fertilization were relatively low (Table 1). These differences were apparently due to different oocyte quality including seasonal variation. Our previous [9] and present data strongly suggest that DTT treatment was a critical factor in the improvement of the efficiency of *in vitro* porcine embryo production mediated by ICSI.

DTT treatment prolonged for 50 min [19] or combined by Triton X-100 [20] induced no improvement of fertilizability and developmental competence. This result would have been induced by excessive DTT treatment, such as prolonged exposure to DTT and combination with Triton X-100. In fact, DTT treatment for 60 min was detrimental to embryonic development up to the blastocyst stage in pigs [9]. We also observed that prolonged DTT treatment impaired sperm's chromosomal integrity in a time-dependent manner in mice [21]. Furthermore, combination of DTT and Triton X-100 impaired chromosomal integrity in mouse spermatozoa [22]. From these observations, therefore, we propose that sperm pre-treatment should be subjected to the 'appropriate' condition for improvement of the fertilizability and developmental competence.

Compared with IVF technique, ICSI may be an unphysiological method, since capacitation and acrosome reaction bypassed spermatozoa are introduced into the ooplasm through the injection procedure. However, ICSI can avoid polyspermy, which is chronic in porcine IVF. Polyspermy involves in embryonic polyploidization, and induces critical damage in *in vitro* embryo production. Therefore, the development of the ICSI technique is essential, and the present study has demonstrated the superiority of DTT treatment as described above. However, it is still unknown whether the method of DTT treatment used in this study is the most appropriate or not. Excessive treatment induced chromosome fragmentation in mice as we reported previously [21]. The chromosome assay of boar spermatozoa using porcine oocytes is very difficult because of their low fertilizability. Additionally, we cannot use mouse oocytes that are able to detect the chromosomal integrity of human spermatozoa [23–25], since the acrosomal enzyme of boar spermatozoa

is a toxin for mouse oocytes [26]. Therefore, we are promoting the development of the chromosome detection method in boar spermatozoa. It is our goal that the most appropriate method of sperm pre-treatment is established in order to improve the production of normal porcine embryos mediated by ICSI.

In conclusion, the most significant finding in the present study was that sperm pre-treatment with DTT facilitated FPN development as well as MPN. Furthermore, the sperm treatment with DTT (5 mM for 30 min) would be appropriate for the production of a high percentage of porcine blastocysts following ICSI.

Acknowledgments

The authors thank Dr. K. Niwa, Okayama University, Japan, for supplying frozen boar semen. We also thank the Hokkaido Meat Board in Obihiro for supplying porcine ovaries. This study was partially supported by the Cooperative Study Program of the National Institute for Physiological Sciences, Japan. H.W. is supported by JSPS Research Fellowships for Young Scientists.

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