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—Original Article—

Pretreatment of bovine sperm with dithiobutylamine (DTBA) significantly improves embryo development after ICSI

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Abstract. We assessed the effect of pretreating sperm with dithiobutylamine (DTBA) to improve embryo development by intracytoplasmic sperm injection (ICSI) in cows. Acridine Orange staining revealed that when applied at different concentrations (2.5, 5, and 10 mM) and exposure times (5 min, 20 min, 1 h, and 2 h), DTBA reduced disulfide bonds in spermatozoa with the highest efficacy at 5 mM for 5 min. DTBA enhanced the percentage of spermatozoa with free protamine thiol groups compared with untreated spermatozoa (control) ($P < 0.05$); however, this result did not differ from that of dithiothreitol (DTT) treatment. The percentage of live spermatozoa after DTBA treatment was identical to that in the control, but significantly higher than that after DTT treatment ($P < 0.05$). After ICSI, DTBA treatment tended to improve male pronuclear formation rate ($P = 0.071$) compared with non-treated sperm injection. Blastocyst formation rate was significantly improved by DTBA treatment compared with that in DTT, control, and sham injection groups ($P < 0.05$). Blastocyst quality in terms of cell numbers and ploidy was not different among these groups. In conclusion, DTBA increases the efficacy of blastocyst production by ICSI even if DTT treatment does not work.

Key words: Bovine, Dithiobutylamine, Dithiothreitol, Intracytoplasmic sperm injection (ICSI)

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Intracytoplasmic sperm injection (ICSI) is an important assisted reproductive technique for overcoming low male fertility in humans [1], livestock, and especially endangered species. Moreover, this technique provides an opportunity to study the mechanisms of fertilization and early embryo development [2]. Since the first successful report of ICSI in hamsters [3], this technique has been applied to produce offspring in several species such as rabbits [4], cats [5], cattle [6], mouse [7], sheep [8], horses [9], pigs [10], and monkeys [11]. Oocytes from different mammalian species seem to differ in their response to the ICSI procedure. In humans [5], mice [7], and rabbits [4], injection of the spermatozoon into the cytoplasm is sufficient for activating the oocyte, and the sperm undergoes decondensation, resulting in the formation of the male pronucleus (PN) which later unites with the female PN to form the embryonic genome. However, in cattle, sperm microinjection alone is not sufficient for activating oocytes, which often results in the failure of male PN formation and thus low embryo developmental rates [6]. Therefore, in cattle, additional oocyte activation is essential after ICSI to ensure embryo development [12–15].

In mammalian spermatozoa, DNA is tightly packed by protamine disulfide bonds. After penetration into the oocyte, the sperm undergoes decondensation by the replacement of sperm protamines with oocyte histones, leading to chromatin relaxation [16]. In bovine oocytes, decondensation of spermatozoa to form the PN is often insufficient after ICSI [12, 17]. To solve this problem, various sperm pretreatment methods have been applied, including immobilizing sperm [18, 19], damaging sperm membranes by freezing and thawing [19, 20], treatment with Triton X-100, an anionic detergent that induces membrane damage and dissolves nuclear proteins [21, 22], or using agents to enhance decondensation of spermatozoa DNA such as heparin-glutathione [23, 24] and dithiothreitol (DTT) [12, 14, 23, 25–28]. DTT is a reducing agent frequently used to reduce disulfide bonds in bovine spermatozoa. Although many studies have determined the optimal DTT concentration for spermatozoa pretreatment to improve the developmental competence of bovine oocytes after ICSI, the results are still controversial [12, 24, 26]. Dithiobutylamine (DTBA) is another reducing agent that reduces disulfide bonds. Its structure is closely related to DTT; the primary amine group is present in DTBA, as opposed to the two hydroxyl

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groups of DTT, leading to a lower ionization constant of an acid (pK_a) values. Under identical pH conditions, the reducing ability of DTBA is more effective than that of DTT [29]. To the best of our knowledge, the efficacy of pretreating sperm with DTBA to improve ICSI efficiency has not been reported to date in any species. Thus, in the present study, we investigated the effects of pretreating sperm with DTBA on the quality of treated spermatozoa, PN formation, blastocyst development, and karyotype of resultant embryos after ICSI of bovine oocytes.

Materials and Methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

Oocyte collection and in vitro maturation (IVM)

Collection and IVM of bovine cumulus oocyte complexes (COCs) were performed as previously described [30]. Briefly, bovine ovaries were collected from a slaughterhouse, transported to the laboratory, washed, and kept in phosphate buffered saline (PBS) supplemented with 100 U/ml penicillin (Meiji Seika Pharma, Tokyo, Japan) and 100 µg/ml streptomycin (Meiji Seika Pharma) at 15°C for approximately 15 h. COCs were aspirated from small follicles (2–8 mm in diameter) using a 10-ml syringe with an 18 gauge needle. The maturation medium was HEPES-buffered TCM 199 medium (Medium 199, 12340-030, GIBCO Invitrogen, Life Technologies, Grand Island, NY, USA) supplemented with 5% newborn calf serum (NCS, S0750-500; Biowest SAS, Nuaillé, France) and 0.2 IU/ml follicle stimulating hormone (FSH; Antrin R10; Kyoritsu Seiyaku, Tokyo, Japan). Only COCs with compacted cumulus cells were selected, washed twice with IVM medium, then cultured in IVM medium under paraffin oil (Paraffin liquid, Nacalai Tesque, Kyoto, Japan) (20 COCs per 100 µl droplet) in 35-mm culture dishes (Nunclon Multidishes; Nalge Nunc International, Roskilde, Denmark) under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 20–21 h. The IVM COCs were gently denuded by repeated pipetting in 0.1% (w/v) hyaluronidase. The oocytes that extruded the first polar body (PB) (known as the metaphase II stage) were selected for these experiments.

Sperm preparation and pre-treatment

A straw of frozen Japanese Black bull semen was thawed by immersion in water at 37°C for 40 sec and then centrifuged in 3 ml of 90% Percoll solution at 740 × *g* for 10 min. The pellet was re-suspended in 5.5 ml of IVF 100 medium (Research Institute for Functional Peptides, Yamagata, Japan) and centrifuged at 540 × *g* for 5 min. The pellet was re-suspended in IVF 100 medium to adjust the final sperm concentration to 6×10^6 sperm/ml. Sperm suspensions were subjected to one of following treatments: (1) 5 mM DTT for 20 min or (2) various concentrations (2.5, 5, and 10 mM) of DTBA for various durations according to experimental design. Following each treatment, spermatozoa were washed twice with IVF 100 medium by centrifugation at 740 × *g* for 5 min. Then, sperm pellets were re-suspended in IVF 100 medium and used for experiments.

Intracytoplasmic sperm injection (ICSI)

ICSI was performed according to a previous report [31] with modifications. Briefly, 3 droplets were prepared on the lid of a 60-mm culture dish; the first was a 10% polyvinylpyrrolidone (PVP; MW = 360,000) solution (FertiCult Flushing medium; Fertipro, 8730 Beemem, Belgium) into which 2 µl of sperm suspension was transferred. The second droplet contained 0.25% trypsin and 1 mM EDTA (for washing the injection and holding pipette), and the third droplet was HEPES-buffered TCM 199 supplemented with 10% NCS for the ICSI procedure. A single spermatozoon was immobilized by pressing the tail against the bottom of the dish with the injection pipette, and then the sperm was loaded tail first into the injection pipette. An oocyte was fixed at the position where the first PB was located at 6 or 12 o'clock, and then an immobilized sperm was injected into the ooplasm at the 3 o'clock position. Sham injections applied the same method as sperm injection but no sperm was loaded into the injection pipette.

Oocyte activation

Within 1 h of injection, oocytes were activated by exposure to 7% ethanol in HEPES TCM 199 supplemented with 10% NCS for 5 min and then subsequently cultured in TCM 199 supplemented with 5% NCS for 3 h to allow extrusion of the second PB. The oocytes showing extrusion of the second PB at 3 h after activation were selected and incubated in Charles Rosenkrans 1 (CR1) medium [32] supplemented with 10 µg/ml cycloheximide under a humidified atmosphere of 5% CO_2 in air at 38.5°C for 5 h.

In vitro embryo culture (IVC)

After oocyte activation, the presumptive zygotes (20 zygotes per 100 µl droplet) were cultured in CR1 medium supplemented with amino acids (CR1aa) [33] and 5% NCS under a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C for up to 9 days. The day of ICSI was considered as day 0.

Assessment of reduced disulfide bonds in spermatozoa by Acridine Orange (AO) staining

The disulfide bond integrity in sperm nuclei was assayed by Acridine Orange (AO) staining according to the method of Tateno and Kamiguchi [25]. Briefly, spermatozoa were smeared on glass slides and fixed overnight in methanol:acetic acid (3:1). Slides were removed from the fixative and allowed to dry for a few minutes before staining. Then, slides were stained with 0.2% AO (Calbiochem, San Diego, CA, USA) (10 ml 1 g/l AO in distilled water, 40 ml 0.1 M citric acid, 2.5 ml 0.3 M Na_2HPO_4) for 5 min and examined with an excitation wavelength of 450–490 nm under an epifluorescent microscope (Olympus, Tokyo, Japan). The sperm nuclei with rich or reduced disulfide bonds fluoresced green or red, respectively. The sperm nuclei of intermediate status fluoresced yellow. In this 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.

Labeling thiol groups in spermatozoa with monobromobimane (mBBr)

To quantify the amount of free thiols in protamines, spermatozoa

were placed on glass slides, air-dried, and labeled with thiol reagent mBBr. A working solution of 0.5 mM mBBr was freshly prepared by diluting a 50 mM stock solution [34] with PBS, and this solution was added to slides and kept in the dark for 10 min at 25°C. Then, the slides were washed twice with PBS, covered with coverslips, and examined under an epifluorescent microscope (Olympus) using a 340–380 excitation filter and an emission filter over 450 nm. The proportion of fluorescence in spermatozoa was determined by Image J (v. 1.40) software [35].

Evaluation of live-dead status of spermatozoa by eosin and nigrosin staining

Eosin and nigrosin staining was carried out according to WHO standards [36]. Briefly, eosin and nigrosin solution was prepared by dissolving 0.2 g of eosin and 2 g of nigrosin in PBS (153 mM NaCl and 9.65 mM NaH_2PO_4, pH 7.4), followed by mixing for 2 h at room temperature and filtration to obtain the staining solution. After washing sperm samples in saline (154 mM NaCl) at 37°C, a drop of the sperm was placed on glass slides, which was mixed with one drop of eosin and nigrosin solution. The mixture was smeared on the glass slides and air dried. The samples were observed under a light microscope. Eosin penetrates into non-viable cells, making them appear red. Nigrosin offers a dark background, facilitating the detection of viable non-stained cells.

Assessment of apoptotic spermatozoa by TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was performed to evaluate DNA fragmentation (a marker of apoptosis) in sperm using a TUNEL assay kit (Apoptosis Detection System Fluorescein; Promega, Mannheim, Germany) according to manufacturer's instructions. Sperm suspensions were centrifuged for 10 min at 300 × *g* at 4ºC. The supernatant was discarded and the remaining pellet was washed in PBS, pH 7.4. A droplet of this sperm suspension was smeared on the slides, air-dried, and fixed by immersion in freshly prepared 4% methanol-free formaldehyde in PBS for 25 min at 4ºC. Then, the slides were washed in fresh PBS for 5 min at room temperature, then treated with 0.2% Triton X-100 in PBS for 5 min, and rinsed twice in PBS for another 5 min at room temperature. Excess liquid was removed by tapping the slides and samples were covered with coverslips. The spermatozoa were then incubated in 50 µl of a mixture containing 5 µl of nucleotide mix, 1 µl of TdT enzymes, and 45 µl of Equilibration Buffer (DeadEnd Fluorometric Tunel System Promega) in humidified chamber for 60 min at 37°C. The reaction was blocked with 2 × SSC for 15 min and then washed 3 times in PBS, 5 min each. To visualize all nuclei, a 1 µg/ml propidium iodide solution in PBS was used. In this assay, apoptotic cells are detected as green whereas PI-stained nuclei are seen as red. The percentage of spermatozoa with fragmented DNA was determined by direct observation of 100 randomly selected spermatozoa using an epifluorescent microscope (Olympus) with the appropriate filters (460–470 nm) at 40 × magnification.

Assessment of PN formation in oocytes after ICSI

At 14–18 h post ICSI, oocytes were fixed with acetic and alcohol (acetic acid:ethanol = 1:3) for at least 3 days, then stained with 1% (w/v) orcein in acetic acid, then rinsed in glycerol:acetic acid:water (1:1:3) and examined under a phase-contrast microscope with 40 × and 100 × objectives. Oocytes with at least one PN were considered to be activated whereas those with two PN without an intact sperm head inside were considered to be normally fertilized. Activated oocytes containing an intact sperm head were considered parthenogenetically activated.

Evaluation of blastocyst cell number

Blastocysts on day 9 were fixed in 99.5% ethanol supplemented with 10 µg/ml Hoechst 33342 (Calbiochem) overnight at 4°C. The total nuclei were counted under UV light with excitation at 330–385 nm and emission at 420 nm under an epifluorescence microscope (Nikon Eclipse E600, Tokyo, Japan).

Blastocyst karyotyping

Chromosomal samples of embryos were prepared and examined as described by Somfai *et al*. [37] with modifications. Briefly, after IVC for 7–8 days, blastocysts were cultured in CR1aa medium containing 5% NCS and 100 ng/ml vinblastine sulfate (Wako Pure Chemical Industries, Osaka, Japan) for 17–20 h followed by incubation in 1% (w/v) sodium citrate solution for 15 min. Each blastocyst was fixed by pouring 0.01 ml acetic methanol (acetic acid:methanol = 1:1) into 0.2 ml of a hypotonic solution of sodium citrate. A blastocyst was placed on glass slide, immediately dropped with acetic acid to separate each embryonic cell and then re-fixed with 2–3 drops of methanol:acetic acid (3:1). After drying completely, blastocyst chromosome samples were overlaid with 80% glycerol (dissolved with ultrapure water) supplemented with 10 µg/ml Hoechst 33342 and covered with a coverslip. Chromosome spreads were observed directly under UV light (405 nm excitation) with a 40 × plain objective.

Experimental design

Experiment 1 investigated the effect of DTBA applied at different concentrations (2.5, 5, and 10 mM) for various exposure times (5 min, 20 min, 1 h, and 2 h) on sperm disulfide bond integrity. Sperm suspensions were treated as described above. Then, the percentages of spermatozoa with intact or reduced disulfide bonds were determined after AO staining. Spermatozoa without any treatment were classified as a control group. Experiments were performed in triplicate.

Experiment 2 investigated the effects of disulfide reducing agents on the quality of and toxicity toward spermatozoa. In Experiment 2-1 the effects of DTBA and DTT on free thiol groups in spermatozoa were investigated by mBBr. Experiment 2-2 investigated the effect of the reducing agents DTBA and DTT on the live/dead status of spermatozoa by Eosin and Nigrosin assay. Experiment 2-3 investigated the effect of DTBA and DTT on DNA fragmentation in spermatozoa by TUNEL assay. In experiments, spermatozoa were pretreated with 5 mM DTBA and 5 mM DTT [28] for 5 min and 20 min, respectively as described above, based on the result of Experiment 1. Spermatozoa without any treatment were used as the control group. Experiments were performed in triplicate.

Experiment 3 investigated the effects of DTBA and DTT on PN formation. After IVM, oocytes were subjected to ICSI using spermatozoa pretreated with 5 mM DTBA and 5 mM DTT for 5 min and 20 min, respectively, as described above. Fourteen to 18 h post ICSI, the oocytes were fixed and stained with orcein. The

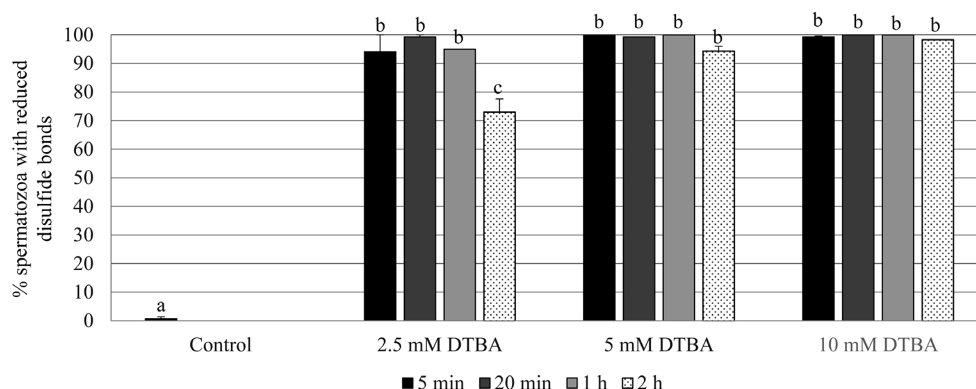

Fig. 1. Reducing effect of DTBA treatment at various concentrations and times on disulfide bonds in spermatozoa assayed using Acridine Orange. Experiments were performed in triplicate. Bars with different superscripts differ significantly (P < 0.05). DTBA: dithiobutylamine.

percentages of oocyte activation and PN formation were compared between treated and non-treated (control) spermatozoa. Experiments were performed in triplicate.

Experiment 4 investigated the effect of sperm pretreatment with DTBA and DTT on embryo development after ICSI. IVM oocytes were subjected to ICSI using spermatozoa pretreated with either 5 mM DTBA or 5 mM DTT for 5 min and 20 min, respectively, as described above. The spermatozoa without any treatment were classified as a control group. Oocytes subjected to injection without spermatozoa were used as a sham injection group. After ICSI, oocytes were subjected to IVC as described above. The cleavage rates, blastocyst formation rates, hatching rates of blastocysts, and blastocyst cell numbers were compared among DTBA, DTT, sham injected, and control groups on day 2, days 7–9, days 8–9, and day 9, respectively. Experiments were performed in quadruplicate.

Experiment 5 investigated the effect of the reducing agents DTBA and DTT on ploidy of the resultant blastocysts. The spermatozoa were pretreated with 5 mM DTBA and 5 mM DTT for 5 min and 20 min, respectively. After IVM, the oocytes were subjected to ICSI and IVC as described above. Karyotyping of blastocysts was performed on days 7 and 8 as described above. Ploidy of embryos in DTBA and DTT groups was compared with those of sham injected and control groups. Experiments were performed in quadruplicate.

Statistical analysis

All data are presented as mean ± SEM. The percentage data were arcsine transformed before analysis. The data were analyzed by one-way ANOVA using software SPSS 17.0 for Windows (SPSS, Chicago, IL, USA) and differences were compared by a post-hoc Fisher's protected least significant difference test. Differences were deemed statistically significant when P values were less than 0.05.

Results

Experiment 1: Effect of DTBA treatment at various concentrations and exposure times of spermatozoa on their disulfide bond integrity

The DTBA pretreatment significantly increased the percentage of sperm with reduced disulfide bonds at all concentrations (2.5 mM, 5 mM, and 10 mM) and exposure time (5 min, 20 min, 1 h, and 2 h) compared with the control group (P < 0.05) (Fig. 1). After 2 h of exposure, the percentage of sperm with reduced disulfide bonds in the 2.5 mM DTBA treated group was significantly lower than other sperm pretreatment groups incubated at the same time (P < 0.05) (Fig. 1).

Experiment 2: The effects of disulfide reducing agents on the quality and toxicity of spermatozoa

2-1. mBBr: In Experiment 2, treatment with DTBA was performed using 5 mM for 5 min, because in Experiment 1 this treatment increased the percentage of sperm with reduced disulfide bonds and maintained this status for 2 h, and the efficiency of reducing disulfide bonds in the 2.5 mM DTBA-treated group was gradually decreased with increased incubation time, while the higher concentration (10 mM) of DTBA affected the stability of sperm suspension which turned cloudy (data not shown). As shown in Table 1, there was no significant difference in the percentage of spermatozoa with free thiol groups in protamine between 5 mM DTBA and 5 mM DTT pretreatments (98.7% *vs*. 91.7%) but both of these values were significantly increased compared with that of the control (0.0%, P < 0.05).

2-2. Eosin and nigrosin: The 5 mM DTBA sperm pretreatment group was not significantly different than the control group in terms of the percentage of live spermatozoa (100% and 100%, respectively) whereas the 5 mM DTT sperm pretreatment group showed a significantly reduced rate of live spermatozoa (94.7%) compared with other groups (P < 0.05) (Table 1).

2-3. TUNEL assay: The percentage of sperm with DNA fragmentation following DTBA and DTT pretreatment were similar (ranging between approximately 12–13.3%) and these values were significantly higher than that recorded in the control group (0.0%, P < 0.05, Table 1).

Experiment 3: Effect of pretreating sperm with disulfide bond reducing agents on PN formation

The mean percentage of activated oocytes was similar among

Table 1. Effects of sperm pretreatment with 5 mM DTT for 20 min and 5 mM DTBA for 5 min on free thiol groups, live/dead status, and DNA fragmentation in spermatozoa

Source of reducing agent used for pretreatment	No. of spermatozoa examined	No. (%) of spermatozoa with free thiol group (Mean ± SEM)	No. (%) of live spermatozoa (Mean ± SEM)	No. (%) of TUNEL-positive sperm (Mean ± SEM)
Control (no treatment)	300	0 (0.0 ± 0.0 ^{a})	300 (100.0 ± 0.0 ^{a})	0 (0.0 ± 0.0 ^{a})
DTT	300	275 (91.7 ± 4.4 ^{b})	284 (94.7 ± 0.3 ^{b})	36 (12.0 ± 3.1 ^{b})
DTBA	300	296 (98.7 ± 0.7 ^{b})	300 (100.0 ± 0.0 ^{a})	40 (13.3 ± 0.9 ^{b})

Experiments were performed in triplicate. Different superscript letters within a column indicate significant differences (P < 0.05). DTT: dithiothreitol; DTBA: dithiobutylamine.

Table 2. Effects of pretreating sperm with 5 mM DTT for 20 min or 5 mM DTBA for 5 min on the developmental competence of bovine embryos after ICSI

Source of reducing agent used for pretreatment	No. of oocytes used for ICSI	No. of activated oocytes # (%Mean ± SEM)	Activated oocytes with (%Mean ± SEM)		
			2 PN	1 PN + sperm head	No sperm head visible
Control (no treatment)	85	75 (88.2 ± 1.2)	26 (34.5 ± 5.5)	44 (58.7 ± 3.3)	5 (6.8 ± 2.8)
DTT	85	76 (89.5 ± 1.8)	31 (40.9 ± 2.4)	45 (59.1 ± 2.4)	0 (0.0 ± 0.0)
DTBA	82	70 (85.5 ± 1.5)	35 (50.1 ± 6.4)	33 (47.1 ± 8.5)	2 (2.8 ± 2.8)
* Sham injection	75	65 (86.7 ± 1.1)	N/D	N/D	N/D

Experiments were performed in triplicate. No significant difference was detected among the treatment groups at P < 0.05. # activated oocytes = oocytes with at least one PN. * Sham injection = the oocytes were injected without sperm. 2 PN = oocytes with 2 pronuclei and no visible sperm; 1 PN + sperm = oocytes with 1 pronucleus and 1 condensed sperm. N/D: not determined. PN: pronucleus; DTT: dithiothreitol; DTBA: dithiobutylamine; ICSI: intracytoplasmic sperm injection.

all groups, ranging between 85.5–89.5%, and was not significantly different among treated spermatozoa, non-treated spermatozoa, and sham injection groups (Table 2). The percentage of PN formation was not significantly different between treated and non-treated spermatozoa. However, the DTBA-treated group showed a tendency toward a higher 2 PN formation rate compared with the control group (P = 0.071).

Experiment 4: Effect of pretreating sperm with disulfide bond reducing agents on embryo development after ICSI of bovine oocytes

As shown in Table 3, the rates of oocytes with extruded second PB in DTT- and DTBA-treated groups were significantly higher than those in the control and sham injection groups (P < 0.05). The lowest cleavage rate was observed in the sham injection group (53.0%), which was significantly lower than rates in the control, DTT, and DTBA sperm pretreatment groups (73.1, 77.8, and 84.1%, respectively, P < 0.05). The percentages of blastocyst formation on days 8 and 9 in the DTBA sperm pretreatment group (26.0% and 26.9%, respectively) were significantly higher than those in other groups (P < 0.05). In contrast, blastocyst formation rates on days 7–9 in the DTT sperm pretreatment group did not differ from those of the control group. In the sham injection group, only 8.5% of oocytes developed to the blastocyst stage, which was significantly lower than blastocyst rates of other groups (P < 0.05). No significant differences (P > 0.05) were found for hatching ability or total cell numbers of blastocysts among all groups (Supplementary Tables 1 and 2: online only).

Experiment 5: Effect of pretreating sperm with disulfide bond reducing agents on ploidy of blastocysts derived from ICSI

As shown in Table 4, the percentage of diploid blastocysts was greatly decreased in the sham injection group (14.6%) compared with the other treatment and non-treatment groups in which approximately 50% of the blastocysts formed were diploid (P < 0.05). The percentage of haploid blastocyst formation in the sham injection group had a tendency to be increased compared with control oocytes, DTT-treated and DTBA-treated groups (P = 0.071, 0.051, and 0.078, respectively); whereas the differences were not significantly different between the control and treatment groups. There was no significant difference in the percentages of embryos with other abnormalities (mixoploidy and polyploidy) among those produced in the control, sperm pretreatment, and sham injection groups.

Discussion

Our study demonstrated for the first time that the efficiency of bovine ICSI could be improved by pretreating sperm with the disulfide-reducing agent DTBA. In this study, DTBA was tested at different concentrations and various treatment intervals, and incubation with 5 mM DTBA for 5 min was found to be the optimal pretreatment method for bovine sperm. This treatment maintained spermatozoa disulfide bonds in a reduced state and maintained the stability of the sperm suspension. Previous studies showed that pretreating sperm with the disulfide reducing agent DTT significantly increased the percentage of reduced disulfide bonds in sperm heads [38, 39], which was similar to the results we obtained with DTBA in the current study.

In previous studies, DTT, a disulfide reducing agent, was applied

Table 3. Effects of pretreating sperm with 5 mM DTT for 20 min and 5 mM DTBA for 5 min on the developmental competence of bovine oocytes after ICSI

Source of reducing agent used for pretreatment	No. of oocytes (%Mean ± SEM)			No. of cultured oocytes cleaved (% Mean ± SEM)	No. of cultured oocytes developed to blastocyst on (% Mean ± SEM)		
	Injected	Extruded second PB	Cultured		day 7	day 8	day 9
Control (no-treatment)	117	97 (83.0 ± 0.9 [a])	97	71 (73.1 ± 2.7 [a])	11 (11.4 ± 1.1 [a])	16 (16.6 ± 2.2 [a])	17 (17.7 ± 1.7 [a])
DTT	117	104 (88.9 ± 0.8 [b])	104	81 (77.8 ± 5.9 [a])	14 (13.4 ± 1.0 [ab])	20 (19.2 ± 1.3 [a])	20 (19.2 ± 1.3 [a])
DTBA	122	108 (88.5 ± 1.7 [b])	108	91 (84.1 ± 2.1 [a])	22 (20.4 ± 0.5 [b])	28 (26.0 ± 1.3 [b])	29 (26.9 ± 1.4 [b])
* Sham injection	125	103 (82.3 ± 2.9 [a])	103	55 (53.0 ± 5.2 [b])	4 (3.8 ± 1.4 [c])	9 (8.5 ± 1.9 [c])	9 (8.5 ± 1.9 [c])

Experiments were performed in quadruplicate. Different superscript letters within a column indicate significant differences ($P < 0.05$). * Sham injection = the oocytes were injected without sperm. Day 0 = the day of ICSI. PB: polar body; DTT: dithiothreitol; DTBA: dithiobutylamine; ICSI: intracytoplasmic sperm injection.

Table 4. Chromosomal analysis of bovine blastocysts derived from ICSI with sperm pretreated with 5 mM DTT for 20 min or 5 mM DTBA for 5 min

Group	No. of blastocysts examined	No. (%) of blastocyst classified as (%Mean ± SEM)			
		haploid	diploid	polyploid	mixoploid
Control (no-treatment)	13	3 (22.5 ± 13.1)	6 (47.5 ± 7.2 [a])	1 (8.3 ± 8.3)	3 (21.7 ± 15.7)
DTT	14	2 (16.7 ± 16.7)	6 (43.3 ± 4.1 [a])	2 (11.2 ± 6.6)	4 (28.8 ± 10.9)
DTBA	18	3 (24.0 ± 11.1)	9 (50.2 ± 10.5 [a])	3 (14.6 ± 8.6)	3 (11.2 ± 6.6)
* Sham injection	10	5 (60.4 ± 15.7)	2 (14.6 ± 8.6 [b])	0 (0.0 ± 0.0)	3 (25.0 ± 14.4)

Experiments were performed in quadruplicate. Different superscript letters within a column indicated significant difference ($P < 0.05$). * Sham injection = the oocytes were injected without sperm. DTT: dithiothreitol; DTBA: dithiobutylamine; ICSI: intracytoplasmic sperm injection.

to bovine and porcine spermatozoa during ICSI to improve the incidence of sperm decondensation [12, 22–24, 26]. However, sperm head decondensation failure is still a major problem in bovine ICSI [12] besides the incomplete oocyte activation. The reason for this problem might be related to the type of protamine, which can be classified as Type I or Type II. Bovine spermatozoa contain only Type I protamine [40], which is rich in cysteine. Each cysteine sulfhydryl is oxidized to form an intra- or intermolecular disulfide bridge [41] which makes bovine Type I protamine more stable compared with human, mouse, and rabbit protamine [17] because most of them contain Type II protamine (containing less cysteine and more histidine) [41]. Our results suggest that DTBA has the same efficiency as DTT in reducing these disulfide bonds in bovine spermatozoa.

Since DTBA is considered a novel agent for application in bovine assisted reproduction, we decided to further test the possible side effects of pretreatment with DTBA in terms of toxicity and resulting sperm quality. The type of sperm injected into the ooplasm during ICSI has been known to affect subsequent embryo development [19, 42]. According to previous reports, immobilization of sperm by scoring its tail could increase the success rate of fertilization during ICSI [43–45]. This phenomenon is attributed to the damage of the plasma membrane of spermatozoa, which enhances nuclear decondensation of the sperm head after ICSI [44]. Furthermore, Yanagida *et al*. [46] reported that immobilized sperm could induce the timing of Ca^{2+} oscillations and release sperm factors which initiate oocyte activation. Moreover, ICSI with immobilized sperm resulted in significantly higher blastocyst development rates compared with ICSI using sperm killed by repeating freeze-thaw cycles without cryoprotectant (0.8%, 20.3%, respectively) [19]. Dead or damaged sperm release substances that are harmful to live sperm and generate reactive oxygen species (ROS) [47, 48], causing DNA fragmentation and suppressing embryo development [48]. In the present study, pretreatment with DTT and DTBA did not reduce sperm viability dramatically. After pretreatment with DTT and DTBA, most spermatozoa were still alive. Although the viability of spermatozoa in the DTT-treated group was significantly reduced compared with the control and DTBA-treated groups, the percentage of live spermatozoa in the DTT-treated group was still very high. This result confirms the safety of sperm pretreatment with DTT and DTBA.

In the present study, we also measured the frequencies of spermatozoa with DNA fragmentation after pretreatment with DTT or DTBA. DNA damage in spermatozoa impairs fertilization and embryo development [49]. However, its impact on embryo development may depend on the proportion of damage [50, 51]. TUNEL assays revealed that pretreating sperm with DTT and DTBA significantly increased the proportion of sperm DNA fragmentation compared with the control group. This result was in concordance with the findings of Sekhavati *et al*. [24] on DTT; however, in that study, the percentage of TUNEL-positive spermatozoa in the DTT-treated group was notably higher (90%) compared with our result (12.0%). This difference may be due to different incubation times with DTT. Sekhavati *et al*. [24] pretreated sperm with DTT for 1 h, whereas we incubated sperm in reducing agents for only 20 min. This discordant result between protocols suggests that longer DTT incubation time is likely increases the percentage of spermatozoa with fragmented DNA, possibly affecting embryo development.

In this study, pretreatment of sperm with DTT had no effect on developmental competence of bovine ICSI oocytes, which is in agreement with several previous reports [14, 24, 26] but in contrast with the study of Rho *et al*. [12]. Our study found that DTT did not significantly increase the percentage of male PN formation, which was in agreement with the findings of Tian *et al*. [22] but contradicted with the reports of Rho *et al*. [12] and Suttner *et al*. [14]. On the other hand, sperm pretreatment with DTBA significantly increased the blastocyst formation rate of bovine ICSI oocytes. Furthermore, DTBA treatment was associated with a tendency to increase normal fertilization as characterized by the presence of 2 PN compared with the control group ($P = 0.071$). Although, DTT and DTBA treatment significantly reduced the disulfide bonds in sperm as demonstrated by red Acridine Orange fluorescence, none of these treatments improved the male PN formation rate. This result is in agreement with that of Cheng *et al*. [23] who reported that reduction of disulfide bonds after sperm pretreatment did not correlate with male PN formation in pigs. These discrepancies may be explained by the fact that reducing agents may elicit harmful effects on bovine sperm chromatin, which in turn causes negative effects on male PN formation. Therefore, we speculate that PN formation in the current ICSI system may be affected by an ooplasmic mechanism(s) other than S-S reduction in the sperm nucleus. Moreover, a significant relationship between the disulfide-rich and free thiol groups and the sperm decondensation rate could not be detected [39]. Previously, an abnormal pattern of sperm decondensation was observed after ICSI in rhesus monkeys [52]. Additionally, Sekhavati *et al*. [24] reported the altered expression of developmentally important genes in the DTT-treated group, which lead to improper embryo formation.

In the present study, sperm pretreatment with DTBA resulted in a higher blastocyst formation rate after ICSI, compared with pretreatment with DTT; however, the reason underlying this difference is unclear. It has been verified that DTT at a concentration of 5 mM for 20 min exerts more toxic effects on sperm than DTBA at a concentration of 5 mM for 5 min (Table 1). This toxic effect on sperm may have been manifested after ICSI, thus lowering embryo development to a level where the advantages of disulfide bond reduction could not be detected, which could be related to the pK_a value. The reactivity of a dithiol depends on the pK_a value. In the reduction of disulfide bonds, the maximum apparent rate of a thiol-disulfide interchange is observed for a thiol whose pK_a is approximately equal to the pH of the solution [53, 54]. The pH of the fertilization medium is approximately 7.8, which is lower than the pK_a of DTT (9.0), whereas the thiol pK_a value of DTBA is approximately 8.2. Thus, the active form of DTT is easily converted to its inactive form. Alternatively, because DTBA has an amino group unlike DTT, DTBA may be retained within the sperm for longer, which may prevent reoxidation of sperm and lead to efficient PN formation. Further research will be required for clarification of this issue. Alternatively, the DTBA-treated group tended to show increased PN formation compared with the control group. Thus, we speculated that the high blastocyst formation rate in the DTBA-treated group may be due to the increased percentage of PN formation. Regarding the efficacy of activation stimuli following ICSI on oocyte activation, no significant difference was observed in the incidence of oocyte activation after ICSI among the treated, non-treated, and sham injection groups. Additionally, some activated oocytes in the sham injection group could develop to the blastocyst stage. According to Li *et al*. [55], such blastocysts may develop owing to parthenogenetic activation caused by the injection and activation protocol. Without blockade of the second PB extrusion, parthenogenetic bovine oocytes can develop into haploid blastocysts, which are characterized by low cell numbers [56]. Because insufficient male PN formation is still frequent in bovine ICSI, the possibility that ICSI-derived blastocysts are a result of parthenogenetic development cannot be excluded.

In this study, to monitor the formation of haploid embryos, blastocysts derived from ICSI were subjected to chromosomal analysis. The percentages of diploid and haploid blastocysts were similar among sperm-injected groups irrespective of pre-treatment, whereas the percentage of diploid blastocysts was significantly lower in sham injection group than in the sperm-injected groups. This result is in agreement with that of a previous study using DTT [12] and suggests that sperm pretreatment with reducing agents does not affect embryo ploidy in bovine ICSI. Nevertheless, the results disagree with the findings of Cheng *et al*. [23] who reported greatly decreased diploid blastocyst formation rates after DTT treatment compared with those in the control group. It was only in the sham injection group that the reduced frequency of diploid embryo formation was associated with an increased tendency towards haploidy, which can be explained by the lack of the fertilizing sperm head. The frequency of blastocysts with other chromosomal abnormalities (such as polyploidy and mixoploidy) was not significantly different among treated, non-treated and sham injection groups. Previous studies have reported that the percentage of chromosomal anomalies were dramatically increased in ICSI blastocysts [12, 23], which was in accordance with the findings of this study that showed a relatively high proportion of mixoploid and polyploid blastocysts. When oocytes are fertilized monospermically, polyploid embryos can be generated by the failure of the second PB extrusion [57], whereas mixoploidy may occur when karyokinesis is not associated with cytokinesis in some blastomeres during early development [58]. Chromosome abnormalities such as polyploidy and haploidy compromise embryo development and may result in pregnancy failure [59, 60]. Nevertheless, mixoploid embryos containing diploid blastomeres may have the potential to develop to term as polyploid cells normally contribute to the formation of the trophectoderm [61, 62].

In conclusion, DTBA pretreatment could reduce disulfide bonds in sperm. Treatment of spermatozoa with 5 mM DTBA for 5 min prior to ICSI significantly enhanced the blastocyst formation rate after ICSI and IVC in bovine oocytes, which was associated with a tendency towards increased percentage of normal fertilization; however, the precise mechanism of action of DTBA is unknown. Although DTT treatment was not effective, DTBA treatment could still improve the embryonic development. DTBA-pretreated sperm could be considered safe and could be used as an alternative for application in bovine ICSI. Further experiments involving embryo transfer into surrogates are required to elucidate whether the pretreatment of sperm with DTBA before ICSI manifests its positive effects on offspring production.

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