

Sperm Pretreatment with Dithiothreitol Increases Male Pronucleus Formation Rates After Intracytoplasmic Sperm Injection (ICSI) in Swamp Buffalo Oocytes

Vibuntita CHANKITISAKUL¹, Nutthee AM-IN¹, Theerawat THARASANIT¹, Tamas SOMFAI², Takashi NAGAI² and Mongkol TECHAKUMPHU¹

¹Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand

²NARO Institute of Livestock and Grassland Science (NILGS), Ibaraki 305-0901, Japan

Abstract. Failure of male pronucleus formation has hampered the success of intracytoplasmic sperm injection (ICSI) in swamp buffalo. The aim of the present study was to improve male pronucleus formation by pretreating sperm with various chemicals before ICSI. In Experiments 1 and 2, sperm were treated according to one of the following protocols: (1) 0.1% Triton-X 100 (TX) for 1 min, (2) 10 μ M calcium ionophore (CaI) for 20 min, (3) freezing and thawing (FT) without any cryoprotectant, or (4) no treatment (control). These sperm treatment groups then either did or did not receive additional sperm treatment with 5 mM dithiothreitol (DTT) for 20 min. Acrosomal integrity (Experiment 1) and DNA fragmentation (Experiment 2) were evaluated in the sperm before ICSI. In Experiment 3, oocytes matured *in vitro* were subjected to ICSI using pretreated sperm as described above and then were cultured either with or without activation. The TX- and CaI-treated sperm caused an increase in the number of acrosome-loss sperm, whereas the FT treatment and control increased the proportion of acrosome-reacted sperm ($P < 0.05$). The DNA fragmentation did not differ among treatments ($P > 0.05$). At 18 h post-ICSI, pronucleus (PN) formation was found only in activated oocytes. The majority of the activated ICSI oocytes contained intact sperm heads. Normal fertilization was observed in the CaI and FT treatment groups and control group when sperm were treated with DTT before ICSI. In conclusion, DTT treatment of sperm with reacted acrosomes before ICSI together with activation of the ICSI oocytes is important for successful male pronucleus formation.

Key words: Decondensation, Dithiothreitol, Intracytoplasmic Sperm injection, Swamp buffalo

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In buffalo, knowledge of intracytoplasmic sperm injection (ICSI) is limited due to few numbers of studies having been published in this field. We have previously reported that an injected sperm itself poorly stimulates the activation process in buffalo oocytes. Although additional chemical activation following ICSI can promote the development of buffalo ICSI oocytes to the blastocyst stage, a failure of male pronucleus formation has been largely observed [1]. A possible reason for this failure may be the lack of an important signaling molecule when a sperm head is injected into an oocyte. In the case of ICSI, the sperm nucleus is injected into ooplasm along with the perinuclear material, acrosome, and plasma membrane [2]. Theoretically, phospholipase C zeta (PLC ζ), a sperm-borne oocyte-activating factor (SOAF) that is localized in the acrosomal and post-acrosomal regions of the perinuclear theca (PT) membrane [3, 4], may not be able to penetrate through the sperm plasma membrane, resulting in a limited amount of PLC ζ available for oocyte activation. This event differs from normal fertilization where the PT is rapidly solubilized following fusion of the sperm plasma membrane with

the oolemma, leading to the release of SOAF and other factors in the ooplasm [5]. Not only sperm factors, but also oocyte contents, especially glutathione, seem to be prerequisite for the reduction of disulfide bonds in the sperm nucleus and promote male pronuclear formation during fertilization [6, 7]. However, it is possible that this disulfide-reducing agent might not affect to sperm nucleus if the sperm plasma membranes are still intact. Therefore, to increase the success rate of ICSI, disintegration of sperm plasma membranes and removal of acrosomes were performed before sperm injection by mechanical stimulation with an injection pipette in humans and certain mammalian species including mice and rabbits [8–10]. These studies have demonstrated that removal of the sperm plasma membrane and acrosome not only accelerates the onset of oocyte activation but also improves embryonic development after ICSI.

Compared with laboratory animals, ICSI is far less efficient in livestock species [11]. Simply put, physical damage seems to be insufficient for disrupting the membrane. As a result, SOAF cannot be released into the ooplasm, and the result is the blockage of sperm head decondensation and oocyte activation [5, 12–14]. This is likely due to a higher rigidity of the PT [4, 15] and a large number of acrosomes and acrosomal enzymes entering the ooplasm after sperm injection [16]. To improve normal fertilization following ICSI, sperm pretreatments before injecting have been successfully performed to improve the developmental rates of ICSI oocytes in cattle [17–19]

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*Correspondence: M Techakumphu (e-mail: tmongkol@chula.ac.th)

and pigs [20–23]. Various sperm pretreatment protocols have been used to disrupt the acrosomal and sperm plasma membrane, such as using Triton-X 100 [20, 23], calcium ionophores [17, 22, 24] and freezing/thawing without a cryoprotectant [25]. Interestingly, it has been suggested that bull and boar sperm chromatin are tightly packaged and more stable than those of other species [26, 27]. Therefore, dithiothreitol (DTT), a chemical that specifically reduces disulfide bonds of sperm protamines, has been used previously to promote the decondensation of sperm chromatin in ICSI oocytes in cattle [19, 28] and to improve oocytes developmental capacity in pigs [23, 29].

Although this approach has not been investigated in buffalo, it seems possible that the presence of a sperm plasma membrane and acrosome, along with tightly packaged sperm chromatin, could contribute to the failure of male pronucleus formation and oocyte activation in swamp buffalo after ICSI. Therefore, the present study was designed to investigate the efficacy of various chemical and physical treatments on sperm prior to ICSI to improve male pronucleus (MPN) formation in buffalo. The quality of the buffalo sperm after each treatment was evaluated in terms of acrosomal integrity and DNA fragmentation. In addition, we examined the effects of the sperm pretreatments on MPN formation in ICSI oocytes.

Materials and Methods

All chemicals used in this study were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise stated.

Oocyte recovery and maturation

Swamp buffalo ovaries were obtained from animals of unknown reproductive status at a local slaughterhouse and were transported to the laboratory within 4 h in 0.9% (w/v) saline supplemented with 100 IU/ml penicillin G and 100 µg/ml streptomycin at 28–30 C. The ovaries were later washed twice in phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin G and 100 µg/ml streptomycin. The oocytes were subsequently aspirated from 2–8 mm antral follicles with an 18-gauge needle attached to a 10 ml syringe. The oocytes were morphologically selected under a stereomicroscope (SMZ645, Nikon, Tokyo, Japan) ×400 magnification. Only cumulus–oocyte complexes (COCs) with homogenous ooplasm that were surrounded by multiple compact layers of cumulus cells were submitted for *in vitro* maturation. Groups of 25–30 COCs were placed in 4-well plastic dishes (Nunc, Roskilde, Denmark), with each well containing 500 µl NaHCO₃ buffered tissue culture medium 199 (with Earle's salts) supplemented with 10% (v/v) fetal calf serum (FCS), 50 IU/ml human chorionic gonadotropin (Intervet/Schering-Plough, Boxmeer, the Netherlands), 1 µl/ml insulin–transferrin–selenium, 0.05 IU/ml recombinant human follicle-stimulating hormone (Organon, Bangkok, Thailand), 100 µM cysteamine, 20 ng/ml epidermal growth factor, 100 IU/ml penicillin G and 100 µg/ml streptomycin. *In vitro* maturation was performed for 21 h at 38.5 C in a humidified atmosphere of 5% CO₂ in air. Then, the cumulus cells were completely removed by gentle repeated pipetting. Only oocytes with a visible first polar body were selected, after which they were maintained in Hepes-buffered synthetic oviductal fluid (H-SOF) until further treatment.

Sperm preparation and treatment

Frozen semen from the same batch of one bull provided by the Thai Swamp Buffalo Conservation and Development Center (TSBCDC; Charoen Pokphand, Thailand) was used in this study. Cryopreservation of sperm was carried out as described previously [30]. Briefly, the ejaculated semen was extended, in one step, in Tris-egg yolk extender plus 8% glycerol to a final concentration of 120×10⁶ sperm/ml. Thereafter the extended semen was slowly cooled to 4 C over a period of 2–4 h. Then the spermatozoa were loaded into 0.25 ml plastic straws and frozen using a programmable biological freezer, with the temperature being decreased at a rate of 18 C/min from 4 C to –40 C and 8 C/min from –40 C to 140 C before the straws were plunged into liquid nitrogen. The semen was thawed at 37 C in a water bath for 30 sec. The thawed semen was then layered on top of two layers of Percoll density gradient consisting of 1 ml each of 45% and 90% Percoll in a 15 ml plastic conical centrifuge tube. The tube was then centrifuged at 800 × g for 15 min, after which the supernatant was removed, leaving only the sperm pellet. The sperm pellet was washed using 1 ml Tyrode's albumin lactate pyruvate (TALP) and centrifuging at 600 × g for 5 min. The supernatant was removed leaving 100 µl containing the sperm suspension in the tube, which was then used for further treatment.

The sperm suspensions were subjected to one of the following treatments: (1) Triton-X 100 (TX) treatment in which 50 µl of sperm suspension was diluted in 50 µl 0.2% (v/v) TX and incubated for 1 min according to Lee and Yang (2004) [20]; (2) calcium ionophore (Cal) treatment in which 50 µl of sperm suspension was exposed to 10 µM CaI for 20 min at 37 C according to Nakai *et al.* (2003) [22]; (3) freezing and thawing (FT) treatment in which sperm were frozen without any cryoprotectant using –20 C refrigerators and then thawed in 37 C water according to Goto *et al.* (1990) [25]; or (4) no treatment in which sperm did not receive any treatments (control). For each group, the sperm were then divided into two subgroups, one of which was treated and one of which was not treated with 5 mM dithiothreitol (DTT) for 20 min at room temperature according to Rho *et al.* (1998) [19]. Following each treatment, the sperm were washed once with 5 ml TALP by centrifugation at 800 × g for 5 min. The sperm pellets were then resuspended in H-SOF and used for either further examination of acrosomal integrity and DNA fragmentation or ICSI, as is described in the experimental design.

Acrosomal integrity of spermatozoa

The integrity of sperm acrosomes was evaluated using fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) staining. Briefly, 10 µl sperm suspension was mixed with 10 µl ethidium homodimer-1 (EthD-1) and incubated at 37 C for 15 min. Then, 5 µl mixture was smeared on a glass slide and air-dried. The samples were then fixed using 95% ethanol for 30 sec and air-dried again. The acrosomes were labeled using 50 µl FITC-PNA (FITC-PNA:PBS dilution = 1:10 (v/v)), and the slides were incubated in a dark moist chamber at 4 C for 30 min. Slides were then rinsed with cold PBS and air-dried. Sperm were assessed under a fluorescent microscope at ×1,000 magnification and categorized into the following three groups: (A) acrosome-intact sperm having bright fluorescence over the entire acrosomal cap, indicating the presence of the outer acrosomal membrane; (B) acrosome-reacted

sperm having a patchy disrupted fluorescence over the acrosome or demonstrating fluorescence limited to segments of the acrosomal cap; and (C) acrosome-loss sperm displaying no FITC-PNA staining but instead showing red fluorescence due to counterstaining with EthD-1 [31].

DNA fragmentation of spermatozoa

DNA fragmentation was assessed using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. In brief, the sperm suspension was first smeared on a glass microscopic slide coated with aminopropyltriethoxysilane and allowed to air-dry at room temperature. Each slide containing sperm was fixed with 4% paraformaldehyde for 30 min. After washing with PBS, the sperm were permeabilized on ice with 0.1% (v/v) Triton X-100 in PBS for 5 min. Detection of DNA fragmentation was performed using an In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the slides containing sperm were washed in PBS and incubated with the TUNEL reaction mixture (TdT enzyme:label solution = 1:10 (v/v)) for 1 h at 37°C in a humidified chamber. Then the chromosomes were labeled using 5 µg/ml of 4',6-diamidino-2-phenylindole (DAPI). An antifade mounting medium (VECTASHIELD™; Vector Laboratories, Burlingame, CA, USA) was used to retard photobleaching that might occur during analysis under the fluorescence microscope. The TUNEL-negative sperm fluoresced red, while the TUNEL-positive sperm fluoresced bright green. TUNEL-positive sperm were classified as apoptotic or DNA-fragmented sperm.

Intracytoplasmic sperm injection (ICSI)

Conventional ICSI was performed using an inverted microscope (IX71; Olympus, Tokyo, Japan) equipped with a micromanipulator (IM-9B; Narishige, Tokyo, Japan). The inner diameter of the injection needle was 9–11 µm (Origio Humagen Pipets, Charlottesville, VA, USA; REF No. MIC-9-30), and the inner diameter of the holding pipette was 10–20 µm. The treated sperm were diluted using 10% polyvinylpyrrolidone in H-SOF. Sperm were injected as described previously [1]. Injection occurred within one hour of each sperm treatment. Sham injection of oocytes was done following the same procedure as sperm injection, except that no sperm was loaded into the injection pipette.

Half of the sperm-injected oocytes and sham-injected oocytes were then exposed to 5 µM calcium ionophore (A23187) in SOF medium supplemented with 3 mg/ml bovine serum albumin (SOF-BSA) [32] for 5 min and incubated for 5 h with 10 µg/ml cycloheximide in SOF-BSA at 38.5°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. The remaining half of the oocytes were not exposed to chemical activation.

After the ICSI procedure was completed, presumptive zygotes were cultured in a 50 µl droplet of SOF supplemented with 3 mg/ml BSA for 18 h at 38.5°C in an atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

In vitro fertilization

After 21 h of IVM, groups of 10 COCs were fertilized with sperm at a final concentration of 2 × 10⁶ sperm/ml in a 50 µl droplet of TALP medium supplemented with 20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine. The semen was prepared with

Percoll as mentioned above. IVF was performed for 12 h at 38.5°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Excessive cumulus cells and sperm were then removed by repeated pipetting in culture medium containing 1 mg/ml hyaluronidase. Ten to fifteen presumptive zygotes were then cultured for 6 h.

Assessment of oocytes

Eighteen hours after culture, the oocytes were fixed and stored in 4% (w/v) paraformaldehyde in PBS until analysis. The chromosomes in the oocytes and the sperm heads were labeled using DAPI staining to precisely examine the nuclear status. The fluorescently labeled samples were then mounted on a glass microscope slide in 2 µl of antifade medium to retard photobleaching and visualized using an epifluorescence microscope (BX51; Olympus). Oocytes with one or two pronuclei and at least one polar body were considered activated, while those with both male and female pronuclei in the absence of a sperm head were considered normally fertilized. An activated oocyte containing a sperm head was considered to have been activated parthenogenetically but not fertilized.

Experimental design

Experiment 1; Effects of the sperm treatments on acrosomal integrity: To assess acrosomal integrity after treatment with protocols 1–4 mentioned above, sperm in each group were divided into two groups and either treated with 5 mM DTT or left untreated, after which they were fixed and evaluated for acrosomal integrity using FITC-PNA labeling. The experiment was replicated at least three times.

Experiment 2; Effects of the sperm treatments on DNA fragmentation: To examine the effect of sperm treatment on DNA fragmentation, DNA fragmentation of the sperm in each group (according to Experiment 1) was evaluated using a TUNEL assay. The experiment was replicated at least three times.

Experiment 3; Effects of the sperm treatments on pronucleus formation: To examine MPN formation in presumptive zygotes obtained by ICSI using pretreated sperm, matured oocytes were subjected to ICSI using sperm pretreatments as described in Experiment 1, with or without subsequent oocyte activation. Nuclear changes of the presumptive zygotes were morphologically evaluated for pronucleus formation using DAPI and epifluorescent microscopy at 18 h post ICSI. The experiment was replicated three times.

Statistical analysis

In Experiments 1 and 2, all of the data on acrosomal and DNA integrity of the sperm from each treatment group before ICSI were tested for normal distribution of residuals from the statistical models using the UNIVARIATE procedure with the NORMAL option. The effects of treatments were examined using the GLM procedure. The LSMEANS statement was used to compare the results among the treatment groups. In Experiment 3, the percentage of MPNs, defined as the number with successful fertilization, was compared among treatment groups by using chi-square and Fisher's exact statistical tests with SAS 9.2 (SAS Institute, Cary, NC, USA). P < 0.05 was defined as statistically significant.

Results

Experiment 1: Effects of the sperm treatments on acrosomal integrity

The distributions of sperm with different acrosome statuses in various pretreatments are shown in Fig. 1. The TX- and CaI-treated sperm caused an increase in the number of sperm without acrosomes (acrosome-loss sperm) compared with sperm treated by FT or the control ($P < 0.05$). Conversely, the proportion of acrosome-reacted sperm was higher in the FT-treated sperm and control groups than in the other groups ($P < 0.05$). DTT had no effect on the acrosomal status of the buffalo sperm ($P > 0.05$). The morphology of the sperm acrosomes is shown in Fig. 2(a).

Experiment 2: Effects of the sperm treatments on DNA fragmentation

The DNA fragmentation following treatment of the sperm in each group was less than 3%, and it did not differ significantly ($P > 0.01$) among the treatments (Fig. 3). The morphology of the sperm DNA fragmentation is shown in Fig. 2(b).

Experiment 3: Effects of the sperm treatments on pronucleus formation

As shown in Table 1, oocyte activation occurred only in the ICSI (+) activation groups and at similar rates (approximately 60–80%). None of the ICSI (–) activation oocytes showed meiotic resumption (data not shown). Irrespective of sperm pretreatment, all of the ICSI activated oocytes contained intact sperm heads when DTT was not applied. Normally fertilized oocytes with 2 PNs (1 MPN) without intact sperm heads were observed in the CaI (+) DTT, FT (+) DTT, and DTT treatment groups (8.9, 23.5 and 31.0%, respectively).

Discussion

Our previous study demonstrated a failure of male pronucleus formation after ICSI in swamp buffalo oocytes [1]. In the present study, we investigated the effects of sperm pretreatments on male pronucleus formation following ICSI. We hypothesized that the presence of a reacted acrosome together with the reduction of disulfide bonds in the sperm nucleus as a result of DTT treatment [19, 33] would promote its decondensation and MPN formation, while the loss of acrosomes, induced by TX or CaI, could not induce male pronucleus formation, even after DTT treatment.

Injection of intact sperm allows decondensation of sperm chromatin in humans and certain other mammalian species [8–10]. In contrast, decondensation of sperm heads has been reported to be very low after injection of untreated sperm in sheep, cattle and pigs [19, 34, 35]. The difference in sperm decondensation in these species has been indicated as being due to a higher rigidity of the sperm plasma membrane surrounding the ooplasm after sperm injection in cattle and pigs [4, 15]. Most previous studies reported an improvement of sperm head decondensation and MPN formation after sperm were freed from the plasma membrane before ICSI, because the damage to the sperm plasma membrane was possibly contributing to the release of factors involved in sperm head decondensation and oocyte activation [17, 36, 37]. In addition, a large amount of acrosomal

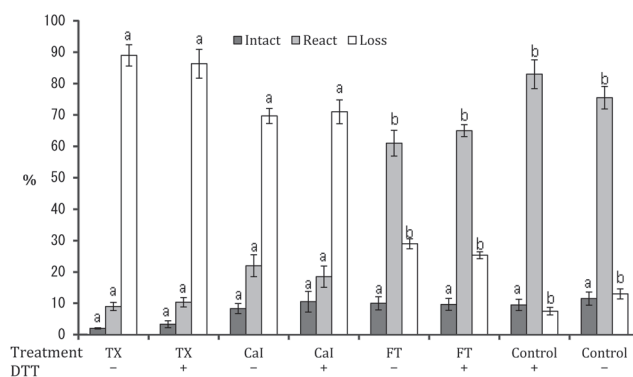


Fig. 1. Percentages of buffalo sperm with various acrosomal configurations in different pretreatments combined with DTT treatment (a, b significantly different within the same color bar, $P < 0.05$). (TX, Triton-X 100; CaI, calcium ionophore; FT, freezing and thawing without any cryoprotectant; Control, untreated sperm; DTT dithiothreitol; +, with DTT; -, untreated DTT).

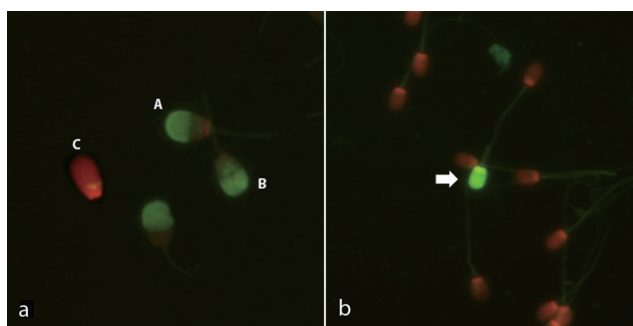


Fig. 2. a: Acrosome (green: FITC-PNA) and DNA (orange: EthD-1) of the buffalo sperm. A: Acrosome-intact sperm show bright fluorescence over the acrosomal cap. B: Acrosome-reacted sperm display patchy disrupted fluorescence over the acrosome. C: Acrosome-loss sperm show no FITC-PNA staining. b: Buffalo sperm stained with TUNEL. DNA fragmentation is identified by bright green fluorescence over the sperm head (arrow).

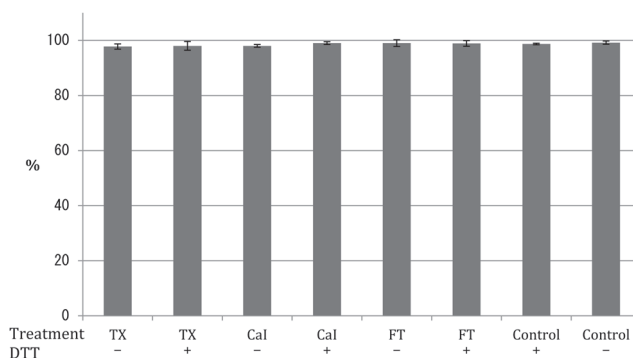


Fig. 3. Percentages of DNA fragmentation in various pretreatments of buffalo sperm combined with DTT (a, b significantly different within the same color bar, $P < 0.05$). (TX, Triton-X 100; CaI, calcium ionophore; FT, freezing and thawing without any cryoprotectant; Control, untreated sperm; DTT dithiothreitol; +, with DTT; -, untreated DTT).

Table 1. Activation and male pronucleus formation of buffalo oocytes injected with sperm after various treatments followed by oocyte activation

Sperm pretreatment	DTT treatment	No. of oocytes injected	No. of oocytes activated ^a	
			Total (%) ^b	With MPN (%) ^c
TX	+	57	42 (73.4)	0 ^d
	-	33	28 (84.8)	0
CaI	+	63	45 (66.2)	4 (8.9) ^{d, e}
	-	45	39 (86.7)	0
FT	+	46	34 (74.0)	8 (23.5) ^{e, f}
	-	64	54 (84.4)	0
Untreated	+	46	29 (63.0)	9 (31.0) ^f
	-	40	32 (80.0)	0
Sham	-	51	41 (80.4)	N/A
IVF	-	56	36 (64.3)	36 (100.0) ^g

^a Oocytes were examined 18 h post ICSI. ^b Percentage of oocytes injected. ^c Percentage of total oocytes activated. ^{d-g} Different superscript letters within a column signify significant differences ($P \leq 0.05$). N/A: no sperm was injected.

contents and enzymes have appeared to be harmful to oocytes in mice, indicating that removal of the acrosome from the sperm before ICSI is essential for successful ICSI [16, 24]. The disintegration of the sperm plasma membrane and removal of the acrosome have been simultaneously investigated using various treatments [16, 17, 28]. In our study, damage to the sperm plasma membrane and acrosome was effectively induced using TX and CaI treatments. Evaluation of sperm acrosomal integrity indicated that sperm treatment with TX and CaI apparently caused a significant increase in the number of acrosome-loss sperm. However removal of the sperm plasma membrane and acrosome did not promote male pronucleus formation and oocyte activation after ICSI in swamp buffalo in this study.

Decondensation of sperm chromatin was enhanced when sperm were treated with DTT before ICSI in the CaI (+) DTT, FT (+) DTT and DTT treatment groups. Dithiothreitol is an agent that specifically reduces disulfide bonds in sperm chromatin. Because the protamine in bull and boar sperm chromatin is a cysteine-rich type 1 protein, resulting in the chromatin configuration being tightly packaged and stable [26], DTT has been suggested for gaining access to the sperm nucleus through the PT by reduction of the protamine disulfide bonds resulting in decondensation of the chromosomes [4, 19]. Interestingly, sperm treatment with DTT following Triton-X (TX (+) DTT) pretreatment could not induce male pronucleus formation in our study. This outcome is not in accordance with previous reports in cattle and pigs, in which TX (+) DTT sperm treatment accelerated sperm head decondensation after injection [38, 39]. Further, in our study we noticed that even though CaI (+) DTT sperm treatment could promote male pronucleus formation at a low rate (8.9%), the majority of activated oocytes contained intact sperm heads. This rate also did not differ significantly from that of TX (+) DTT. Conversely, when treatment groups containing high rates of sperm with reacted acrosomes (i.e., the control and FT groups) were treated with DTT, the highest MPN formation rates were obtained after ICSI. The high rate of sperm with reacted acrosomes in the control group in this study can be explained by damage to the sperm plasma membrane during freezing and thawing, even in diluents containing

cryoprotectant under standard procedures for frozen semen at TSBCDC. Therefore, we speculated that the status of acrosomal integrity had an effect on male pronucleus formation after ICSI.

Our results demonstrate that the proportion of sperm without acrosomes is significantly increased by TX (+) DTT and CaI (+) DTT treatments but not by FT (+) DTT and DTT treatments. Although suppressed MPN formation by TX treatment could theoretically be caused by a side effect on the nuclear structure, TUNEL staining demonstrated that no difference existed for DNA integrity among the treatment groups. It is therefore possible that the methods for buffalo sperm plasma membrane and acrosomal damage using TX and CaI, at least in our study, might have either caused the premature release of the acrosomal matrix containing specific substances or the removal of certain extranuclear components that participate in the activation process. It is important to note that the amount of PLC ζ in treated boar sperm was significantly lower than in whole untreated sperm, resulting in a weakened sperm activation signal [40]. It is possible that such side effects may lead to the failure of sperm chromatin decondensation [41, 42].

Although DTT promoted male pronucleus formation in this study, the oocytes injected with DTT-treated sperm in the non-activated group did not undergo meiotic resumption. Because it has been reported that sperm introduced by ICSI in oocytes generated insufficient calcium oscillation to activate the oocytes [43], additional activation may be required to compensate for insufficient stimulation of the buffalo oocytes to lead to pronucleus formation. Regarding the effects of additional activation following ICSI on oocyte activation in sheep [35], cattle [38, 44] and pigs [34, 45], however, the normal fertilization (2 PN, without intact sperm) rate still remained low. Additionally, it was noticed that the incidence of activation at 18 h post ICSI in the experimental and control groups (sham injection) was similar to that following a sham injection. From our previous findings, we inferred that additional chemical activation is needed to elevate cytosolic calcium and inhibit the activity of mitogen-activated protein kinase and maturation-promoting factor. These factors are necessary during fertilization and pronucleus formation [18, 46]. However, because these chemicals predominantly stimulated female pronucleus formation rather than sperm head decondensation or MPN formation, we should consider parthenogenesis in activated ICSI oocytes.

In conclusion, we improved the efficiency of ICSI in buffalo using a combination of sperm treatments and oocyte activation. These results show that disintegration of the sperm plasma membrane and removal of the acrosome in buffalo sperm can be performed by various methods that do not significantly affect DNA integrity. We report for the first time that buffalo sperm pretreatments, together with DTT following additional activation, promoted male pronucleus formation. Quality control of ICSI-derived buffalo blastocysts may be achieved with improved MPN formation as a result of sperm DTT treatment. The high potential of such blastocysts to develop into live offspring should be confirmed in the near future.

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