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Procedure for Bovine ICSI, not Sperm Freeze-drying, Impairs the Function of the Microtubule-organizing Center

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Abstract. This study was designed to investigate whether freeze-dried (FD) bull spermatozoa maintained the function of the microtubule-organizing center (MTOC) after rehydration and intracytoplasmic sperm injection (ICSI). In a preliminary attempt, the cleavage and blastocyst formation rates in FD-ICSI zygotes (36 and 1%, respectively) were found to be considerably lower than those in control ICSI zygotes (67 and 21%, respectively) or in IVF zygotes (78 and 43%, respectively). An alkaline comet assay indicated that the DNA fragmentation index (length of comet tail $\times$ % DNA liberated) was not significantly different between fresh and FD spermatozoa. In the main experiment, formation of sperm-asters in the FD-ICSI oocytes 7 h postinsemination occurred at a similar rate when compared with the control ICSI oocytes (41 vs. 49%). Among the oocytes exhibiting sperm aster formation, the extent of microtubule network assembly was comparable between the FD-ICSI and control ICSI groups. However, the MTOC of the ICSI oocytes was not as functional as that of IVF oocytes in terms of the aster formation rate (97%) and the fluorescent intensity of the microtubule network (2.0 folds). These results suggest that the freeze-drying process *per se* had no adverse effect on maintaining the MTOC function in bull spermatozoa.

Key words: Bovine Intracytoplasmic sperm injection (ICSI), Freeze-drying, Microtubule-organizing center (MTOC), Sperm-aster

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A centrosome is composed of a pair of centrioles surrounded by pericentriolar materials such as γ -tubulin, centrin and pericentrin. Since an interphase network of microtubules and the mitotic bipolar spindle are nucleated from the centrosome, the centrosome is considered to be the microtubule-organizing center (MTOC) [1]. During fertilization in most mammalian species, the centrosome brought into an oocyte by a spermatozoon plays a critical role in assembly of the microtubule network that brings both male and female pronuclei to the center of the newly formed zygote, as reported in humans [2], rhesus monkeys [3], rabbits [4], pigs [5] and cattle [6]. Interestingly, paternal inheritance of the MTOC does not occur in the mouse [7] and rat [8], and the microtubule network developed from multiple cytoplasmic asters, instead of a single sperm-aster, is involved in the migration of pronuclei [9].

Freeze-drying has been proposed as an alternative method to preserve mammalian spermatozoa [10], although freeze-dried (FD) spermatozoa after rehydration lose their motility and application of intracytoplasmic sperm injection (ICSI) technique is necessary. Rodent spermatozoa can be stored practically at refrigeration temperatures [11–13]. However, in large domestic species, *in vitro* production of blastocysts derived from FD-ICSI is still considered

to be a challenging endeavor [14–16]. We have recently reported that the freeze-drying protocol slightly reduced the ability of bull spermatozoa to induce calcium oscillations [17] and that it had no adverse effect on the active demethylation dynamics of the paternal genome [18]. Reports on the MTOC function of FD sperm are available only in rabbits [19] and primates [20]. In those reports, sperm preservation by freeze-drying had no adverse effects on the frequency of ICSI oocytes forming a sperm-aster. Since the MTOC function of FD bull spermatozoa is not well-understood, this study was conducted to investigate whether FD bull spermatozoa maintained the MTOC function after rehydration and ICSI.

In a preliminary part of this study, developmental potential of bovine zygotes derived from FD-ICSI, control ICSI and conventional IVF was compared (Table 1). There was no significant difference in the proportion of oocytes extruding the second polar body between the FD-ICSI and control ICSI groups (74%, 130/176, vs. 73%, 98/135). Culture of presumptive zygotes that survived FD-ICSI resulted in a lower cleavage rate (36%) when compared with the cleavage rates of zygotes derived from control ICSI (67%) and IVF (78%). Only one blastocyst was obtained from culture of 176 FD-ICSI zygotes for 7 days (<1%), while 21 and 43% of the control ICSI- and IVF-derived zygotes developed into blastocysts during the culture period, respectively. Although no analyses for karyotype or ploidy were performed with these blastocysts, the supplementary activation regimen for FD-ICSI and control ICSI oocytes employed here (ionomycin plus ethanol at a 4-h interval)

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Table 1. *In vitro* development of bovine oocytes injected with freeze-dried spermatozoa

	No. (%) of oocytes			
	Inseminated	Survived	Cleaved	Developed to blastocysts
Control ICSI	161	135 (84) ^a	91 (67) ^b	29 (21) ^b
FD-ICSI	193	176 (91) ^a	63 (36) ^a	1 (1) ^a
IVF	145	145 (100) ^b	113 (78) ^b	62 (43) ^c

^{a-c} Significantly different within columns (P<0.05).**Table 2.** DNA damage in freeze-dried bull spermatozoa estimated by an alkaline comet assay

Spermatozoa	Comet parameters		
	Comet tail length: pixel	DNA liberated: %	Moment ¹
Fresh	12.1 ± 2.6 ^a	23.3 ± 6.6 ^a	4.6 ± 0.6 ^a
Freeze-dried	16.4 ± 6.8 ^a	21.7 ± 6.3 ^a	5.4 ± 2.8 ^a
H ₂ O ₂ -treated	404.8 ± 64.0 ^b	63.7 ± 5.0 ^b	282.9 ± 62.9 ^b

¹ Moment = Comet tail length × % DNA liberated. ^{a,b} Significantly different within columns (P<0.05).

was unlikely to produce parthenogenetically developing blastocysts [21]. An alkaline comet assay indicated that the DNA fragmentation index (moment: length of comet tail × % DNA liberated) was not significantly different between fresh and FD spermatozoa, while a significant increase of the index was detected in H₂O₂-treated positive control spermatozoa (Table 2). In contrast, a harmful effect of the freeze-drying protocol was detected by chromosomal analysis [22] and sperm chromatin structure assay [23] in mouse spermatozoa, which may suggest the presence of species difference, the application of an FD regimen suitable for bull sperm and/or the lower sensitivity of the alkaline comet assay. Considering the poor developmental potential of FD-ICSI oocytes, it was investigated whether the centrosome in bull spermatozoa could be a possible target for detrimental injuries induced by the freeze-drying process.

Function of the MTOC in the FD bull spermatozoa was assessed by immunostaining against α -tubulin (counterstained with DAPI). The proportion of FD-ICSI oocytes exhibiting sperm-aster formation (41%, 14/34) was comparable with that of control ICSI oocytes (49%, 16/33), both of which were significantly lower than that of IVF oocytes (97%, 31/32), as shown in Fig. 1A. Relative fluorescent intensity of the aster network (defined the mean value in control ICSI group as 1.0) was calculated using a stacked image taken by confocal microscope. The fluorescent intensity of the aster network was highly variable but did not differ between the FD-ICSI (relative value, 0.9) and control ICSI groups, as shown in Fig. 1B. On the other hand, the relative fluorescent intensity of the aster network in the IVF group was 2.0-folds higher than that in the control ICSI group. Taken together, the poor developmental potential of FD-ICSI bovine oocytes to the early cleavage or blastocyst stage was unlikely to be caused by detrimental damage in the centrosome of the FD sperm. The target of the FD damage that is more responsible for the poor developmental competence in the FD-ICSI oocytes may be DNA and/or related proteins [13, 22–25], although it is possible that several small amounts of functional damage may

have accumulated to disturb a series of postfertilization events. As the freeze-drying protocol proven effective for rodent spermatozoa [13] seems not to be applicable to bull spermatozoa, overall improvement of the freeze-drying protocol may be required. On the other hand, the difference in the MTOC function between ICSI- and IVF-derived zygotes may explain the difference in their developmental competence, suggesting a further requirement of improving the bovine ICSI protocol.

Hamano *et al.* [26] reported successful calf production following ooplasmic injection of flow cytometrically sorted sperm heads. Ultrasound sonication was applied to prepare the sperm heads in the above study, but it was unclear whether the spermatozoal centrosome was dissociated from the heads by this treatment. Goto *et al.* [27] reported in cattle that blastocyst yields from oocytes injected with sperm heads were lower than those with whole sperm. Even though a sperm-aster failed to function as the MTOC in the oocytes injected with sperm heads, a microtubule network developed from multiple cytoplasmic asters may be involved in the migration of pronuclei [9]. A few weak points of our experimental design regarding the MTOC functional analysis include the injection of FD spermatozoa without dithiothreitol (DTT) treatment and the penetration of capacitated / acrosome-reacted spermatozoa into IVF oocytes, both of which were impossible to control. The DTT facilitates not only destabilization of nuclear packaging in the sperm head but also organization of γ -tubulin in the sperm centrosome, from which microtubules are nucleated [28]. However, ICSI using bull sperm with or without DTT pretreatment resulted in similar blastocyst yields (14 vs. 11%; our unpublished data). Since the effect of acrosomal contents brought nonphysiologically into the oocyte on MTOC function remains unclear, further investigation is required to clarify this interaction.

In conclusion, the process of freeze-drying and rehydration for bull spermatozoa had no adverse effect on MTOC function (sperm-aster formation and microtubule network assembly), suggesting that the poor blastocyst yield from the FD-ICSI-derived oocytes

Fig. 1. MTOC function in FD bull spermatozoa. (A) Proportions of FD-ICSI, control ICSI and IVF oocytes exhibiting sperm-aster formation. The asterisk shows a significant difference from the value in the control ICSI group ($P < 0.05$). (B) Boxplots for overall fluorescent intensities of the MTOC network in the aster-forming oocytes. The asterisk shows a significant difference from the value in the control ICSI group ($P < 0.05$).

was not due to impaired MTOC function in FD sperm. On the other hand, the significant difference in MTOC function observed in the control ICSI and IVF oocytes may be responsible for the lower yield of blastocysts from ICSI-derived oocytes compared with IVF-derived ones.

Methods

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Spermatozoa were prepared from commercially available frozen semen of a Japanese Black bull. Thawed semen was washed by centrifugation at 700 g for 20 min in a 90%/45% density gradient of Percoll in modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan). A part of the sperm pellet was subjected to a freeze-drying regimen as follows. The sperm pellet was resuspended in EGTA buffer (10 mM Tris-HCl, Wako, Osaka, Japan; 50 mM EGTA; and 50 mM NaCl, pH 8.0) and washed twice with the same buffer. Aliquots (300 μ l) of the sperm suspension (1×10^7 sperm/ml) were divided into 3-ml volume glass vials (No.1; Maruemu, Osaka, Japan) and then frozen at a depth of 1 cm in liquid nitrogen. The frozen samples were transferred onto the shelf (-30°C) of a programmable freeze-dryer (ALPHA 2-4; Christ, Harz, Germany). Primary drying of the samples was performed for 14 h at 0.37 hPa with the shelf temperature increased to $+30^\circ\text{C}$ at the end of this stage, and secondary drying was performed for 3 h at 0.001 hPa. After filling the vials with inactive N_2 gas and sealing them with rubber caps and aluminum stoppers, the vials were stored in a refrigerator ($+4^\circ\text{C}$) for 1 week. The FD samples were rehydrated by adding 300 μ l of ultrapure Milli-Q water and then washed twice with mBO medium at 300 g for 5 min each [17, 18].

Zygotes were produced *in vitro* either by conventional IVF or

Fig. 2. An immunostained bovine oocyte 7 h after fertilization. Male and female pronuclei were identified by DAPI staining, and a microtubule network developed from the MTOC (microtubule-organizing center: adjacent to the male pronucleus) was labeled by α -tubulin antibody. MPN: male pronucleus. FPN: female pronucleus. The scale bar represents 10 μm .

ICSI using control or FD spermatozoa. Cumulus-oocyte complexes were recovered from ovaries stored for 1 day and cultured for 22 h in TCM199 supplemented with 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.002 AU/ml FSH and 1 $\mu\text{g}/\text{ml}$ estradiol 17- β at 38.5°C under 5% CO_2 in air. After removal of cumulus cell layers by a brief vortex-mixing in 1,000 IU/ml hyaluronidase solution, matured oocytes extruding the first polar body were harvested. (1) For control ICSI, a Percoll-washed sperm pellet was treated with 5 mM DTT in mBO medium for 20 min at 37.0°C and then washed twice with mBO medium at 300 g for 5 min each. The ICSI was performed with a piezo-driven micromanipulator (PMAS-CT150; Prime Tech, Ibaraki, Japan) in M2 medium [29] containing 8% polyvinylpyrrolidone (PVP), as described previously [21]. The ICSI oocytes were first treated with 5 μM ionomycin in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS for 5 min and incubated in TCM199+5% FBS for 4 h. Next, the oocytes were treated with 7% ethanol in TCM199+1 mg/ml PVP for 5 min. (2) For FD-ICSI, sperm cells were prepared as in control ICSI, except that the rehydrated and washed samples were not treated with DTT because aggregation of DTT-treated FD sperm made it difficult to handle a single spermatozoon. After the FD-ICSI, oocytes were activated with ionomycin and ethanol as described above. (3) For IVF, the sperm pellet was resuspended in the mBO medium supplemented with 5 mM theophylline and washed twice. The sperm pellet was resuspended in mBO medium supplemented with 5 mg/ml BSA and 10 $\mu\text{g}/\text{ml}$ heparin (fertilization medium) to yield a concentration of 2.5×10^7 sperm/ml. Twenty μl of the sperm suspension was added to 80 μl of the fertilization medium containing 10–12 matured oocytes (final sperm concentration, 5×10^6 sperm/ml) and kept for 6 h at 38.5°C under 5% CO_2 in air.

Developmental potential of FD-ICSI, ICSI and IVF oocytes into blastocysts was investigated according to the method described previously [21] as a preliminary part of this experiment. Briefly, ≤ 30 presumptive zygotes (4 h after FD-ICSI/ICSI or 6 h after IVF) were transferred to 250- μ l microdrop of modified synthetic oviduct fluid (mSOF) [30] supplemented with 30 μ l/ml essential amino acids solution ($\times 50$, Gibco-11130), 10 μ l/ml nonessential amino acids solution ($\times 100$, Gibco-11140) and 5% FBS and cultured at 39.0 C under 5% O₂, 5% CO₂ and 90% N₂ for 8 days. Extrusion of second polar body (ICSI and FD-ICSI groups only), cleavage and appearance of expanding blastocysts were recorded 4 h, 3 days and 8 days after insemination, respectively.

An alkaline comet assay [31] was used to estimate the DNA damage in FD spermatozoa in comparison with fresh control spermatozoa. Fresh spermatozoa treated with 10 mM H₂O₂ for 20 min at 4 C served as a positive control. According to the operation manual of the CometAssay[®] kit (Trevigen, Helgerman, CT, USA), aliquots of spermatozoa (1×10^4 cells/10 μ l) were mixed with 0.5% low-melting agarose gel (90 μ l), added to agarose slides, treated with lysis solution for 3 h (including 10 mM dithiothreitol for 0.5 h and 4 mM lithium diiodosalicylate for 1.5 h) and then processed with electrophoresis under a pH>13 alkaline condition (15 V, 20 min). The slides were stained with SYBR Green, and the captured BMP images of the comet (50 comets per sample) were analyzed by the Comet Score software. The length of the comet tail (pixel) was measured from the center of gravity of the head, and the DNA content in the comet tail was determined with the relative fluorescent intensity and defined as % DNA liberated. The DNA fragmentation index (Moment) was calculated as the length of the comet tail $\times$ the % DNA liberated.

To assess MTOC function in fertilized oocytes, FD-ICSI, control ICSI and IVF oocytes were subjected to immunostaining [32]. For FD-ICSI and control ICSI, injected oocytes were activated with ionomycin and ethanol at a 4-h interval and further cultured in TCM199+5% FBS for 3 h at 38.5 C under 5% CO₂ in air. Then, the zygotes were sampled for immunostaining (time equivalent to 7 h after insemination). For IVF, the period for coincubation of oocytes with spermatozoa (preincubated for 2 h in the fertilization medium) was shortened to 2 h in order to restrict the fertilization window. The inseminated oocytes were further cultured in TCM199+5% FBS for 6 h (6–8 h after sperm penetration). As it was defined that sperm penetration occurred in the middle of the coincubation period, oocytes were sampled 8 h after initiation of coincubation. All the samples at 7 h postfertilization were extracted for 15 min by buffer M (25% glycerol, 50 mM KCl, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA and 50 mM imidazole hydrochloride, pH 6.8) containing 5% (v/v) methanol and 1% (v/v) Triton X-100 detergent after the zonae pellucidae had been removed with 0.75% protease in M2 medium. The zygotes were then fixed with cold methanol for 10 min and permeabilized overnight in 0.1 M Ca²⁺/Mg²⁺-free PBS containing 0.1% (v/v) Triton X-100. Microtubules were labeled with a monoclonal antibody against α -tubulin (T5168; diluted 1:2000). The primary antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (F1010; diluted 1:200). Nuclear DNA was visualized by counterstaining with 2.5 μ g/ml DAPI. The prepara-

tions were mounted with coverslips in 100 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO) dissolved in glycerol:PBS (9:1; v/v) and examined using a confocal laser scanning microscope (FV1000-D; Olympus, Tokyo, Japan). Polyspermically penetrated zygotes and unfertilized oocytes were discarded, and at least 30 normally fertilized zygotes were assessed in each group. Digital images were collected at a distance of 2 μ m. To analyze the size of the aster network, fluorescent intensity of the images were stacked and measured by a software freely available online, ImageJ (National Institutes of Health, Bethesda, MD, USA). A photograph of an oocyte with a developing sperm-aster is shown in Fig. 2.

Experiments were repeated at least 4 times in each group. Percentage data in each replicate were arcsine transformed and subjected to one-way ANOVA. Differences among means in the three groups were analyzed by a Bonferroni test. Values for size of sperm-aster were compared by the Student's *t*-test. A value of *P*<0.05 was chosen as an indication of statistical significance.

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