Revised

The Ability of Freeze-dried Bull Spermatozoa to Induce Calcium Oscillations and Meiosis Resumption

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Abstract

This study was designed to investigate the ability of freeze-dried (FD) bull spermatozoa to induce calcium oscillations in mouse oocytes and meiosis resumption in in-vitro-matured bovine oocytes after intracytoplasmic sperm injection (ICSI). Bull spermatozoa were freeze-dried and stored for one year at +25 ◌̊C, +4 ◌̊C, or –196 ◌̊C. In the first experiment, rehydrated sperm heads were microinseminated into hybrid mouse oocytes loaded with fluo-3/AM, and the kinetics of intracellular calcium concentration was monitored for 1 h. Repetitive increases of intracellular calcium concentration were recorded in the majority of injected oocytes, with exception of a few oocytes injected with FD sperm heads stored at +4 ◌̊C (11%) and +25 ◌̊C (8%) that exhibited a single increase or no response (non-oscillated). Proportion of oocytes oscillated with high frequency (≥10 spikes per hour) was significantly higher ($P < 0.05$) in the non-dried control group (79%) than those in the FD groups (58, 55 and 54% for storage at –196, +4, and +25 ◌̊C, respectively). In the second experiment, control and FD spermatozoa were microinseminated into in-vitro-matured, denuded bovine oocytes. The oocytes were fixed and stained 12 h after ICSI. Significantly higher proportion of bovine oocytes injected with control spermatozoa (70%) resumed meiosis than those injected with +25, +4 and -196 ◌̊C-stored FD spermatozoa (53, 48 and 57%, respectively). The proportion of ICSI oocytes that developed to the pronuclear-stage (complete activation) was significantly higher in the control group (64%) than those in all the FD groups (34, 27, and 28% for storage at –196, +4, and +25 ◌̊C, respectively). Thus, the ability of bull spermatozoa to induce frequent intracellular calcium spikes in mouse oocytes was impaired by the process of freeze-drying, probably resulting in lower proportion of bovine oocytes that resumed meiosis and/or developed to the pronuclear-stage.

Keywords: Bovine; Calcium oscillations; Freeze-drying; Homogenous ICSI; Interspecies ICSI

1. Introduction

Bull spermatozoa cryopreserved in a solution containing glycerol and egg yolk are conveniently used for offspring production either via artificial insemination or in vitro fertilization. As an alternative method for cryopreservation, freeze-drying or lyophilization which is widely used for preservation of biological or pharmaceutical materials is possible candidate. If freeze-dried (FD) spermatozoa could be preserved at ambient or refrigeration temperatures, the cost and possible risk for maintenance and shipping of spermatozoa would be reduced because neither dry ice nor liquid nitrogen (LN_2) will be required. Freeze-drying depends on direct conversion of ice into vapor, sublimation, which occurs when heat is supplied to frozen samples under very low vacuum pressure. Since the freeze-drying process causes functional losses of sperm cells such as immobilization, the rehydrated spermatozoa need to be injected into oocytes (ICSI: intracytoplasmic sperm injection). Offspring derived from the intracytoplasmic injection of FD spermatozoa have been reported firstly in mouse [1], followed by rabbit [2] and rat [3]. To date, preservation of FD spermatozoa for extended periods (≥1 year) was valid only at a refrigeration temperature in mouse [4, 5, and 6], rabbit [2] and rat [7]. On the other hand, blastocyst development after ICSI with FD sperm heads was reported in large domestic animals including cattle [8, 9] and pig [10].

At fertilization, the earliest function of penetrated sperm is to induce repetitive increases in the concentration of calcium ions in the ooplasm ($[Ca^{2+}]i$), the so-called calcium oscillations. The lasting of calcium oscillations for a long time is essential for regulating various post-fertilization events such as resumption of the second meiosis and extrusion of the second polar body [11], exocytosis of cortical granules [12], pronucleus formation [13] and developmental performance [14]. In bovine in-vitro fertilization, calcium oscillations were found to last until pronuclear migration and nuclear envelop breakdown [15, 16]. Treatment of oocytes with some chemicals which can induce calcium oscillations has been reported to produce parthenotes in many species including cattle [17, 18]. Moreover, developmental arrest of bovine ICSI embryos has been attributed to the inability of bull sperm to induce appropriate calcium oscillations [19]. The process of egg activation extends from the release from second metaphase to pronuclear formation, which is considered as an excellent criterion for completion of meiosis and entry to

first interphase of development. The degree of oocyte activation has been related to the frequency, amplitude and duration of calcium spikes [20-22]. A possible candidate of sperm-borne oocyte-activating factor (SOAF) is phospholipase C-zeta or substance that acts upstream of egg phospholipase C-zeta [23, 24].

The FD spermatozoa undergo exposure to and freezing in cryoprotectant- and calcium-free buffer, complete dehydration, long-term storage and rehydration, which may adversely affect either structural or functional sperm characteristics. To our knowledge, It has been reported that bull spermatozoa freeze-dried under a single vacuum pressure and rehydrated immediately after freeze-drying have similar calcium oscillation-inducing ability to the non-dried sperm in mouse oocytes [25], without examining the ability of such sperm to induce activation of bovine oocytes. The present study was designed to investigate the ability of bull spermatozoa freeze-dried and stored for one year at three different temperatures (–196, +4 and +25 ◌̊C) to induce calcium oscillations in mouse oocytes (by interspecies ICSI), as well as to induce meiosis resumption in bovine oocytes (by homogenous ICSI).

2. Materials and methods

2.1. Chemicals and media

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA). Buffer for freeze-drying of bull spermatozoa consisted of 10 mM Tris-HCl (Wako, Osaka, Japan), 50 mM EGTA and 50 mM NaCl (pH 8.0). Medium used for temporal culture of mouse oocytes under 5% CO_2 in air was Chatot, Ziomet and Bavister (CZB) medium [26] with some modifications by removal of EDTA and supplementation with 5 mg/mL bovine serum albumin (BSA), referred as to CZB-BSA medium. Medium used for handling mouse oocytes under air condition (oocyte collection, sperm injection and observation of $[Ca^{2+}]i$ kinetics) was Hepes-buffered CZB medium in which 20 mM Hepes instead of sodium bicarbonate and 0.01 mg/mL polyvinylalcohol (PVA) instead of BSA were supplemented to the CZB medium, referred as to HCZB-PVA medium. For in vitro maturation of bovine oocytes, 25 mM Hepes-buffered TCM-199 (Earle's salt; Gibco BRL, Grand Island, NY, USA) supplemented with 0.2 mM sodium pyruvate, 50 μg/mL gentamycin sulfate, 10% fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.002 AU/mL FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan) and 1 μg/mL 17ß-estradiol was used. TCM-199 supplemented with 5% FBS, and modified synthetic oviduct fluid (mSOF) supplemented with 30 μL/mL EAA, 10μL/mL NEAA and 5% FBS [27] were used for short-term culture of bovine pre- and post-ICSI oocytes.

2.2. Freeze-drying of bull spermatozoa

Commercially available frozen semen from a Japanese Black bull was used. After thawing in water bath at 37 ℃ for 30 sec, content of 0.5-mL straw was layered on the top of percoll density gradient consisting of 2 mL of 45% percoll above 2 mL of 90% percoll and centrifuged for 20 min at 700 g. The sperm pellet was resuspended in freeze-drying buffer and washed twice for 5 min each at 300 g. The sperm suspension (3-5 x 10^6 sperm/mL) was divided to 200 μL aliquots in 5-mL volume glass vials (no.2; Maruemu, Osaka, Japan), and then frozen by plunged into LN_2 of 1-cm depth. The frozen samples were

transferred onto the shelf (–30 ℃) of programmable freeze-dryer (ALPHA 2-4; Christ, Harz, Germany), and dried primary for 14 h at 0.37 hPa and secondary for 3 h at 0.001 hPa, as described previously [7]. During the process of primary drying, the shelf temperature was controlled to increase to +30 ℃. After fulfilling with inactive nitrogen gas and sealing the vials with rubber caps and parafilm, the dried samples were transferred to a conventional glass desiccator (5-L volume) at 25±5 ℃, a refrigerator (300-L volume) at +4 ℃, or a LN_2 tank (30-L volume) at –196 ℃, and then stored for 1 year under dark conditions.

2.3. Interspecies ICSI

In the first experiment, bull spermatozoa freeze-dried and stored for 1 year at three different temperatures as well as non-dried control spermatozoa from the same bull and batch were microinseminated into hybrid mouse oocytes, and then kinetics of intracellular calcium ions was monitored. Our animal experimentation was reviewed and approved by Animal Care and Use Committee of the Shinshu University, and all procedures for handling and treatment of the animals were conducted according to the Guidelines of the Committee.

2.3.1. Collection of mouse oocytes

Specific pathogen-free / virus antibody-free B6D2F1/Crlj female mice (8-12 weeks old; Charles River Japan, Kanagawa, Japan) were superovulated by intraperitoneal injection of 7.5-10 IU equine chorionic gonadotropin (eCG; Aska Pharmaceutical, Tokyo, Japan) and 7.5-10 IU human chorionic gonadotropin (hCG; Mochia Pharmaceutical, Tokyo, Japan) at an interval of 48 h. Cumulus-oocyte complexes (COCs) were collected into a droplet of HCZB-PVA medium supplemented with 440 IU/mL hyaluronidase, 14 h after hCG injection. After removal of cumulus cells by gentle pipetting, denuded oocytes were washed several times with HCZB-PVA medium and twice with CZB-BSA medium. The oocytes were cultured in the same CZB-BSA medium at 37 ℃ under 5% CO_2 in air until use.

2.3.2. Microinsemination

Bull spermatozoa freeze-dried and stored for 1 year were rehydrated by adding 500 μL of ultrapure milli-Q water to the vial, washed twice with HCZB medium for 5 min each at 300 g, and then sonicated for 20-30 sec using ultrasonic glassware cleaner. Non-dried control motile spermatozoa were separated by the percoll-washing and washed twice with HCZB medium for 5 min each at 300 g. Control spermatozoa were also sonicated as above, to dissociate the head from tail.

Oocytes were treated with a Ca^{2+}-sensitive fluorescent dye, fluo-3-acetoxymethyelester (fluo-3/AM; Molecular Probes, Eugene, OR, USA), at a concentration of 22 μM in HCZB medium for 30 min at 37 ℃. Immediately before ICSI, 10 oocytes were placed in 10-μL microdrop of HCZB-PVA medium, and 2 μL of sperm suspension was mixed with 8 μL of HCZB medium supplemented with 12% polyvinylpyrrolidone (PVP). Then, one sperm head was deposited into ooplasm with a blunt-ended injection pipette with an outer diameter of 7-8 μm using a piezo-driven micromanipulator (PMM-150FU; PrimeTech, Ibaraki, Japan), as described previously [28].

2.3.3. Observation of $[Ca^{2+}]i$ kinetics

Intracellular Ca^{2+} level was measured by a Ca^{2+}-imaging method described previously [29] with some minor modifications. Briefly, the ICSI oocytes were placed to 5 μL microdrop of HCZB-PVA medium in a glass slide chamber, and transported to another laboratory kept at 30 ℃. The kinetics of $[Ca^{2+}]i$ was monitored for up to 1 h at 20 sec intervals using a confocal laser-scanning microscope (Bio-Rad 2100; Nippon Bio-Rad Laboratories, Tokyo, Japan) equipped with an image processor. Records from oocytes showing sings of degeneration (lysed or contracted) at the end of monitor period were discarded. Based on the oscillation pattern recorded, oocytes were classified into one of five categories; Pattern-A: oocytes oscillating with short cycles (≥10 spikes per hour, Fig. 1a), Pattern-B: oocytes oscillating with moderate intervals (2 to 9 spikes per hour, Fig. 1b), Pattern-C: oocytes exhibiting delayed response followed by highly repetitive, low amplitude spikes (Fig. 1c), Pattern-D: oocytes exhibiting single rise (Fig. 1d), and Pattern-E: oocytes without response.

2.4. Homogenous ICSI

In the second experiment, bull spermatozoa freeze-dried and stored for 1 year at three different temperatures as well as non-dried control spermatozoa from the same bull and batch were microinseminated into in-vitro matured bovine oocytes. The ICSI oocytes were fixed and stained to determine their activation status.

2.4.1. In vitro maturation of bovine oocytes

Abattoir-derived bovine ovaries were transported to the laboratory in 10-12 ◌°C saline within 24 h from slaughter. The COCs were collected by follicle aspiration, and those surrounded with at least two layers of compact cumulus cells were matured for 22 h at 38.5 ◌°C under 5% CO_2 in air. Oocytes were freed from cumulus cells by a brief Vortex-mixing in the TCM-199 containing 3 mg/mL BSA and 1000 IU/mL hyaluronidase. Oocytes with an extruded first polar body were defined as matured oocytes and cultured in the TCM-199 + 5% FBS until the use for ICSI (<4 h).

2.4.2. Microinsemination

Control and FD bull spermatozoa were prepared as described in the interspecies ICSI experiment, except for using modified Brackett and Oliphant (BO) solution (IVF100; Institute for Functional Peptides, Yamagata, Japan) instead of HCZB medium and skipping ultrasonic treatment. Matured oocytes were placed in 10-µL microdrop of TCM-199 + 5% FBS, and 2 µL of sperm suspension was mixed with 8 µL of M2 medium [30] supplemented with 10% PVP. Then, single spermatozoon was aspirated tail first into a blunt-ended injection pipette with an outer diameter of 7-9 µm and deposited into the ooplasm with a help of piezo-driven micromanipulator, according to the method described by Horiuchi et al. [31]. In case of the control ICSI, spermatozoon was immobilized by application of several piezo pulses (speed 2, intensity 2) to the mid piece. The ICSI oocytes were cultured for 4 h in TCM-199 + 5% FBS at 38.5 ◌°C under 5% CO_2 in

air, and then cultured for additional 8 h in the mSOF medium at 39 ℃ under 5% CO_2, 5% O_2 and 90% N_2.

2.4.3. Nuclear staining

Twelve hours after ICSI, number of oocytes extruding second polar body was recorded under phase contrast inverted microscope at a magnification of x 400. Then, the zonae pellucidae were removed by digesting in phosphate-buffered saline (PBS) containing 0.25% pronase, because the oocytes were used for another immuonstaining that requires removal of zona pellucida (data not shown). The zona-free oocytes were fixed with 4% paraformaldehyde overnight at 4 ℃ and then permeabilized by 0.5% Triton X-100 in PBS for 30 min. Finally, the oocytes were stained with 5 μg/mL propidium iodide (PI) for 30 min, and were observed under a confocal laser-scanning microscope. Activation status of the oocytes was classified as followings; non-activated oocytes, those still arrested at metaphase stage (Fig. 2a), partially activated oocytes, those resumed meiosis but arrested again in chromosome condensation without pronucleus formation (Fig. 2b and 2c), fully activated oocytes, those developed to pronuclear stage (Fig. 2d, 2e, 2f).

2.4.4. Assessment of pronuclear symmetricity

In the zygotes with two pronuclei, digital optical sections for the area containing the two pronuclei were collected using a Z-series scanning feature of confocal laser scanning microscope at 2 μm distance. With the assistant of Image J software (National Institutes of Health, Bethesda, MD, USA; http://rsb.info.nih.gov/ij/), all the slices were converted to one stalk and size of each pronuclear area was measured. The size ratio of the larger pronucleus to the smaller pronucleus was calculated.

2.5. Statistical analysis

Statistical analysis was performed using online available program http://www.physics.csbsju.edu/stats/Index.html. Differences among groups regarding proportions of oocytes showed different calcium oscillation pattern (experiment-1) or different activation status (experiment-2)

were analyzed using Fisher's exact probability test. Differences in number of calcium spikes and pronuclear symmetricity among groups were analyzed using Student's t-test. P values less than 0.05 were considered significant.

3. Results

3.1. Interspecies ICSI to monitor [Ca^{2+}]i kinetics

Response of mouse oocytes to the injected bull sperm heads was shown in Table 1. Repetitive increases of intracellular calcium concentration were recoded in the majority of injected oocytes (89 to 100%). However, proportion of oocytes oscillated with high frequency (pattern-A) was higher in the non-dried control group (79%) than those in the FD groups (58, 55 and 54% for storage at –196, +4 and +25 ºC, respectively). On the other hand, some of the oocytes injected with FD sperm, but not control sperm injected oocytes, showed a different pattern of oscillations (pattern-C). Proportion of pattern-C oscillated oocytes was significantly higher in oocytes injected with FD spermatozoa stored at –196 ºC (26%) than in those injected with FD spermatozoa stored at +4 ºC (8%) and +25 ºC (10%), . Moreover, a few oocytes injected with FD spermatozoa stored at +4 ºC (11%) and +25 ºC (8%) exhibited a non-oscillating single increase (pattern-D) or no response (pattern-E). The average number of the spikes in pattern-A oscillated oocytes (19.3, 21.4, 19.7 and 21.6 spikes in control, FD/–196 ºC-stored, FD/+4 ºC-stored, and FD/+25 ºC-stored groups, respectively) and pattern-B oscillated oocytes (5.6, 8.3, 5.8, and 5.7 spikes in control, FD/–196 ºC-stored, FD/+4 ºC-stored, and FD/+25 ºC-stored groups, respectively). Additionally, the overall average numbers of calcium spikes in oscillating oocytes (pattern-A plus pattern-B) were comparable (16.9±8.2, 18.9±8.6, 15.7±10.0, and 16.3±9.1 spikes in control, FD/–196 ºC-stored, FD/+4 ºC-stored, and FD/+25 ºC-stored groups, respectively).

3.2. Homogenous ICSI to examine activation status

Nuclear configuration in bovine zygotes after ICSI was shown in Table 2. The total proportion of activated oocytes was significantly higher after injection of control sperm (70%) than after injection of FD sperm stored at -196, +4 and +25 ºC (57, 48and 53%, respectively). Moreover, significantly higher proportions of oocytes injected with FD sperm stored at –196, +4 and +25 ºC (23, 21 and 25%, respectively) were re-arrested at a condensation stage without progressing to pronuclear stage (Fig. 2b and

2c) when compared to those injected with control sperm (6%). Accordingly, the proportion of oocytes fully activated to allow pronuclear formation in the control group (64%) was higher than those in the FD groups (34, 27 and 28% for –196, +4 and +25 ºC stored groups, respectively). Proportions of oocytes extruded second polar body in all groups were slightly lower than those resumed meiosis even after oocytes showed three pronuclei (presumably due to inhibition of second polar body extrusion) were excluded. Within the fully activated oocytes with two pronuclei, severe asynchrony in the size of the two pronuclei has been observed frequently in zygotes developed after injection of FD sperm stored at +25 ºC (ratio of larger to smaller pronucleus, 3.1±1.8) rather than those developed after injection of control, FD sperm stored at +4 or –196 ºC (1.8±0.6, 1.8±0.8 and 1.5±0.5, respectively). Pronuclear size ratio in bovine zygotes with two pronuclei was calculated and the distribution was shown as box-plots in Fig. 3.

4. Discussion

Intracytoplasmic injection of freeze-dried spermatozoa has been resulted in successful production of live offspring in mouse [1, 4-6, 32], rat [3, 7, 33] and rabbit [2], and blastocyst-stage embryos in cattle [8, 9] and pig [10]. However, long-term storage of FD spermatozoa at room temperature has not yet been achieved in any mammalian species. Since the stability of sperm function after freeze-drying and long-term storage has not been fully investigated, the first experiment of the present study was designed to examine the ability of FD bull spermatozoa to induce calcium oscillations using interspecies ICSI assay. The interspecies ICSI assay using hybrid mouse oocytes, that was originally developed by Rybouchkin et al. [34], has been used to investigate the SOAF activity of different spermatogenic cells in hamster, rat, rabbit, human [35] whale [29] and cynomolgus monkey [36] and chemically membrane-damaged spermatozoa in cattle, pig and human [37]. The advantages of using B6D2F1 mouse oocytes are their abilities to be loaded easily with calcium-sensitive fluorescent dye and their resistance to sham-injection treatment in term of oocyte activation.

Pattern-B oscillation (Fig. 1b) is the common response recorded after injection of mouse oocytes with different stages of human, mouse, hamster, rat and rabbit spermatogenic cells [34, 35]. On the other hand, pattern-A oscillation (Fig. 1a) may reflect a qualitatively or quantitatively higher SOAF activity in each spermatozoon, because such a short-cycled oscillation pattern has been reported when a non-physiologically larger volume of porcine [38, 39] and equine [40] sperm extracts, or isolated sperm heads from bulls and whales but not from mice [29] were microinseminated into mouse oocytes. Moreover, human spermatozoa treated with lysolecithin induced calcium oscillations with higher frequent spikes [37], so the frequency of calcium oscillation may be related to factors controlling release of SOAF into the ooplasm. The process of SOAF release into ooplasm requires destruction of plasma membrane and exposure of perinuclear theca [41]. In the present study, significantly lower proportions of pattern-A oscillation after injection of FD bull sperm compared to control sperm (Table 1) suggest that the freeze-drying process of bull spermatozoa including rehydration process may result in a partial loss of SOAF activity present in submembrane or perinuclear loci [42, 43]. Moreover, higher storage temperatures (+4 and +25 ℃) may have an additional adverse effect on stability of the SOAF activity of FD bull spermatozoa, because there were some non-oscillating oocytes in

these groups. There is only one publication investigated calcium oscillation-inducing ability of FD bull spermatozoa [25], in which similar average numbers of calcium spikes during the first 1 hour were detected in mouse oocytes injected with FD versus control sperm (14±3 versus 13±3 spikes, respectively), with a conclusion that the freeze-drying process does not affect the SOAF activity. Similarly, the overall average numbers of calcium spikes during the first hour in our study were not different between control and all FD groups, as ranged from 15.7 to 18.9; however the proportions of pattern-A oscillating oocytes differed significantly. Theoretically, interaction of oocytes with perinuclear theca that contains the SOAF [44] may be facilitated by destruction of sperm membrane induced by freeze-drying. Otherwise, pattern-C oscillation (Fig. 1c) was observed only in the oocytes injected with FD sperm heads (Table 1). The pattern-C proportion after injection of FD sperm stored at –196 ºC was significantly higher than those in the other two FD groups. This suggests that freeze-drying process may modify the perinuclear theca by the way that interferes with SOAF release into the ooplasm, and that direct contact of the freeze-dried sperm to liquid nitrogen may enhance this effect.

Transient increases of intracellular calcium level were considered to be responsible for initiation of meiosis resumption and completion of cell cycle, but the number of calcium spikes required for completion of cell cycle is higher than those required for resumption of meiosis [21]. Application of a single calcium pulse to mouse oocytes has resulted in resumption of meiosis without pronuclear formation; instead the oocytes were re-arrested at condensation stage after extrusion of the second polar body [45]. The progression to pronuclear-stage in cell cycle is controlled by activation of calmodulin-dependent protein kinase-II (CaMKII) that leads to activation of the anaphase-promoting complex/cyclosome (APC/C) and subsequent inactivation of the metaphase-promoting factor (MPF) through degradation of securin and cyclin-B [46, 47]. To make relation between the calcium oscillation-inducing ability of FD bull spermatozoa and their ability to induce bovine oocyte activation, the FD spermatozoa were injected into bovine oocytes without any exogenous activation stimuli. The freeze-drying process of bull spermatozoa decreased the proportion of activated and fully-activated bovine oocytes after ICSI (Table 2). Adverse effect of drying on the ability of sperm cells to induce pronuclear formation has been previously reported after freeze-drying of bull [8,9] and porcine sperm [10] and after heat-drying of bull sperm [48]. Moreover, the adverse effect of freeze-drying was notable in the higher proportions of partially activated bovine oocytes after ICSI with FD

versus control bull spermatozoa (Table 2). Such a re-arresting in the condensation stage has been reported to occur more frequently in bovine ICSI oocytes after weaker activation, single inonmycin treatment, compared to stronger activation as multiple ionomycin treatments or ionomycin plus 6-DMAP treatment [49, 50]. Not only long-lasting but also highly frequent calcium oscillations may be required for the full activation of bovine ICSI oocytes.

Pronuclear asymetricity was frequently observed in bovine zygotes 12 h after injection of FD sperm stored at +25 ℃ (Fig. 3), which may indicate an additional adverse effect of higher storage temperature upon the FD bull sperm. Asynchrony between chromatin remodeling has been reported in mouse oocytes after ICSI [51] and bovine oocytes fertilized in vitro with spermatozoa from thermally insulated testis [52]. Alterations in sperm chromatin and/or DNA damage might result in defective decondensation and a delay in the formation of the male pronucleus [53, 54]. A prolonged storage of FD spermatozoa at ambient temperatures may induce such alterations and/or DNA damage. We have previously reported that one-year storage of FD rat spermatozoa at a room temperature has resulted in a higher incidence of chromosomal aberrations compared to those stored in LN_2 or refrigerator [7]. This has been accompanied with great reduction of cleavage rate after injection of FD sperm stored at +25 ℃ (1%) compared to 36-38% after injection of FD sperm stored at +4 or –196 ℃. Further research to clarify the effect of higher storage temperature on function of FD bull spermatozoa would be required.

In conclusion, the ability of bull spermatozoa to induce frequent intracellular calcium spikes in mouse oocytes was impaired by the process of freeze-drying, probably resulting in lower proportion of bovine oocytes either resuming meiosis or developing into pronuclear stage. Although routine bovine ICSI technique includes application of exogenous stimuli for oocyte activation [55, 56], stronger stimuli may be required when the freeze-dried bull spermatozoa are used for bovine embryo production by ICSI.

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Figure legends

Fig. 1. Calcium oscillation patterns induced in mouse oocytes after interspecies ICSI with bull sperm heads. (a) Pattern-A: oocytes oscillating with short cycles (≥10 spikes/h); (b) Pattern-B: oocytes oscillating with moderate intervals (2-9 spikes/h); (c) Pattern-C: oocytes exhibiting delayed response followed by highly repetitive, low amplitude spikes; (d) Pattern-D: oocytes exhibiting single rise.

Fig. 2. The PI-staining of bovine oocytes 12 h after injection of bull spermatozoa. (a) Non-activated oocyte with sperm head and second metaphase plate; (b) Partially activated oocyte with single condensed chromatin set; (c) Partially activated oocyte with double condensed chromatin sets; (d) Fully activated oocyte with sperm head and female pronucleus; (e) Fully activated oocyte with severely asynchronized two pronuclei in size; (f) Fully activated oocyte with well-synchronized two pronuclei in size.

Fig. 3. Box-plots showing a variation of pronuclear size ratio in bovine ICSI oocytes. The area of larger pronucleus was divided by the area of smaller pronucleus. Closed circles indicate mean value. * $P < 0.05$.

Table 1. Ability of bull spermatozoa freeze-dried and stored for one year at three different temperatures to induce calcium oscillations in interspecies ICSI assay using mouse oocytes.

Groups	No.of oocytes examined	Oscillating (%)				Non-oscillating (%)		
		Pattern-A	Pattern-B	Pattern-C	Subtotal	Pattern-D	Pattern-E	Subtotal
Control	38	30 (79) [a]	8 (21)	0 (0) [b]	38 (100)	0 (0)	0 (0)	0 (0)
FD/–196 ℃-stored	38	22 (58) [b]	6 (16)	10 (26) [a]	38 (100)	0 (0)	0 (0)	0 (0)
FD/+4 ℃-stored	38	21 (55) [b]	10 (26)	3 (8) [b]	34 (89)	4 (11)	0 (0)	4 (11)
FD/+25 ℃-stored	39	21 (54) [b]	11 (28)	4 (10) [b]	36 (92)	2 (5)	1 (3)	3 (8)

See Fig. 1 for classification of oscillation pattern.

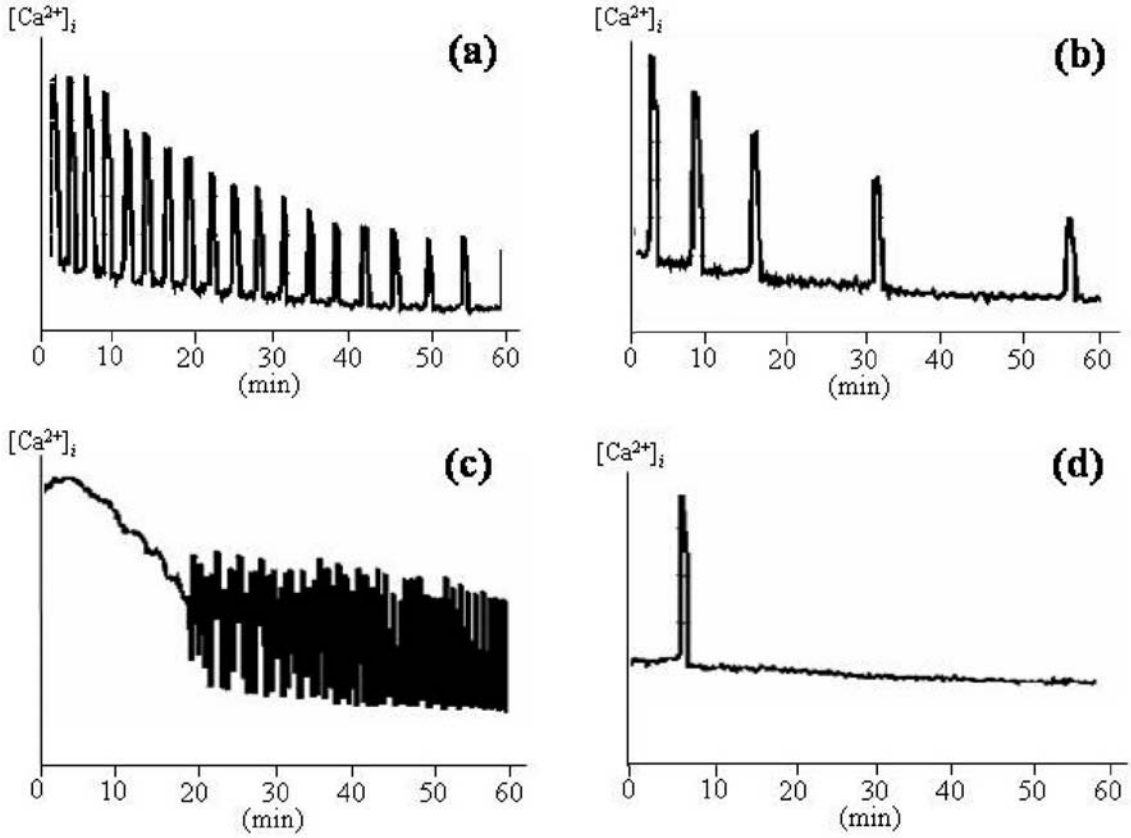
Six to eight replicates in each group. Values with different superscripts within columns are significantly different ($P<0.05$).

Table 2. Nuclear configuration of bovine oocytes 12 h after injection with bull spermatozoa freeze-dried and stored for one year at three different temperatures.

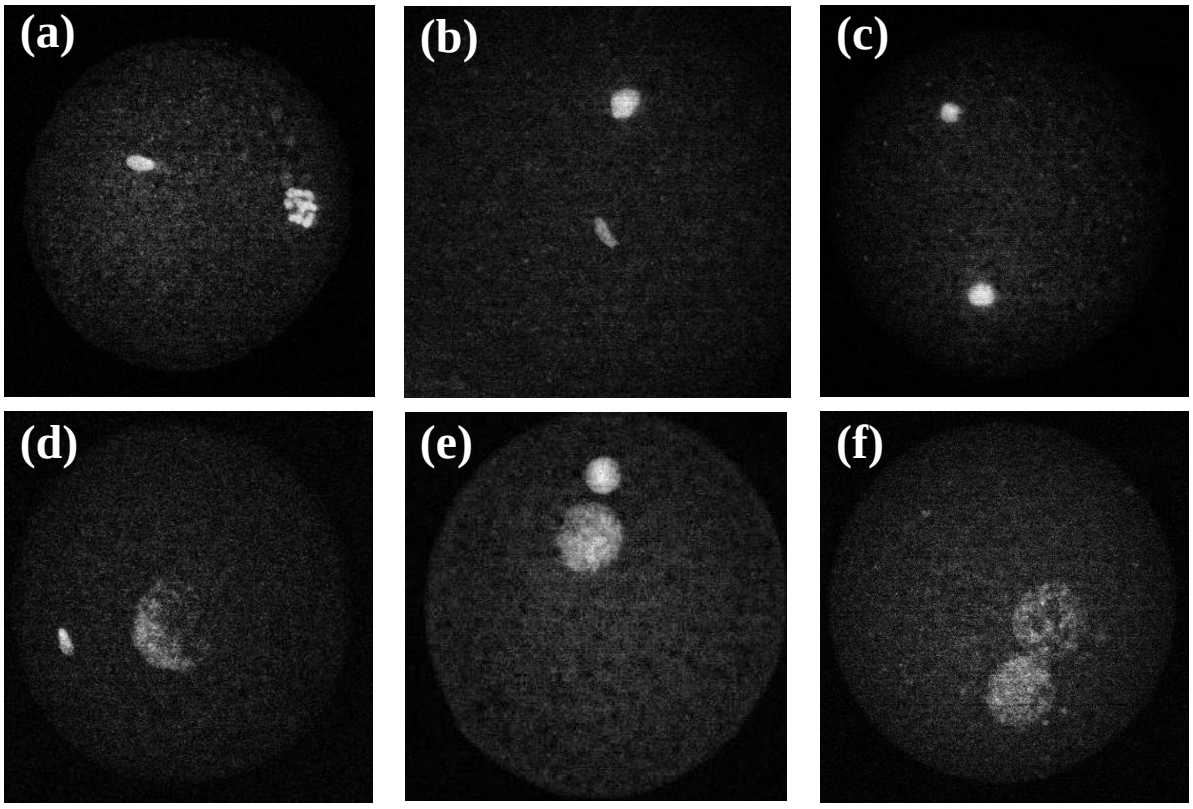
Groups	No. of oocytes examined	2nd PB extruded (%)	Arrested (%)	Resumed meiosis: Activated (%)							Total
				Partially activated			Fully activated				
			SPH+MII	SPH+CCH	2CCHs	Subtotal	SPH+PN	2PN	3PN	Subtotal	
Control	115	75 (65) [a]	34 (29) [b]	6 (5) [ab]	1 (1) [b]	7 (6) [b]	18(15) [a]	55 (48) [a]	1 (1)	74 (64) [a]	81 (70) [a]
FD/–196 ºC-stored	130	69 (53) [b]	56 (43) [a]	2(2) [bc]	28 (21) [a]	30 (23) [a]	4 (3) [b]	37 (29) [b]	3 (2)	44 (34) [b]	74 (57) [b]
FD/+4 ºC-stored	130	57 (44) [b]	67 (52) [a]	0 (0) [c]	28(21) [a]	28 (21) [a]	2 (2) [b]	29 (22) [b]	4 (3)	35 (27) [b]	63 (48) [b]
FD/+25 ºC-stored	90	44 (49) [b]	42 (47) [a]	7 (8) [a]	16 (17) [a]	23 (25) [a]	2 (2) [b]	23 (26) [b]	0 (0)	25 (28) [b]	48 (53) [b]

PB: polar body; SPH: sperm head; MII: metaphase-II plate; CCH: condensed chromatin; PN: pronucleus.

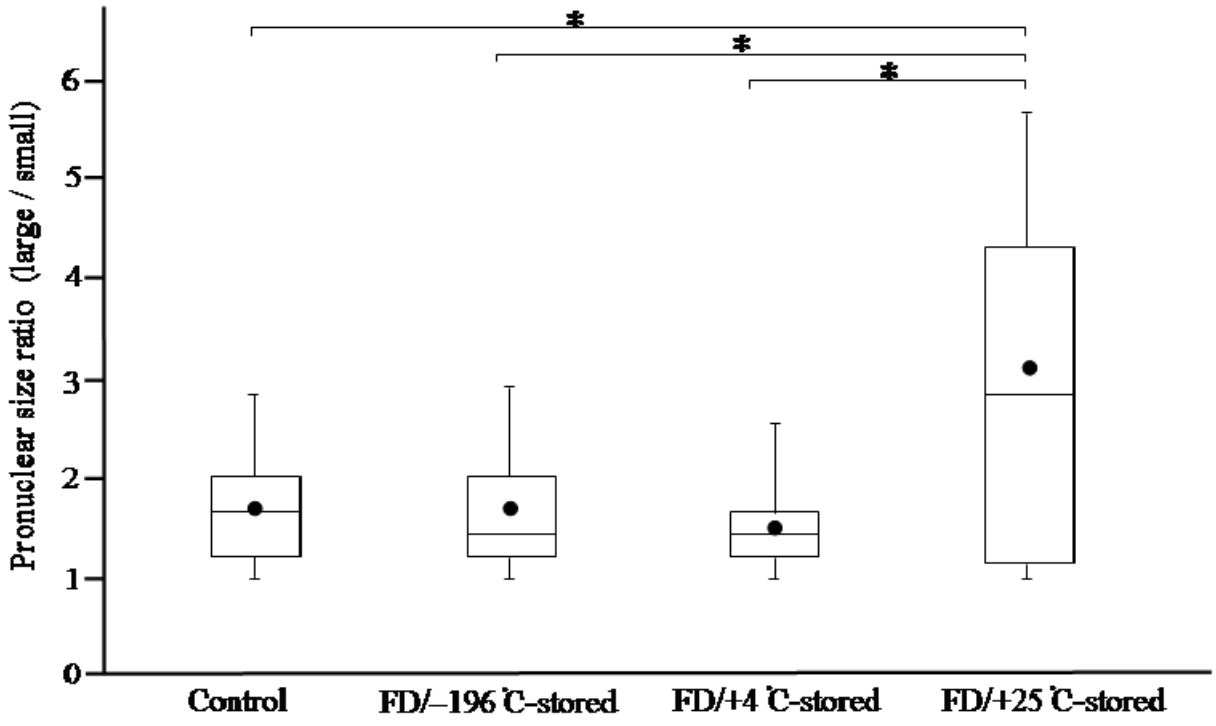
Six replicates in control, FD/–196 ºC-stored, and FD/+4 ºC-stored groups, and five replicates in FD/+25 ºC-stored group. Values with different superscripts within columns are significantly different ($P<0.05$).


[Ca2+]i
(a)
0 10 20 30 40 50 60
(min)
[Ca2+]i
(b)
0 10 20 30 40 50 60
(min)
[Ca2+]i
(c)
0 10 20 30 40 50 60
(min)
[Ca2+]i
(d)
0 10 20 30 40 50 60
(min)

(Fig 1)



(Fig. 2)


Pronuclear size ratio (large / small)
0
1
2
3
4
5
6
*
*
*
Control
FD/–196 ℃-stored
FD/+4 ℃-stored
FD/+25 ℃-stored

(Fig. 3)