Effects of freeze-drying on cytology, ultrastructure, DNA fragmentation, and fertilizing ability of bovine sperm

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Abstract

Freeze-drying sperm is an alternative to cryopreservation. Although sperm from various species has been freeze-dried, there are few reports for bovine sperm. The primary objective of this study was to evaluate the protective effect of various freeze-drying media on the structural and functional components of bovine sperm. The media tested were composed of TCM 199 with Hanks salts supplemented with 10% fetal calf serum (FCS) and TCM 199 with Hanks salts supplemented with 10% FCS and 0.2 M trehalose and EGTA solution. The efficiency of each medium on the preservation of freeze-dried sperm structures was evaluated with conventional and electron microscopy, DNA integrity was analyzed by a TUNEL assay, and fertilizing ability of lyophilized sperm was determined with ICSI. Although the plasma membrane was damaged in all media tested, mitochondria were similarly preserved in all freeze-drying treatments. The acrosome was best preserved in the media that contained trehalose (other treatments also conserved this structure). In contrast, media containing EGTA or trehalose most effectively preserved the nuclei in freeze-dried sperm, with only 2 and 5%, respectively, of cells with fragmented DNA. Furthermore, sperm conserved with these media also had higher ($P < 0.05$) rates of sperm head decondensation (32.5 and 27.5%), pronucleus formation (37.5 and 45.0%) and blastocyst formation (19.4 and 18.3%) than medium supplemented with FCS (15.0, 20.0 and 10.2%, respectively). In conclusion, media with EGTA and trehalose adequately protected bovine sperm during freeze-drying by preserving the viability of their nuclei.

Keywords: Bovine; ICSI; Freeze-drying; Sperm preservation; TUNEL

1. Introduction

Freeze-drying (lyophilization), was developed to preserve bioactive molecules (DNA, enzymes, and proteins), pharmaceuticals products (antibiotics) and other delicate, solvent-impregnated materials [1] and [2]. It has also been used to preserve cells, due to its ability to restrict active water by ice sublimation [3].

Freeze-drying sperm has been the focus of many research groups. Relative to conventional cryopreservation, freeze-drying is cheaper, uses no liquid nitrogen, requires less space for gamete storage, and is an easier and cheaper method of transporting sperm. The first attempt to preserve mammalian sperm by dehydration was reported by Polge et al. [3] using fowl sperm; after rehydration, the sperm were motile but their fertilizing ability was not

evaluated. Subsequent efforts to lyophilize human and bovine sperm yielded very poor results [4] and [5]. In 1957, Yushchenko [6] reported for the first time the birth of a domestic animal following AI of freeze-dried rabbit sperm. Subsequent production of offspring with freeze-dried sperm has only been reported following the use of ICSI [7] and [8]. These results provided new possibilities to store and transport dehydrated gametes at room temperature or at 4 °C, with many benefits for preservation of male gametes from laboratory and farm animals [9].

One of the main concerns with any preservation method is the extent of cellular damage. Regardless of the specific protocol used, cryopreservation of sperm reduces both motility and fertilizing ability [10]. Although freeze-drying is much more deleterious to sperm (than cryopreservation) and induces substantial loss of fertilizing ability, live offspring were obtained when freeze-dried sperm from mouse [7] and rabbit [8] were used for ICSI. Therefore, despite substantially reduced motility, cells remained viable, based on the fact that sperm nucleus and centrosome integrity are essential for the success of ICSI [11].

Efforts to preserve sperm structural integrity during cryopreservation have been made by testing various protector substances, e.g. albumin [7] and [12], EGTA solution [13] and [14] and trehalose [15]. However, it is not clear how freeze-drying affects specific components of sperm cells. The primary objective of the present study was to determine how the structural and functional components of bovine sperm were affected when various freeze-drying media containing these protective substances were used.

2. Materials and methods

2.1. Animals

All semen samples were obtained from one Nelore bull with semen previously tested for in vitro fertilization in our laboratory. In a pre-experiment, semen samples from three bulls were tested for lyophilization. Freeze-dried sperm with different lyophilization media were evaluated by the acridine orange test (AOT) for chromatin integrity and by trypan blue-giemsa stain for acrosome integrity. Since sperm characteristics were similar for all three bulls (Table 1), to facilitate comparisons of embryo development, we selected one bull as the source of all semen used in the experiment.

Table 1
Percentage of bovine sperm cells with intact chromatin and an intact acrosome in semen from three bulls that was freeze-dried in TCM Hank's supplemented with fetal calf serum (T1), trehalose (T2), and EGTA solution (T3)

Bull	Intact chromatin (%)			Intact acrosome (%)		
	T1	T2	T3	T1	T2	T3
1	97	100	100	88	90	89
2	95	100	100	83	91	87
3	98	100	100	85	93	90

There were no significant differences among bulls or treatments

2.2. Solution for freeze-drying sperm

All reagents, unless otherwise stated, were purchased from Sigma Chemicals (St. Louis, MO, USA). The different freeze-drying media tested were: Medium 1: TCM 199 with Hank's salts (Gibco Life Technologies Inc., Grand Island, NY, USA) supplemented with 10% (v/v) FCS (Gibco Life Technologies Inc.); Medium 2: TCM 199 with Hanks salts supplemented with 10% (v/v) fetal calf serum and 0.2 mol/L trehalose; Medium 3: 10 mmol/L Tris–HCl buffered supplemented with 50 mmol/L of each of NaCl and EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N'N'-tetraacetic acid] and pH of final solution adjusted to 8.2. Media 1, 2 and 3 were considered as Treatments 1, 2 and 3, respectively.

2.3. Experimental design

Immediately after collection, motility was evaluated subjectively using light microscopy, morphology by phase contrast microscopy, and concentration by counting cells in a hemocytometer. Only semen samples with >80% motility and <10% morphologically abnormal sperm were used for the experiment. Each ejaculate was divided into two portions. One portion was cryopreserved with egg yolk Tris glycerol extender [16] with a total concentration of 30 × 106 sperm/0.5 mL and was used as the control group. The other portion was centrifuged in a percoll gradient (45–90%), to remove seminal plasma. Subsequently, sperm was washed twice in Tyrode's albumen lactate pyruvate (TALP) [17], and allocated into the three freeze-drying treatments (Treatments 1, 2 and 3). Samples from all treatments were submitted to freeze-drying process under the same conditions, were stored for 3 months at 4 °C, and then rehydrated and sperm were evaluated by light, electron and fluorescence microscopy. In addition, sperm from different treatments were microinjected into matured oocytes to evaluate embryo developmental potential.

2.4. Sperm freeze-drying

For all treatments, the concentration was adjusted to 10 × 106 sperm/100 µL freeze-drying solution. Samples were diluted, placed in tubes of 1.5 mL and kept at room temperature for 30 min. Then, sperm were cooled in liquid nitrogen vapor (approximately −80 °C for 1 h), by keeping the tubes at a distance of 5 cm from liquid nitrogen surface before plunged into it. Frozen samples were immediately inserted into the freeze-drying machine (Thermo Savant, Holbrook, NY, USA), previously stabilized at −40 °C and 350 × 10−3 Mbar pressure. After 12–16 h of freeze-drying, the tubes containing the samples were well covered with aluminum foil and stored for 3 months at 4 °C.

2.5. Rehydration

Freeze-dried sperm samples were rehydrated by adding 100 µL of milli-Q water at room temperature.

2.6. Plasma membrane and acrosome integrity

Before and after freeze-drying, integrity of the sperm membrane and acrosome was assessed by light microscopy following Didion et al. [18] with slight modifications. A sample of 20 µL from each treatment suspension was incubated with 20 µL of trypan blue stain (0.2%) at 37 °C for 10 min, and then centrifuged twice at 700 × g for 6 min. The pellet was re-suspended with 0.5 mL of TALP, three smears were made from each sample, fixed with methanol for 5 min, dried and stained overnight with Giemsa (10%). Slides were evaluated by counting 200 cells in bright field microscopy and the results were expressed as percentage of cells with an intact acrosome.

2.7. Ultrastructural assessment

To confirm the observations obtained in light microscopy and to evaluate sperm ultrastructure, transmission electron microscopy was done on samples from all treatments. Sperm of each treatment were fixed for 3 h at room temperature in a solution containing 2% glutaraldehyde, 2% paraformaldehyde, 5% sucrose and 5 mM CaCl2 in 0.1 M sodium cacodylate buffer, pH 7.2. After fixation, the specimens were rinsed in buffer, and post-fixed (1

h) in 1% osmium tetroxide, 0.8% potassium ferricyanide, 5 mM CaCl2 in 0.1 M sodium cacodylate buffer. Dehydration was carried out in acetone and embedding in Epon 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a JEOL 1011 (JEOL, Tokyo, Japan) transmission electron microscope, operating at 80 kV. All electron microscopy reagents were purchased from Electron Microscopy Sciences (Ft. Washington, PA, USA).

2.8. Evaluation of chromatin integrity by acridine orange test

For acridine orange staining, three smears from each sample were prepared on glass slide and air-dried. Each smear was fixed overnight in Carnoy's solution, freshly prepared with methanol and glacial acetic acid (3:1 ratio). The slides were air-dried again, and incubated in tampon solution (80 mmol/L citric acid and 15 mmol/L Na2HPO4, pH 2.5) at 75 °C for 5 min to test the chromatin stability. Then, the slides were stained with acridine orange stain (0.2 mg/mL). The slides were washed with water to remove background staining and while still wet, they were covered with coverslips and evaluated using epifluorescence microscope (Axiophot Zeiss; 490 nm/530 nm excitation/barrier filter). A total of 100 cells were analyzed for each treatment slide. Sperm with normal DNA content had green fluorescence, whereas sperm with an abnormal DNA content emitted fluorescence in a spectrum varying from yellow-green to red. The percentage of sperm with intact chromatin was calculated by dividing the number of green-stained sperm by the total number of sperm and multiplying by 100.

2.9. Detection of DNA fragmentation by TUNEL assay

For the TUNEL technique, we used the in situ Cell Death Detection Kit, fluorescein, carried out according to manufacturer's (Roche Diagnostics GmbH, Mannheim, Germany) protocol with slight modifications. Sperm were washed in 100 µL of phosphate-buffered saline (PBS) supplemented with 0.1% polyvinylpyrrolidone (PVP). Sperm suspension was fixed with 100 µL of 4% paraformaldehyde for 1 h at room temperature. Cells were washed again in PBS supplemented with PVP 0.1% and permeabilized with 0.5% Triton X-100 in 0.1% sodium citrate for 1 h on ice. Then, permeabilized sperm were washed once in PBS supplemented with PVP 0.1% and incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) plus dUTP label in the dark at 37 °C for 1 h. After labeling, the cells were washed in PBS with PVP 0.1% and counterstained with 5 µg/mL Hoechst 33342 to visualize

total DNA. Negative (omitting TdT from the reaction mixture) and positive (using only DNAse I, 1 mg/mL for 30 min at room temperature) controls were performed in each sample. For each sample tested, at least 100 cells were analyzed using a fluorescence microscope. Each spermatozoon was assigned to contain either a normal (blue nuclear fluorescence due to Hoechst 33342) or a fragmented DNA (green nuclear fluorescence). The final percentage of sperm with fragmented DNA was referred to as TUNEL positive (%).

2.10. Gametes preparation for ICSI

Oocytes were recovered from abbatoir-derived ovaries and matured for 22 h in medium composed of TCM 199 with Earle's salts, supplemented with FCS 10% (v/v), 24 IU/mL of LH, 10 μg/mL of FSH and antibiotics (50 IU/mL penicillin and 50 μg/mL streptomycin). Matured oocytes were then exposed to 1 mg/mL of bovine hyaluronidase for 5 min to remove the cumulus cells. Denuded oocytes with evident polar body were transferred back to maturation medium and maintained in the incubator until microinjection.

Freeze-dried sperm samples were rehydrated and washed twice by centrifugation for 3 min at 700 × g in TALP medium, where they remained until their use for ICSI. The frozen semen samples used as a control were prepared by centrifugation for 20 min at 700 × g in a percoll gradient (45 to 90%) [17]. The selected sperm obtained after percoll were capacitated by incubation with 200 μg/mL of heparin for 1 h, before being used for ICSI.

2.11. Sperm microinjection

Sperm microinjection was performed by using a micromanipulator (Narishige Instrument, Tokyo, Japan) connected to an inverted microscope (Nikon, Japan) in a 200× magnification. Dishes for microinjection were prepared by adding 10 drops of 20 μL of TCM 199 with Hanks salts and 25 mM HEPES, FCS (10%, v/v) and antibiotics in the periphery of the dish and 4 drops of 10% polyvinylpyrrolidone (PVP) solution (Irvine Scientific, Santa Ana, CA, USA) containing the sperm in the center of the dish. The selected spermatozoon had its tail broken by pressing the injection pipette against the bottom of the Petri dish. The spermatozoon with a broken tail was then aspirated into the injection pipette and transferred to the drop containing the oocytes. The tip of the pipette was introduced into the perivitelline space and the oolemma was ruptured by gently aspirating small amounts of the ooplasm into the pipette. Then, the spermatozoon and the small amount of the aspirated cytoplasm were

inserted back into the oocyte with a minimal volume of PVP solution. Sham injections were performed in a similar manner for the parthenogenetic control.

2.12. Oocyte activation and embryo culture

All microinjected oocytes from each treatment were cultured for 1 h in SOF medium [19], until activation time. For activation oocytes were exposed to a 5 µM of ionomycin solution for 5 min and then were transferred to SOF medium supplemented with 6 mg/mL of BSA fatty acid free, where they remained for 5 min to stop activation. After activation oocytes were cultured in SOF medium for 4 h for extrusion of the second polar body and finally incubated in a solution of 1.9 mM 6-dimethylaminopurine (DMAP) for 4 h. Activated oocytes were cultured in SOF medium, using a co-culture with cumulus cells under mineral oil at 39 °C with 5% CO2 in air.

2.13. Evaluation of decondensed sperm head, pronucleus formation and embryonic development

A total of 165 oocytes were removed from culture at 16–18 h post-ICSI, fixed overnight in methanol: acetic acid (3:1, v/v) and stained with 1% of acetic acid lacmoid. Then, the oocytes were examined with phase-contrast microscopy to verify the presence of a decondensed sperm head and pronucleus formation. Embryonic development was assessed 2 days (cleavage) and 7 days (blastocyst rate) after the start of culture.

2.14. Statistical analysis

All data were submitted to a normality test before analysis. One-way ANOVA was used to compare the effects of various freeze-drying treatments. The data are given as mean values ± S.D. When ANOVA revealed a significant effect, the treatments were compared by a Holm-Sidak test using Sigma Stat for Windows Version 3.11 (Systat Software, Inc., Richmond, California, USA). A difference of $P < 0.05$ was considered significant.

3. Results

3.1. Pre-experiment results

All sperm (100%) from the three bulls evaluated had a damaged plasma membrane, regardless of the protector medium utilized. There were no differences ($P > 0.05$) among bulls or treatments for chromatin and acrosome integrity (Table 1).

3.2. Evaluation of optical microscopy

Although fresh semen had an average of 90% motile sperm, dilution with freeze-drying media reduced sperm motility. Ten minutes after dilution, motility was 80, 30 and 40% for treatments Treatments 1, 2 and 3, respectively. After 30 min, no further change in the motility for Treatment 1 was noted, whereas in Treatments 2 and 3, all cells were immotile. Indeed after freeze-drying and rehydration, all sperm were immotile and were stained as dead (by the trypan blue-giemsa stain), due to breakage of the plasma membrane.

Sperm from Treatments 1 and 2 were easily diluted and dispersed by the use of milli-Q water. In contrast, those from Treatment 3 were strongly agglutinated, being very difficult to dissolve, even after repeated pipetting.

Although there was a tendency ($P < 0.10$) for the percentage of sperm with tail separated from the head to be higher in Treatment 1 (8%) versus Treatment 2 (5%), Treatment 3 (6%) and control (3%), no differences were detected among then ($P > 0.05$). The percentage of sperm cells with an intact acrosome in Treatment 2 (92%) was similar ($P > 0.05$) to the control group (95%), but higher ($P < 0.05$) than in Treatments 1 (75%) and 3 (85%).

3.3. Ultrastructural assessment

Electron microscopy (Table 2) confirmed the results obtained by optical microscopy in regard to plasma membrane and acrosome integrity. The sperm cell component most affected by freeze-drying was the plasma membrane (Fig. 1A–D), which was damaged in all treatments, differing from the control (Fig. 1E and F). Microtubules organization was also compromised in the majority of the sperm from Treatments 2 and 3 (Fig. 1), diverging from Treatment 1 (Fig. 1B) and control (Fig. 1F), in which microtubules were intact. Conversely, the acrosome and

mitochondria were well preserved in all treatments; in that regard, sperm preserved with trehalose had more intact acrosomes (Fig. 1A–F).

Table 2
Ultrastructural evaluation of the principal components of bovine sperm, following freeze-drying using dilution media supplemented with fetal calf serum, trehalose, or EGTA solution (Treatments 1, 2 and 3, respectively)

Treatment	Membrane	Acrosome	Mitochondria	Microtubules
1	Damaged	Intact	Intact	Intact
2	Damaged	Intact	Intact	Damaged
3	Damaged	Intact	Intact	Damaged
Control	Intact	Intact	Intact	Intact

The control treatment was frozen-thawed semen from the same bull.

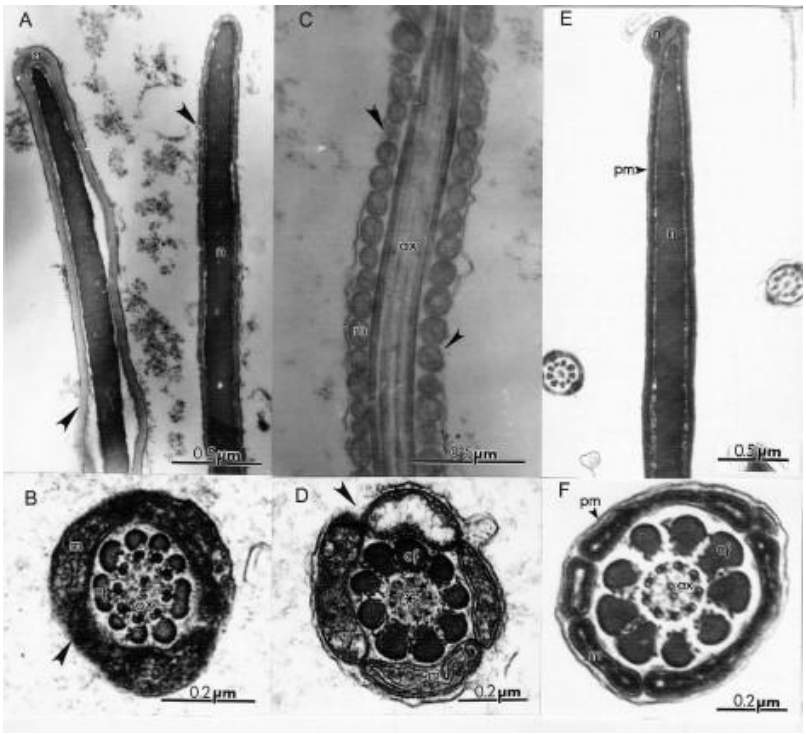

Fig. 1. Electron micrographs showing freeze-dried sperm: (A) sperm from T3 with damaged plasma membrane (arrowhead) and intact acrosome (a) and nucleus (n); (B) sperm from Treatment 2 with damaged plasma membrane (arrowhead), presence of intact mitochondria (m) and axoneme (ax); (C) sperm from Treatment 1 with damaged plasma membrane (pm, arrowhead), presence of intact mitochondria (m), coarse fibers (cf) and axoneme (ax); (D) sperm from Treatment 2 with disorganized microtubules (*); (E and F) frozen sperm (control) with well-preserved ultrastructure.

3.4. DNA fragmentation assay

The TUNEL assay was used to assess differences in DNA damage among treatments. Treatment 3 had the lowest rate of DNA fragmentation (2%), not significantly different from the control. Treatment 2 had 5% of the cells with DNA fragmentation, which was higher than

both Treatment 3 and the control, but lower than Treatment 1. The highest ($P < 0.05$) DNA damage rate (14%) was present in Treatment 1.

3.5. Development of oocytes after ICSI

The functionality of the freeze-dried sperm nucleus (assessed by ICSI) is shown in Table 3 and Table 4. When matured oocytes were microinjected with sperm from Treatments 2 and 3, the proportion of oocytes with sperm head decondensation and pronuclear formation, was similar ($P > 0.05$), but superior ($P < 0.05$) to Treatment 1. Similarly, the rate of development to the blastocyst stage was similar ($P > 0.05$) for the control and Treatments 2 and 3, but superior ($P < 0.05$) to Treatment 1 and the parthenogenetic control (sham injection).

Table 3
Number and percentage of bovine oocytes (combined for five replicates) with an intact sperm head (ISH), sperm head decondensation (DH) and pronucleus formation (PN) after ICSI using frozen-thawed spermatozoa (control) and spermatozoa lyophilized with freeze-drying media supplemented with fetal calf serum, trehalose, or EGTA solution (Treatments 1, 2 and 3, respectively)

Treatment	No. of oocytes	ISH	ISH + 1PN	DH + 1PN	2PN
1	40	9 (22.5%)	17 (42.5%)	6 (15.0%)^{b}	8 (20.0%)^{a}
2	40	5 (12.5%)	6 (15.0%)	11 (27.5%)^{a}	18 (45.0%)^{b}
3	40	10 (25.0%)	2 (5.0%)	13 (32.5%)^{a}	15 (37.5%)^{b}
Control	45	11 (24.4%)	4 (8.8%)	5 (11.1%)^{b}	25 (55.5%)^{c}

(a and b) Within a column, values without a common superscript differ ($P < 0.05$).

Table 4
Embryonic development (combined for 10 replicates) after ICSI using frozen-thawed spermatozoa (control), sham injection (parthenogenetic control) and spermatozoa lyophilized with freeze-drying media supplemented with fetal calf serum, trehalose, or EGTA solution (Treatments 1, 2 and 3, respectively)

Treatment	No. of oocytes	No. of cleaved (%)	No. of blastocysts (%)
1	185	67 (36.2%)^{a}	19 (10.2%)^{a}
2	185	93 (50.2%)^{b}	34 (18.3%)^{b}
3	180	104 (57.7%)^{c}	35 (19.4%)^{b}
Frozen-thawed semen	185	99 (53.5%)^{c}	38 (20.5%)^{b}
Sham injection	160	52 (32.5%)^{a}	13 (8.1%)^{a}

(a–c) Within a column, values without a common superscript differ ($P < 0.05$).

4. Discussion

In the present study, we have generated novel data regarding the extent that various freeze-drying media preserved the structural and functional characteristics of the bovine sperm. We demonstrated for the first time that EGTA solution, which is widely used in mice [2], [3], [4], [5], [6], [7], [8], [9], [10], [11], [12], [13] and [14], efficiently preserved the nucleus,

acrosome and mitochondria of bovine sperm. In addition, we showed that presence of trehalose in medium supplement with FCS provided better protection to bovine sperm than the medium with FCS alone. The presence of a decondensed sperm head, pronucleus and blastocyst formation after ICSI demonstrated that freeze-dried sperm using EGTA solution and medium supplemented with FCS and trehalose were able to fertilize matured oocytes.

The long-term goal of research on freeze-drying sperm is to preserve its motility and fertilizing capability, enabling the sperm to subsequently be used in AI or IVF. However, this goal has not been achieved in any of the species studied. As expected, in the present investigation, sperm were immotile after freeze-drying in all treatments. Indeed, damage to the sperm plasma membrane of all cells analyzed was verified by exclusion stain and confirmed by electron microscopy. In that regard, the plasma membrane is highly susceptible to damage, due to loss of water during dehydration. According to Crowe et al. [20], water loss from phospholipid headgroups in cell membranes may lead to lateral phase separation and cause extravasation of intracellular contents.

The acrosome is another sperm component that has been widely reported to be substantially damaged during the freeze-drying process. However, >75% of the sperm had an intact acrosome, regardless of the medium used. The lower percentage ($P < 0.05$) of cells with an intact acrosome in Treatments 1 and 3 (75 and 85%) suggested that the protection of the acrosome structure was less pronounced when FCS and EGTA solution were present in the lyophilization. Although there is no report in cattle, the results of the present study differed from those described in mice [7] and rabbits [8] and are similar to those in rats [21]. It is important to point out that electron microscopy confirmed that the acrosome was not lost and no changes in its morphology were detected freeze-dried sperm as well as in the control group (Fig. 1A and E). We inferred that the presence of protective media during freeze-drying provided higher acrosome retention and minimized morphological alterations.

Separation of the tail from the head of the lyophilized sperm has been mentioned as a common finding in sperm from pigs [9], rabbits [8] and in mice when Dulbecco's Modified Eagle Medium (DMEM) [7] or EGTA medium [13] and [14] was used. Although there were slight differences among media used, in general we observed a low degree of head–tail separation. Media containing trehalose and EGTA had the lowest rate of tail separation. Perhaps bovine sperm has a greater stability in the connection region than other species, which could account for the low rate of loss of tail after freeze-drying when compared to data from other species. Based on electron microscopy, in all treatments the mitochondria were preserved (Fig. 1C) after freeze-drying, however, microtubules remained intact only in the media containing FCS and in the control group (Table 2; Fig. 1B and F). Therefore, we

hypothesize that the media with EGTA and trehalose, due to their hypertonicity, could affect microtubule integrity.

Although the objective of freeze-drying was to completely preserve structural and functional sperm characteristics, an intact sperm nucleus is the essential prerequisite for successful embryo development [7] and [14]. Mammalian sperm nuclei are very stable and highly condensed with a unique DNA organization [22], 6-fold more compact and 40-fold lower DNA volume than somatic cells [23] and [24]. This unique DNA packing is essential to protect the cell and minimize damages caused by exogenous agents before fertilization. It has been suggested that the bovine sperm have a more stable nuclear packing than other species [25]. Although this has caused some difficulties for sperm head decondensation in the ooplasm when bovine sperm are used for ICSI, it facilitates preservation of bovine sperm by freeze-drying.

Sperm DNA can be damaged during freeze-drying and especially during storage if the adequate protection is not provided. It is well known that DNA damage can be caused by activation of endogenous nucleases, which always occurs after freeze-drying. In the present investigation, DNA damage during freeze-drying and storage were evaluated by TUNEL. Sperm preserved in media containing trehalose and EGTA stored for 3 months at 4 °C had lower sperm DNA fragmentation than the medium containing FCS.

It was not known why sperm agglutination occurred in the medium with EGTA; although it hindered the manipulation in ICSI, it did not affect the results. Therefore, the nuclear function of freeze-dried sperm (evaluated by ICSI) corroborated the structural evaluation by TUNEL; sperm lyophilized with media containing EGTA or trehalose had the highest rates of sperm head decondensation, formation of a pronucleus, and production of a blastocyst (Table 3 and Table 4).

Although solutions containing EGTA or trehalose adequately preserved bovine sperm viability during the freeze-drying process, the mechanism and function of these solutions in protecting the sperm are not clear. It is believed that EGTA, a calcium chelator, either prevents or decreases the activity of calcium-dependent endonucleases [14] by limiting the availability of the circulating calcium; therefore, EGTA is recommended to minimize chromosome breakage [26]. Conversely, trehalose, a disaccharide of glucose is present in many organisms that are able to survive complete dehydration, a phenomenon known as anhydrobiosis [20] and [27]. It is hypothesized that during sperm freeze-drying, trehalose binds to the sperm membranes and makes them more stable (reduced molecular mobility), which would increase biological stability. Another possibility is that trehalose could replace the water and cause stabilization due to the formation of hydrogen bridges with components of the cells when the

water is removed [28]. Therefore, trehalose has been successfully used to protect liposomes [29], bacteria [30], yeast [31], retroviruses [32], proteins [33], chromatin [34], and cell lines in vitro [35], [36] and [37].

In conclusion, we demonstrated that the medium TCM 199 with Hanks salts supplemented with FCS and 0.2 M of trehalose and the medium containing EGTA solution were more efficient in avoiding damage to components of bovine sperm, especially the nuclei. Therefore, the medium used for freeze-drying process directly affected sperm nuclear integrity. Considering the beneficial effects of trehalose and EGTA, perhaps a combination of the two would confer even better protection to sperm during the freeze-drying and especially during storage.

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