Title: Adverse Effect of Cake Collapse on the Functional Integrity of Freeze-Dried Bull Spermatozoa

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Keywords: Bull spermatozoa; Cake collapse; Freeze-drying; Glass transition temperature; ICSI; Tg'

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Title:

Adverse Effect of Cake Collapse on the Functional Integrity of Freeze-Dried Bull Spermatozoa

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ABSTRACT

Under optimal freeze-drying conditions, solutions exhibit a cake-like porous structure. However, if the solution temperature is higher than the glass transition temperature of the maximally freeze-concentrated phase ($T_g'$) during drying phase, the glassy matrix undergoes viscous flow, resulting in cake collapse. The purpose of the present study was to investigate the effect of cake collapse on the integrity of freeze-dried bull spermatozoa. In a preliminary experiment, factors affecting the $T_g'$ of conventional EGTA buffer (consisting of Tris-HCl, EGTA and NaCl) were investigated in order to establish the main experimental protocol because EGTA buffer $T_g'$ was too low (-45.0°C) to suppress collapse. Modification of the EGTA buffer composition by complete removal of NaCl and addition of trehalose (mEGTA buffer) resulted in an increase of $T_g'$ up to -27.7°C. In the main experiment, blastocyst yields after ooplasmic injection of freeze-dried sperm preserved in collapsed cakes (drying temperature: 0 or -15°C) were significantly lower than those of sperm preserved in non-collapsed cake (drying temperature: -30°C). In conclusion, freeze-dried cake collapse may be undesirable for maintaining sperm functions to support embryonic development, and can be inhibited by controlling both $T_g'$ of freeze-drying buffer and temperature during the drying phase.

Keywords: Bull spermatozoa; Cake collapse; Freeze-drying; Glass transition temperature; ICSI; $T_g'$
Although cryopreservation is the most common method for sperm preservation, liquid nitrogen use is associated with various problems such as high maintenance cost and risk of accidental loss of frozen cell stock. To overcome these problems, freeze-drying has been proposed as an alternative method for sperm preservation. Wakayama and Yanagimachi [36] first reported that freeze-dried mouse sperm stored at refrigerator temperature could support full-term development after intracytoplasmic sperm injection (ICSI). Since then, several studies have been conducted to improve the developmental ability of oocytes injected with freeze-dried spermatozoa, paying special attention to buffer composition [13,14,18,24]. A relatively simple buffer consisting of Tris-HCl, EGTA and NaCl (defined hereafter as EGTA buffer) has been used for suspending and freeze-drying spermatozoa [6,14,15,18,24]. Until now, successful birth of live offspring derived from freeze-dried spermatozoa has been reported in several mammalian species including mouse [13,14,18,19,36], rat [8,9], hamster [26] and rabbit [21]. However, practically acceptable offspring rates were achieved only with rodents. For large domestic species including cattle and pigs, blastocysts have been harvested from ICSI oocytes with freeze-dried sperm cells [16,20,24].

In addition to the buffer composition, the drying conditions are also important for pharmaceutical protein production [12]. Frozen samples are generally dried at the maximum allowable product temperature ($T_{\text{max}}$) to acquire the highest dehydration speed [29]. For a solute system which forms amorphously after freezing, the theoretically optimal value of $T_{\text{max}}$ depends on the glass transition temperature of the maximally freeze-concentrated phase ($T_g^*$). The solution exhibits a cake-like porous structure under the optimal drying conditions. But, if the product temperature is higher than the $T_g^*$ during the drying phase, the glassy matrix will undergo viscous flow, resulting in loss of its porous structure. This loss is defined as a collapse.
phenomenon [27,33]. Collapsed cakes contain high amounts of residual water [37], and prolonged reconstitution time may be required during rehydration due to reduced surface area [1,35]. In freeze-drying of pharmaceutical proteins, collapse can adversely affect acellular product stability [12,23,28]. However, it remains unclear whether collapse incidence in freeze-dried sperm suspensions has a detrimental effect on rehydrated cell function. In previous studies, we used freeze-drying conditions for rat and bull spermatozoa which did not consider this phenomenon, and we recently observed that those conditions can produce cake collapse (unpublished observation). Additionally in somatic cell freeze-drying for nuclear transfer in sheep, collapse can be confirmed qualitatively in figures which depict its typical characteristics [22].

The purpose of the present study was to investigate the effect of cake collapse on the functional integrity of freeze-dried and rehydrated bull spermatozoa. Since it was found that $T_g'$ of the EGTA buffer was too low to suppress collapse, in a preliminary experiment (Experiment 1), factors affecting $T_g'$ (including composition of the EGTA buffer and cooling rate during freezing) were examined using calorimetric measurements in order to establish the subsequent experimental protocol. In the main experiment (Experiment 2), collapsed and non-collapsed cakes were produced by drying bull sperm suspensions in modified EGTA buffers at different temperatures. The functional integrity of the rehydrated sperm cells was assessed by ICSI, alkaline comet assay and transmission electron microscopic observation (TEM).
Materials and methods

Experimental design

In Experiment 1, the $T_g'$ of the EGTA buffer conventionally used for sperm freeze-drying was determined by differential scanning calorimetry (DSC), and the effect of buffer composition modification (removal of NaCl, and addition of trehalose at different concentrations) on $T_g'$ was investigated. Effects of three different cooling rates were also investigated. In Experiment 2, bull spermatozoa were resuspended in the modified EGTA buffer and freeze-dried under three different temperatures. Both collapsed and non-collapsed cakes were used for measurements of residual water content and glass transition temperature ($T_g$). The functional integrity of the rehydrated sperm cells was assessed by blastocyst yield 8 days after ICSI, followed by alkaline comet assay and TEM.

Measurement of $T_g'$ and $T_g$ by DSC

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). The EGTA buffer consisted of 10 mM Tris-HCl, 50 mM EGTA and 50 mM NaCl (pH 8.0), and was modified by complete removal of NaCl and/or addition of trehalose to give a final concentration of 0.01, 0.05, 0.1, 0.5 and 1.0 M. Fifteen µL of the EGTA buffer with or without the above-mentioned modifications was transferred to an aluminum pan and cooled to -80°C in DSC (Seiko Instruments Inc., Chiba, Japan). The aluminum pan was then scanned at a rate of 10°C/min until it reached 20°C, using an empty aluminum pan as a reference. Otherwise, the cooling rate was adjusted to be -1 or -20°C/min in DSC, or a presumed < -1,000°C/min by direct plunging into liquid nitrogen (5 min). In this series, NaCl-free EGTA buffer supplemented with 0.5 M trehalose, defined hereafter as “mEGTA buffer”, was used.
\[ T_g \] of sperm suspension cakes was measured to reflect freeze-drying completeness. Cakes (> 1 mg) were collected and transferred to aluminum pans in a glove box filled with nitrogen gas and were then hermetically sealed. Cooling rate of the cakes in DSC was \(-20^\circ C/min\) and the scanning rate was \(10^\circ C/min\). The second scanning was conducted after annealing at 110-130\(^\circ C\) for 5 min to eliminate interference from enthalpy relaxation \([34]\), because the first DSC thermogram of the cake was too unclear to determine \(T_g\).

Freeze-drying of bull sperm

Commercially available frozen semen from a Japanese Black bull was thawed in a water bath at 37\(^\circ C\) for 30 sec. The contents of a 0.5 mL straw was layered on the top of Percoll density gradient consisting of 2 mL of 45% (v/v) Percoll/TALP above 2 mL of 90% (v/v) Percoll/TALP in a 15 mL conical tube, and centrifuged for 20 min at 700\(g\). The sperm pellet was re-suspended in 4 mL of mEGTA buffer and then washed twice (5 min at 300\(g\) each). Fifteen \(\mu L\) of sperm suspension at a concentration of \(2-4 \times 10^7\) sperm/mL was placed in a 5-mL volume glass vial and transferred onto shelf (pre-cooled to -30\(^\circ C\)) of a programmable freeze-dryer (ALPHA 2-4; Christ, Harz, Germany). After freezing for 30 min (the cooling rate between +25 and -25\(^\circ C\) was estimated as \(-20^\circ C/min\)), the sperm suspension was dried for 6 h at 0, -15 or -30\(^\circ C\) relative to the shelf temperature. The chamber pressures employed during the drying phase were determined as one-third of the vapor pressure of ice at each temperature \([27]\); they were 1.98, 0.57 and 0.12 hPa for drying at 0, -15 and -30\(^\circ C\), respectively. After sealing of vials with rubber caps and aluminum stoppers, the samples were stored overnight at -20\(^\circ C\). Residual water contents (% [w/w]) in freeze-dried cakes were calculated gravimetrically, using reference weights of sperm suspension before freeze-drying (=100% content) and after heating in oven at 96\(^\circ C\) for 24 h (=0% content).
**Blastocyst production by ICSI**

Abattoir-derived bovine ovaries were transported to the laboratory in saline (maintained at 10 to 12°C) within 24 h after slaughter. The contents of 2-8 mm follicles were aspirated and oocytes surrounded with at least two layers of compact cumulus cells were collected from the follicular fluid. Maturations were conducted in Hepes-buffered TCM-199 (Earle’s salt; Gibco BRL, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; SAFC Biosciences, Lenexa, KS, USA), 0.2 mM sodium pyruvate, 0.02 AU/mL FSH (Kawasaki-Mitaka Pharmaceutical, Kanagawa, Japan), 1 µg/mL 17β-estradiol, and 50 µg/mL gentamycin sulfate for 22 h at 38.5°C under 5% CO₂ in air. Next, cumulus cells were removed by a brief vortex-mixing in the Hepes-buffered TCM-199 supplemented with 3 mg/mL bovine serum albumin (BSA), 0.2 mM sodium pyruvate, 1,000 IU/mL hyaluronidase and 50 µg/mL gentamycin sulfate. Matured oocytes extruding the first polar body were used for the following experiment.

Freeze-dried spermatozoa were rehydrated with 15 µL of ultrapure water immediately before ICSI, and non-dried control spermatozoa were those after Percoll-washing. Each sperm suspension was washed twice with modified Brackett and Oliphant (mBO) medium (IVF100; Institute for Functional Peptides, Yamagata, Japan) at 300g for 5 min. Two µL of sperm suspension was mixed with 8 µL of M2 medium [30] containing 10% (w/v) polyvinylpyrrolidone (PVP), and then ICSI was performed with a piezo-driven micromanipulator (PMAS-CT150; Prime Tech, Ibaraki, Japan) as described previously [11]. The ICSI oocytes prepared within 1 h of rehydration were treated with 5 µM ionomycin in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS) for 5 min and incubated in Hepes-buffered TCM-199 supplemented with 5% (v/v) FBS, 0.2 mM sodium pyruvate and 50 µg/mL gentamycin sulfate.
gentamycin sulfate at 38.5°C under 5% CO₂ in air for 3 h. Next, the oocytes were treated with 7% (v/v) ethanol in Heps-buffered TCM-199 supplemented 1 mg/mL PVP for 5 min and subsequently 2 mM 6-dimethylaminopurine (6-DMAP) in modified synthetic oviduct fluid (mSOF) [10], supplemented with 30 µL/mL essential amino acids (× 50 solution, Gibco-11130), 10 µL/mL non-essential amino acids (× 100 solution, Gibco-11140) and 5% (v/v) FBS (defined hereafter as mSOFaa/FBS) at 38.5°C under 5% CO₂ in air for 3 h. The presumptive zygotes were cultured in a 250 µL microdrop of the mSOFaa/FBS at 39.0°C under 5% CO₂, 5% O₂ and 90% N₂ for 8 days. Cleavage rate and blastocyst formation rate were determined after 2 and 8 days of culture, respectively.

**Alkaline comet assay**

To estimate DNA damage in the freeze-dried spermatozoa, alkaline comet assay [32] was conducted according to the operation manual of the Comet Assay kit (Trevigen, Helgerman Ct, USA) with some modifications. Briefly, spermatozoa freeze-dried at 0 and -30°C, as well as non-dried control spermatozoa (10 µL of 1 × 10⁶ cells/mL suspension) were mixed with 1% (w/v) low-melting agarose gel (90 µL), added onto agarose-covered 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 in the latter two-third) and then processed with electrophoresis under a pH>13 alkaline condition (10 V, 20 min). Half of the sperm suspension was treated with 10 mM H₂O₂ for 20 min at 4°C before being mixed with low-melting agarose gel. Sperm DNA were stained with SYBR Green, and the captured BMP images of the comet (> 100 comets per sample) were analyzed by the Comet Score software. The DNA fragmentation index (tail moment) was calculated as the length of comet tail (pixel) × the % DNA librated.
**Ultrastructural analysis**

TEM was applied to the spermatozoa freeze-dried at 0 and -30°C and non-dried control spermatozoa. Sperm cells were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M PBS (pH 7.4) at 4°C. After centrifugation at 300g for 5 min, the sperm pellets were post-fixed with 2% (w/v) osmium tetroxide in the same buffer at 4°C for 1 h. The sperm cells were subsequently dehydrated in the graded series of ethanol, substituted with the propylene oxide and embedded in epoxy resin (Okenshoji Co., Tokyo, Japan). Ultrathin sections were cut on an ultra-microtome (Leica Microsystems, Wetzlar, Germany) at 0.1 µm thicknesses, mounted on copper grids and stained with 1% (w/v) uranyl acetate and lead citrate. Electron micrographs were taken with a transmission electron microscope (JEOL Ltd., Tokyo, Japan). The number of sperm with membrane gaps was recorded.

**Statistical analysis**

Arcsine-transformed percentage data in residual water content, cleavage and blastocyst yield were compared using one-way ANOVA. Values of $T_g'$ in freeze-drying buffers, $T_g$ of freeze-dried cakes and tail moment were also compared using one-way ANOVA. When ANOVA reached significance, differences among means were analyzed using post hoc Tukey’s tests. Incidences of membrane damage in TEM were compared using a chi-square test with a Bonferroni correction. P values less than 0.05 were considered significant.
**Results**

**Experiment 1**

$T_g'$ of the conventional EGTA buffer was as low as -45.0 ± 0.61°C (mean ± SD) in DSC measurement (Fig. 1 and 2). Complete removal of NaCl from the EGTA buffer resulted in a $T_g'$ increase to -41.0 ± 0.59°C (Fig. 2). Supplementation of trehalose to the NaCl-free EGTA buffer (0.01 to 0.5 M) made it possible to further increase the $T_g'$ (up to -27.7 ± 0.36 °C; Fig. 1 and 2), but supplementation of 1.0 M trehalose had no further effect on $T_g'$ increase and rendered post-centrifugation sperm retrieval difficult due to the high specific gravity. The $T_g'$ of mEGTA buffer frozen rapidly in liquid nitrogen (-29.4 ± 0.08°C) was significantly lower than when frozen slowly (-27.2 ± 0.94 and -27.6 ± 0.05°C at -1 and -20°C/min, respectively).

**Experiment 2**

Freeze-dried cake collapse was observed when the drying phase temperature was higher than the $T_g'$ of mEGTA buffer (Fig. 3A and B). On the other hand, when processed at -30°C the sperm suspension cake exhibited a silky porous structure without any signs of collapse (Fig. 3C). Collapsed cakes contained a higher amount of residual water when compared with non-collapsed cakes (3.3 to 3.6% vs 0.7%), as shown in Table 1. $T_g$ values of collapsed cake were significantly lower than that in their non-collapsed counterparts (13.5 to 14.1°C vs 50.1°C; Table 1).

Embryonic cleavage ability with freeze-dried spermatozoa was comparable regardless of collapse incidence, but blastocyst yields after injection of sperm dried at 0 and -15°C were lower than those when dried at -30°C (0.7 to 3.7% vs 14.2%; Table 2). Both cleavage rate and blastocyst yield in the non-dried control group were significantly higher than those in freeze-dried groups. Alkaline comet assay revealed that the freeze-drying process did not induce
sperm DNA damage when the baseline of tail moment was not stimulated by H$_2$O$_2$ treatment (Table 3). After application of the H$_2$O$_2$ treatment, harmful effects of freeze-drying on sperm DNA integrity were clearly detected (almost double with respect to control), but there was no significant difference in the values of tail moment between sperm samples dried at 0 and -30°C. Analysis of TEM images revealed that sperm with severe or moderate membrane gaps (Fig. 4A and B) were frequently observed after drying at 0°C versus -30°C (43.6%, 140/321 versus 28.7%, 90/314; P < 0.05). These proportions were significantly higher than those in non-dried control sperm (10.9%, 36/330).
Discussion

In sperm preservation by freeze-drying, maintenance of cellular function can be influenced by drying conditions. However, few studies have focused on the drying condition for mammalian sperm freeze-drying. Kawase et al. [15] reported that the ability of mouse spermatozoa to support full-term development was better maintained when the sperm were dried under a chamber pressure of 0.37 hPa versus 1.03 and 0.04 hPa, but did not include information regarding the drying temperature. In freeze-drying of macroscopic samples, it is known that chamber pressure is associated with drying temperature [17]. Dehydration speed is dramatically decreased if the chamber pressure is higher than the vapor pressure of ice at the product temperature; on the other hand, excessively low chamber pressure induces low product temperature, resulting in dehydration speed decreases [5,27]. It has therefore been recommended that the chamber pressure should be set to the one-quarter to one-half of the vapor pressure of ice at the product temperature [27]. In the present study, chamber pressures were fixed to one-third of the vapor pressure at the shelf temperature in order to clarify the effect of collapse on sperm function independent of chamber pressure.

Collapse of freeze-dried cake is believed to harm the stability of acellular pharmaceutical protein products [12,23,28]. Since the conventional EGTA buffer has very low \( T_g \)’ value (-45°C; Fig. 1 and 2), it may be reasonable to assume that conventional freeze-dryers (without drying temperature control) and even programmable freeze-dryers (the lowest shelf temperature of our programmable freeze-dryer was -30°C) could have produced collapsed cake in previous studies. In order to suppress collapse, the EGTA buffer composition was modified to yield a higher \( T_g \)’.

NaCl removal and trehalose addition resulted in \( T_g \)’ increases from -45°C to -28°C (Fig. 1 and 2). Electrolytes such as NaCl increase the quantity of unfrozen water in the freeze-concentrate, where the unfrozen water acts as a plasticizer [7]. Trehalose is a disaccharide which is known to
facilitate glass formation, and whose solution T_g’ has been reported to be between -22 and -32°C [4]. Rapid cooling with liquid nitrogen, routinely employed for sperm suspension freezing prior to drying [6,14,36], was also found to affect the T_g’ of mEGTA buffer. Such small decreases in T_g’ may be the result of glass transition prior to sufficient freeze-concentration during rapid cooling. In viral vaccine freeze-drying, the importance of freezing conditions has been described [38].

Collapse of sperm suspension cake was dependent on drying temperatures, as expected from a correlation between T_g’ of mEGTA buffer and drying temperature (Fig. 3). This allowed an empirical investigation of the correlation between collapse incidence of freeze-dried sperm suspension and function of rehydrated sperm cell. Drying the sperm samples at -40°C was not impossible if our programmable freeze-dryer was run in a cold room (+4°C), but the drying process seemed to be insufficient after the scheduled 6 h running (data not shown). The ability of rehydrated sperm to support blastocyst development was impaired when the sperm were preserved in collapsed cakes (Table 2). High residual water content was characteristic of collapsed cakes (Table 1), consistent with previous results [37]. It is known that high residual water content decreases the T_g of freeze-dried cake. If T_g is lower than the storage temperature, the sample stability would decrease with residual water content due to increased molecular mobility [2]. However, the residual water would not be responsible for the lower blastocyst yields in the present study, because T_g of collapsed cake (approximately +14°C) was higher than the storage temperature (-20°C). Since collapse leads to increased product resistance to water vapor flow and decreased sublimation rates, collapse may cause loss of sperm function during the drying phase. These results suggest that collapse should be avoided in order to recover the functional sperm cells after rehydration.

In standard alkaline comet assay procedures, H_2O_2 treatment serves as positive control
reference. In this study, this standard procedure failed to detect differences in DNA damage, both between freeze-dried and non-dried spermatozoa, and between spermatozoa dried at 0 and -30 °C (Table 3). However, when the baseline of tail moment was stimulated by H$_2$O$_2$ treatment, freeze-drying was found to harm sperm DNA integrity. Nevertheless there was no significant difference in the moment values between spermatozoa dried at 0 and -30°C. Since membrane permeability of H$_2$O$_2$ is enough to neglect influence [3], the present results may suggest the higher sensitivity of freeze-dried sperm to oxidative stress which is likely to deteriorate sperm DNA damage caused by reactive oxygen species present in oocytes.

TEM image analysis indicated that freeze-dried sperm damage was localized to their plasma membrane (Fig. 4A and B), especially in sperm dried at 0 versus -30°C. Comparable cleavage rates after ICSI of sperm dried at 0 and -30°C contradict the hypothesis that active sperm-borne oocyte activating factor flows out through damaged plasma membranes. The damaged membrane resulted in the increase of sperm stickness and rendered the ICSI operation difficult (data not shown). Although TEM unexpectedly failed to detect other ultrastructural damage, the nuclear matrix was reported to be essential for paternal DNA replication [31]. Lower blastocyst yield after ICSI with freeze-dried bull sperm, compared with previous reports [16,24], may be caused by low quality of oocytes retrieved from 1-day-stored ovaries [25] and/or suboptimal method to activate ICSI oocytes with freeze-dried spermatozoa. Blastocyst-to-calf developmental potential needs to be further investigated by transfer to recipients, following proof of normal karyotype [16].

In conclusion, freeze-dried cake collapse may adversely affect sperm function. Since temperatures higher than the $T_g$ of freeze-drying buffer induce cake collapse, controlling both the buffer $T_g$ and the drying-phase temperature can reduce collapse incidence.
Acknowledgements

We thank Dr. Musubu Ichikawa for use of glove box in $T_p$ measurement and Ms. Makiko Shinotsuka for her technical assistance in TEM.
References


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Figure Captions

Fig. 1. DSC thermogram of EGTA buffer and mEGTA buffer. The arrow indicates the glass transition temperature of the maximally freeze-concentrated phase (T_g'). Data are expressed as the mean ± SD of 3 replicates in each group.

Fig. 2. Effect of EGTA buffer composition on the glass transition temperature of the maximally freeze-concentrated phase (T_g'). Squares with solid lines represent EGTA buffers, and circles with dotted lines represent NaCl-free EGTA buffers.

Fig. 3. Freeze-dried cakes appearance. (A) Collapsed cake dried at 0°C, (B) Collapsed cake dried at -15°C, (C) Non-collapsed cake dried at -30°C, (D) Zoomed-out view of the glass vial containing a cake. Scale bars represent 2 mm (A-C) and 2 cm (D).

Fig. 4. Ultrastructural damage observed in sperm plasma membrane. (A) Sperm with severe membrane gaps, (B) Sperm with moderate membrane gaps, and (C) Intact spermatozoa. Arrows indicate break of sperm plasma membranes. Scale bar represents 0.4 µm.
Table 1 Residual water contents and glass transition temperature ($T_g$) of freeze-dried cakes.

<table>
<thead>
<tr>
<th>Temperature during drying phase (°C)</th>
<th>Residual water contents (% [w/w])</th>
<th>$T_g$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.6 ± 0.1 $^a$</td>
<td>14.1 ± 3.3 $^a$</td>
</tr>
<tr>
<td>-15</td>
<td>3.3 ± 0.2 $^a$</td>
<td>13.5 ± 2.1 $^a$</td>
</tr>
<tr>
<td>-30</td>
<td>0.7 ± 0.1 $^b$</td>
<td>50.1 ± 1.0 $^b$</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of 3 replicates in each group.

$^a,b$ Superscripts represent significantly different groups (P < 0.05).
Table 2 Developmental potential of bovine oocytes injected with bull spermatozoa dried at different temperatures.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Oocytes (n [%])*</th>
<th>Developed to blastocysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freeze-drying during drying phase (ºC)</td>
<td>Sperm-injected</td>
<td>Cleaved</td>
</tr>
<tr>
<td>+ 0</td>
<td>173</td>
<td>67 (37.9 ± 5.3)a</td>
</tr>
<tr>
<td>+ -15</td>
<td>173</td>
<td>70 (39.1 ± 4.0)a</td>
</tr>
<tr>
<td>+ -30</td>
<td>172</td>
<td>68 (41.5 ± 3.1)a</td>
</tr>
<tr>
<td>- -</td>
<td>149</td>
<td>89 (60.5 ± 2.4)b</td>
</tr>
</tbody>
</table>

Percentages are expressed as the mean ± SEM of at least 4 replicates in each group. Blastocyst yields are calculated from the number of cleaved oocytes.

a,b,c Superscripts represent significantly different groups within columns (P < 0.05).
Table 3 DNA damage of freeze-dried bull sperm detected by alkaline comet assay.

<table>
<thead>
<tr>
<th>Freeze-drying during drying phase (ºC)</th>
<th>Temperature</th>
<th>Values of tail moment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard protocol</td>
<td>H₂O₂-treated</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>6.8 ± 0.5</td>
</tr>
<tr>
<td>+</td>
<td>-30</td>
<td>6.3 ± 0.7</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>6.9 ± 0.3</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD of 3 replicates in each group.

<sup>a,b</sup> Superscripts represent significantly different groups (P < 0.05).
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Figure 1

EGTA buffer

$T_g' = -45.0 \pm 0.6 \, ^\circ C$

mEGTA buffer

$T_g' = -27.7 \pm 0.4 \, ^\circ C$