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Cytoskeletal proteins F-actin and β-dystrobrevin are altered by the cryopreservation process in bull sperm ☆

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ABSTRACT

The cryopreservation process has an important impact on sperm structure and physiology. The negative effects have been mainly observed on the plasma membrane, which is directly stabilized by the cytoskeleton. Since cytoskeleton proteins are osmosensitive and thermosensitive, the aim of this study was to evaluate the damage caused to the bull sperm cytoskeleton by cryopreservation (freezing–thawing). Fresh and frozen-thawed bull semen samples were exposed to a treatment with the neutral detergent Brij 36-T. Electron microscopy evidenced important damages at the sperm perinuclear theca after the protein extraction protocol; the perinuclear theca was partially solubilized, the perinuclear theca substructure disappeared in the cryopreserved samples. Furthermore, the sperm head's shape was significantly altered on the cryopreserved samples. Fluorescence analysis showed a decrease of the intensity of actin and dystrobrevin on the frozen-thawed samples. Western blot assays revealed a stronger signal for actin and β-dystrobrevin in the frozen-thawed sperm samples than in the fresh ones. Our results suggest that the cryopreservation process highly alters the sperm cytoskeleton stability, causing its proteins to become more fragile and therefore more susceptible to be extracted.

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Introduction

Cryopreservation protocols of mammalian spermatozoa always result in injuries during freezing and thawing [11,44]. These cryoinjuries include the loss of membrane fluidity and integrity, oxidative stress leading to lipid peroxidation [24], DNA fragmentation [50], cytoskeleton modifications [31,15,12,20,25], reduction of the percentage of motile, live and acrosome-intact sperm, additionally it favors nuclear decondensation and decreases the sperm ability to fertilize [36,40]. During the freezing process, ice formation in the extracellular solution causes solutes and suspended materials, including cells, to migrate and localize into freeze-concentrated compartments [41]. This process exposes cells to a hypertonic environment which leads to an osmotic loss of water [44]. Glycerol was the first cryoprotectant used to preserve bull sperm, since it prevents fracture of frozen solutions reducing the total ice volume expansion during water solidification [22,55]. However, glycerol causes osmotic and toxic effects on cryopreserved cells of different species [19,27,8].

Structural elements of the cytoskeleton are responsible for the appropriate cell volume regulation. Research done on different mammalian sperm species confirmed that cell volume regulation is crucial for the success of cryopreservation [53,60]. Furthermore, an intact cytoskeleton is required to regulate sperm volume [51]. It is noteworthy that an increase in the proportion of sperm showing postacrosomal actin immunoreactivity has been reported in cryopreserved cells [31,12,20]. In addition the amount of α-tubulin recovered from the cryopreserved groups increased in comparison to the control [15]. Both changes suggest that important alterations of sperm cytoskeleton occurred during the cryopreservation process.

The sperm nucleus is surrounded by a complex protein structure, named perinuclear theca (PT), whose proteins and cytoskeleton are characterized by being highly resistant to neutral detergent extraction and high salt buffers [39,47]. Recently, studies in frozen-thawed bull spermatozoa revealed the structural damage caused to

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the cytoskeleton associated to the perinuclear theca (PT) and PT substructure due to cryopreservation, which alter nuclear decondensation [25,40]. The PT is the main cytoskeletal element, it surrounds the sperm nucleus as a helmet, except at the tail insertion [45]. A vast net of proteins conform the PT and their distribution has been reported in previous studies [47,45,37,13]. While some PT proteins are located only at the subacrosomal or postacrosomal region, other are distributed in both regions [45]. Among these proteins F-actin and dystrobrevin are importantly distributed at the PT as well as others sperm structures. In addition F-actin has been found associated to the sperm plasma membrane [28]. F-actin and dystrobrevin, a cytoskeletal protein associated to F-actin, are also associated with the PT and flagellum [13,43,21,29]. Many different functions are attributed to the PT such as the volume regulation [52], capacitation [6] and the early steps prior to fertilization [62,65,34].

Dystrobrevin is a cytoplasmatic component of the dystrophin associated protein complex (DPC), which links the cortical actin cytoskeleton with the extracellular matrix and serves as a scaffold for signaling proteins such as protein kinase A. Dystrobrevins are the products of two different genes coding for two highly homologous proteins, α- and β-dystrobrevin, the first is predominantly expressed in skeletal muscle, heart, lung and brain, whereas β-dystrobrevin is considered as non-muscle, and it is abundant in brain, lung, kidney and liver [57]. Different isoforms of dystrobrevins are present in guinea pig sperm, being β-dystrobrevin the most abundant and it is found associated to the perinuclear theca and flagella [29].

Considering that the cytoskeleton is resistant to be solubilize by neutral detergents [7,61,48], the aim of this study was to evaluate the morphological damage caused to the bull sperm cytoskeleton by the cryopreservation process as well as the alterations of some its constituent proteins (F-actin and β-dystrobrevin). In the present study, fresh and frozen-thawed semen samples were exposed to neutral detergent extraction, and assessment of their protein content was performed. Sperm treated with neutral detergent and the proteins solubilized were evaluated by electron microscopy, immunodetection and SDS–PAGE. We also determined the effects that the cryopreservation process had on the stability of the PT cytoskeleton and PT-substructure in order to investigate the potential mechanism for cell damage.

Materials and methods

Most reagents used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA), Merck (Whitehouse Station, NJ, USA), and Bio-Rad (Cambridge, MA, USA), unless otherways stated. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Jackson Immunoresearch (West Grove, PA, USA).

Semen collection and cryopreservation

Semen was collected from eight Holstein–Frisian bulls by the use of an artificial vagina. Bulls were between 2 and 3 years of age with a known reproductive history and freezability. Progressive movement of all semen samples used was above 70%. An aliquot of fresh semen was kept as a control; the rest of the ejaculate was processed as previously described [40]. Briefly, skimmed milk and antibiotics (lincomycin and gentamycin) were added as first semen extender, and then temperature was lowered at 4 °C. After dilution with the second extender containing glycerol plus the ingredients mentioned before, the extended semen samples were left at 4 °C during 4 h for equilibration. Then, semen was loaded into straws and frozen using automated equipment. Then straws were plunged into liquid nitrogen for storage. After a freezing period of 2 weeks, straws were thawed in a water bath at 35 °C for 20 s.

Cytoskeleton exposure

Fresh and frozen-thawed sperm were washed by centrifugation at 1000 rpm for 5 min at 4 °C. Then sperm cytoskeleton was exposed by resuspending the sperm (35×10^6 sperm/ml) in a solution of Brij 36T (1.2%) diluted in PBS (cytoskeleton buffer); the sperm were incubated for 10 min, at 4 °C. Then the samples were centrifuged at 5000 rpm for 5 min at 4 °C, the supernatants were recovered and treated with SDS–PAGE. Pellets, containing cytoskeleton exposed sperm were washed three times by centrifugation at 5000 rpm for 5 min at 4 °C, and fixed for either immunofluorescence or electron microscopy.

Perinuclear theca and flagella solubilization by DTT–SDS treatment

After treatment with cytoskeleton buffer, sperm were resuspended in Tris–HCl buffer (50 mM, pH 9.0) containing 25.4 mM DTT (final concentration) and incubated for 15 min on ice. Thereafter, samples were added with 1.0% SDS (final concentration) [19,34], and incubated for 5 min on ice. Immediately, sperm were washed three times with Tris–HCl buffer and fixed for posterior observation using negatively stained electron microscopy.

Electron microscopy

Sperm cytoskeletons and sperm heads from fresh or frozen-thawed samples were fixed to assess their ultrastructure by electron microscopy observation. As mentioned elsewhere [40], aliquots containing 35×10^6 sperm were suspended in 1 ml of saline solution (154 mM NaCl), fixed in Karnovsky solution [35] for 1 h, washed three times at 1500 rpm for 3 min each with saline solution plus three more washes with distilled water. The final pellet was resuspended in filtered distilled water, drops of 100 μl samples were mounted over 300 mesh cooper grids. Then they were stained with phosphotungstic acid (0.002%, final concentration, Merck Darmstadt, Germany) before being examined with a JEOL 2000EX transmission electron microscope (Akishima, Japan).

Electrophoresis and Western blot (Wb)

Sperm (500×10^6) were resuspended in lysis buffer (50 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM PMSF. Complete, 1 mg/ml aprotinin, 10 mM sodium orthovanadate, 25 mM sodium fluoride, and 1% Triton X-100) as previously reported [49]. Samples were then centrifuged at 14,000 rpm for 5 min at 4 °C, supernatants were collected and protein concentration was determined [5]. Whole sperm proteins samples and proteins extracted by cytoskeleton buffer were then boiled for 5 min in sample buffer [38]. Then proteins were loaded in 10% SDS–PAGE and transferred to nitrocellulose membranes [63]. Membranes were blocked using Phosphate-buffered saline (PBS) containing 5% dried fat-free milk and 0.1% Tween-20. Membranes were then incubated overnight at 4 °C with the corresponding antibody (anti-actin 1:1000 and anti-β-dystrobrevin 1:1000) and membranes were washed and incubated with the appropriate HRP-labeled secondary antibody (1:10,000). Finally, immuno-reactive proteins were detected by chemiluminescence using an ECL Western blot detection kit (Amersham).

Immunofluorescence procedures

Cells were fixed in 1.5% formaldehyde in PBS, permeabilized using acetone at −20 °C for 7 min and washed three times in PBS and once in distilled water. Water-resuspended cells were used

to prepare smears, which were air-dried at room temperature and rinsed with PBS. Smears were then incubated with the primary antibody dilutions (anti-β-dystrobrevin 1:100) in blocking solution (1% bovine serum albumin in PBS), under glass-cover slides for 12 h at 4 °C in humid conditions. Exhaustive PBS-washes were carried out, then, cells were incubated for 1 h at 37 °C under humid conditions with the appropriate TRITC-labeled secondary antibodies. In all cases, smears were exhaustively washed with PBS and mounted under glass-covers for their observation using gelvatol.

F-actin detection

The F-actin cytoskeleton was revealed using TRICT-labeled phalloidin (30 μM) for 45 min at 37 °C keeping humid conditions. Smears were exhaustively washed with PBS and mounted as mentioned above. Images were acquired using an Olympus BX50 photomicroscope equipped with phase contrast and epifluorescence or using a Leica TCS SP2 confocal microscope as required.

Densitometry analysis

To quantify Western blot band density, we used the software Image J 1.44 (open source, public domain, developed by The National Institute of Health, USA). All densitometry values obtained were calculated from non-saturated signals and were normalized with respect at the total amount of actin or β-dystrobrevin detected in whole sperm proteins samples. Results were analyzed by paired Student's *t*-test and expressed as Relative Intensity Normalized.

Statistical analysis

All results are representative of at least three independent experiments and were expressed as average ± S.E.M. Results comparing two samples were analyzed by paired Student's *t*-test. Significance levels were set at $P < 0.05$.

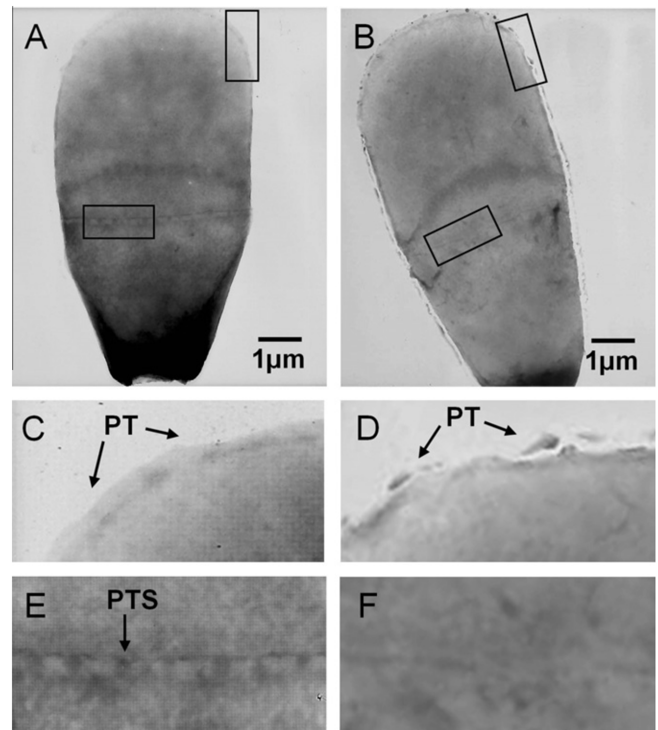

Fig. 1. Cryopreservation effects on the perinuclear cytoskeleton. Sperm were treated with the nonionic detergent Brij 36-T to remove plasma membrane, acrosome and nuclear membranes; they were whole-mounted in cooper grills for electron microscopy and then were negatively stained. (A) Electron micrograph showing PT and PT-substructure morphology of non-cryopreserved sperm. (B) Electron micrograph showing the alteration in PT and PT-substructure morphology on the frozen-thawed sperm. (C) and (D) are high-magnification of PT from NC-sperm and FT-sperm respectively (upper rectangles). (E) and (F) show high-magnification from the equatorial segment (lower rectangles), arrows indicate the presence of PT-substructure (PTS). These images represent three independent experiments.

Results

Cryopreservation alters the perinuclear theca (PT) cytoskeleton

In order to investigate the effect that cryopreservation had on the cytoskeleton, the presence of the PT and PT-substructure in frozen-thawed bull sperm (FT-sperm) and non-cryopreserved sperm (NC-sperm) previously treated with Brij 36-T was analyzed by electron microscopy. After exposure to cytoskeleton buffers, NC-sperm showed the PT associated around the nucleus (Fig. 1A and C), and its PT-substructure was observed linked to the equatorial segment (Fig. 1A and E). FT-sperm treated with cytoskeleton buffer showed that the PT was detached from the nucleus and partially solubilized (Fig. 1B and D), as well as a complete absence of PT-substructure over the equatorial segment was observed (Fig. 1B and F). In spite of the damage to FT-sperm, nuclear decondensation was not detected.

The sperm cytoskeleton is destabilized by cryopreservation

In order to search in detail the cryopreservation effects on bull sperm cytoskeleton, we analyzed two important cytoskeletal elements present in mammalian sperm, F-actin and β-dystrobrevin. In NC-sperm labeled with phalloidin-TRITC (see Materials and methods), F-actin was observed in the whole sperm head, while the middle piece displayed an intense fluorescence, a pale fluorescence was observed on the principal piece (Fig. 2A). When NC-sperm were treated with the cytoskeleton buffer, F-actin was observed mainly over the equatorial segment, postacrosomal region and middle piece (Fig. 2C). In contrast, FT-sperm showed a uniform florescence throughout the entire cell (Fig. 2B). However, when these cells were treated with the cytoskeleton buffer, a pale fluorescence was observed on the postacrosomal region and the middle piece (Fig. 2D). Immunolocalization of β-dystrobrevin in NC-sperm showed fluorescence throughout the entire head, however, higher fluorescence was detected at the equatorial segment and along the flagellum (Fig. 3A). Nevertheless, when NC-sperm were treated with cytoskeleton buffer, β-dystrobrevin was found at the subacrosomal and equatorial regions, as well as along the flagella (Fig. 3C). In contrast, in FT-sperm β-dystrobrevin was located throughout the whole sperm without a defined pattern (Fig. 3B). Moreover, after these sperm were treated with cytoskeleton buffer, β-dystrobrevin fluorescence decreased within the flagella, and fluorescence was very faint over the head (Fig. 3D). These results suggest that an important amount of F-actin and β-dystrobrevin were solubilized by the cytoskeleton buffer in the FT-sperm.

To corroborate the above results, the amount of actin and β-dystrobrevin was analyzed by Western blot in the solubilized proteins extracted with the cytoskeleton buffer on FT and NC-sperm samples. Proteins extracted from approximately 20×10^6 NC-sperm or FT-sperm were treated for Wb (see Materials and methods). Anti-actin antibody revealed a protein band with a RM of 45 kDa, which was more intense on the FT-sperm samples than on the NC (Fig. 4A). When anti-β-dystrobrevin antibody was used, a protein band with a RM of 66 kDa was detected (Fig. 4A), its intensity was lower in NC-sperm that in FT-sperm (Fig. 4A). The amount of actin and β-dystrobrevin detected by Wb from the solubilized

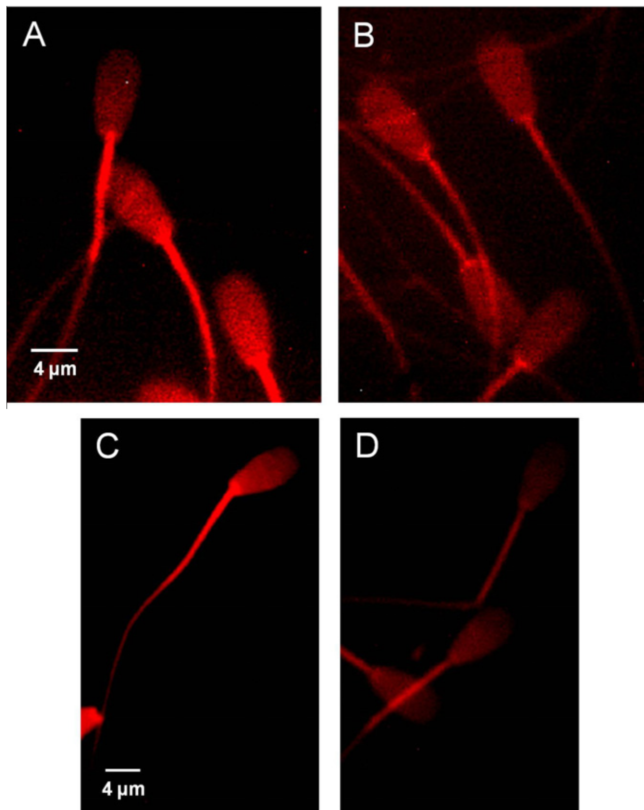

Fig. 2. Cryopreservation effects on the F-actin cytoskeleton. Sperm were treated with the nonionic detergent Brij 36-T, washed with PBS and then fixed. F-actin was detected using Phalloidin-TRITC. (A) and (C) NC-sperm before and after Brij 36-T treatment, respectively. (B) and (D) FT-sperm before and after Brij 36-T treatment, respectively. These images represent three independent experiments.

proteins by the cytoskeleton buffer was analyzed by densitometry and compared with the total actin and β-dystrobrevin detected in 20×10^6 NC-sperm or FT-sperm (Fig. 4B). This analysis showed no difference in the total amount of actin and β-dystrobrevin contained in NC-sperm and FT-sperm (Fig. 4B); however when the sperm cytoskeleton was extracted significant differences were obtained between actin and β-dystrobrevin from NC-sperm and FT-sperm (Fig. 4B).

Effect of PT solubilization on the sperm nucleus

It has been reported that cryopreservation produces different damages on the perinuclear theca [40]. Therefore, in order to investigate whether the sperm nucleus was affected by cryopreservation after treatment with cytoskeleton buffer, the PT was solubilized using the DTT–SDS treatment (see Materials and methods), procedure that at short times of incubation solubilizes the PT and PT substructure, only maintaining the integrity of the nuclear matrix as well as the nuclear cytoskeleton [30,46]. Transmission electron microscopy analysis showed that the PT and PT-substructure were totally solubilized from NC-sperm and FT-sperm (Fig. 5A and B, respectively); however, an important increase in the nucleus size of FT-sperm treated with DTT–SDS was observed (Fig. 5B), thus, the area displayed by the nucleus was evaluated. The mean nucleus area from FT-sperm resulted to be significantly greater than the nucleus area of NC-sperm (Fig. 5C) ($P < 0.05$). Among the FT-sperm, only 18.9 ± 1.9% ($n = 3$) showed a nucleus area similar to the mean displayed by NC-sperm. These results suggest a possible alteration in the nuclear cytoskeleton produced by the cryopreservation process.

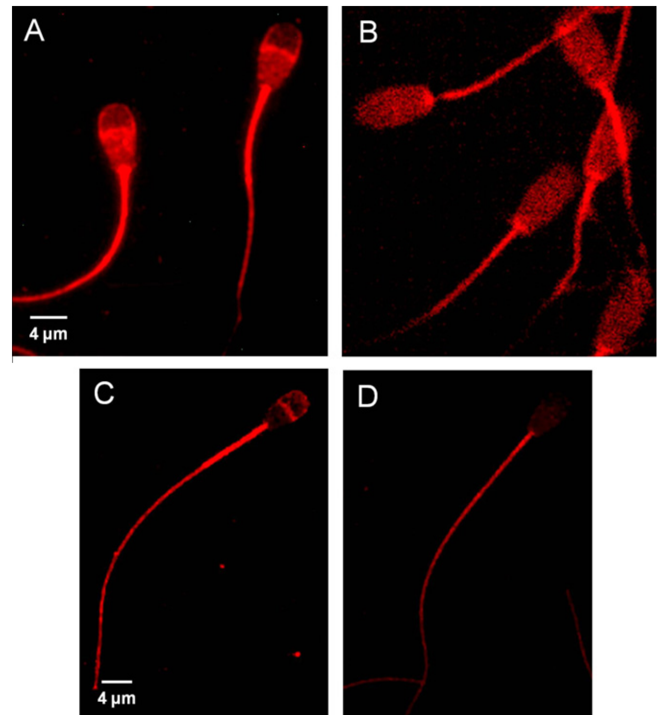

Fig. 3. Cryopreservation effects on β-dystrobrevin. Sperm were treated with the nonionic detergent Brij 36-T, washed with PBS and then fixed. β-Dystrobrevin was located using a polyclonal antibody anti-β-dystrobrevin, which was reveled with a secondary antibody labeled with TRITC. (A) and (C) NC-sperm before and after Brij 36-T treatment, respectively. (B) and (D) FT-sperm before and after Brij 36-T treatment, respectively. These images represent three independent experiments.

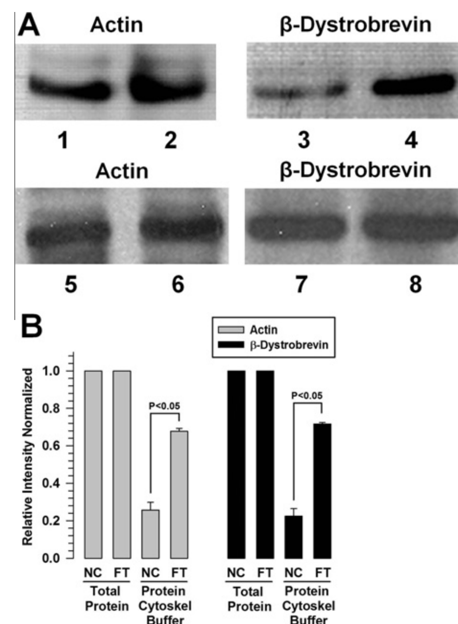

Fig. 4. Brij 36-T effects on cytoskeletal proteins from non-cryopreserved and cryopreserved sperm. Sperm were treated with the nonionic detergent Brij 36-T, and the solubilized proteins were treated with SDS–PAGE and transferred to nitrocellulose membranes. (A) Immunodetection of actin and β-dystrobrevin by Western blot. (1–4) Detection of actin and β-dystrobrevin solubilized by Brij 36-T treatment. (5–8) Detection of whole actin and β-dystrobrevin. (1, 3, 5 and 7) Non-cryopreserved sperm (NC). (2, 4, 6 and 8) Frozen-thawed sperm (FT). (B) Densitometric analysis of the immunodetected actin and β-dystrobrevin. The graph represents the mean ± S.E. of three independent experiments.

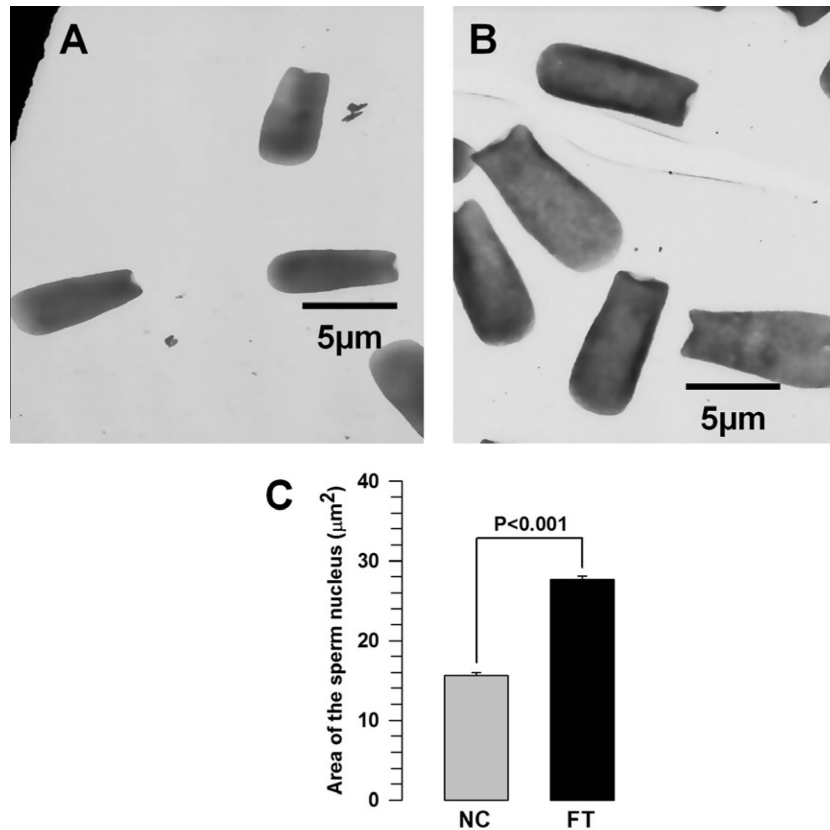

Fig. 5. Cryopreservation effects on nuclear cytoskeleton. Brij 36-T sperm were treated with DTT–SDS, the sperm heads were whole-mounted on cooper grills for electron microscopy, and then were negatively stained. (A and B) Sperm heads from NC-sperm and FT-sperm (respectively) after DTT–SDS treatment. (C) The area of sperm heads was measured, the graphic represents the mean ± S.E. of three independent experiments, where *n* = 30 sperm heads measured by experiment.

Discussion

The freezing/thawing process produces different cryoinjuries on living bull sperm that decreases the sperm ability to fertilize [36,40]. Other effects of cryopreservation can be observed when sperm membranes are solubilized with the non-ionic detergent Brij 36-T, increased the number of sperm with disrupted or absent PT-substructure [40]. The results presented in this study support this last observation (Fig. 1), besides we also found that in bovine sperm, the PT is partially solubilized by Brij 36-T treatment, suggesting that PT proteins and cytoskeleton were damaged by the cryopreservation process. These changes may become highly significant for the sperm ability to fertilize, since the sperm PT plays an important role during fertilization. It can be appreciated from the observation that oocyte activation can be fully triggered by intracytoplasmic injection of a sperm head with the nucleus and PT presents [36]. Furthermore, intracytoplasmic injection of the PT-associated protein PAWP (postacrosomal sheath WW domain binding protein) promotes meiotic resumption, pronuclear development and intracellular calcium release [65,1]. Therefore, important PT cryoinjuries may negatively affect the sperm ability to activate the oocyte.

Cryopreservation also causes an extreme fluctuation in osmolarity in sperm, resulting in sublethal and lethal injuries to the cells. It was previously hypothesized that the actin cytoskeleton might be damaged during sperm cryopreservation [64]. Alterations in the localization pattern of actin and F-actin have been reported in ram and boar sperm after cryopreservation [31,20,25]. In this work, we found similar changes in the network formed by F-actin in bovine sperm, and additionally, we found that β-dystrobrevin has also undergone changes in its cellular localization pattern. These changes suggest that the stability of the sperm cytoskeletal elements such as F-actin and β-dystrobrevin is altered by cryopreservation; probably the freezing/thawing process produces depolymerization or fracturing of the networks formed by F-actin, causing them to become susceptible to solubilization by neutral detergents such as Brij 36-T. This hypothesis is supported by different facts: (1) after freezing/thawing an increase in G-actin was detected in the postacrosomal region of ram sperm [12], (2) cryopreserved sperm, after Brij 36-T treatment, displayed low fluorescence for both F-actin and β-dystrobrevin, (3) increased actin and β-dystrobrevin solubilized by Brij 36-T was observed in cryopreserved sperm as compared to the corresponding non-cryopreserved control. This last result is not in agreement with the results obtained by Flores et al. Flores et al. [20], since they reported a clear decrease in the amount of actin solubilized after the freezing/thawing process which was greater in FT-sperm; this discrepancy could be explained by the methodology used to solubilize actin, (4) interestingly, there are reports about damages occurring to the mitochondrial sheath and matrix [58], as well as in the axoneme. In this regard Courtens and Paqiugnon [10] have reported that temperature reduction induced loss of axonemal organization, which was possibly produced by depolymerization of tubulin causing a great reduction of sperm motility, process that could be reverted (within 3–5 min) after sperm were rewarmed, thus recovering motility [10]; besides the recovery of motility could be accelerated when sperm are thawed in presence of ATP [32]. In agreement with the Courtens and Paqiugnon [10] results, an increase of α-tubulin solubilized from post-cryopreservation of human sperm was reported [15]. A major support for our hypothesis are the following important facts: hyposmotic stress drastically reduces F-actin in the macaque sperm [9]; previous studies on other cell types such as PC12 cells, astrocytes, HL-60 cells, chondrocytes and annulus fibrous cells, have demonstrated that

hyposmotic stress disrupted cortical F-actin in a dose-dependent manner [9,54,26,18]. Furthermore, the freezing–thawing process markedly altered the viscoelastic properties of the actin cytoskeleton in cryopreserved mouse oocytes, producing depolymerization of the F-actin network, which is reversible when the oocytes are treated with latrunculin A, suggesting that F-actin is more sensitive that G-actin to the freezing/thawing process [33]. So, the facts mentioned above, support our hypothesis about the freezing/thawing process which produces depolymerization or fracturing of the network formed by F-actin, changing of the viscoelastic properties of the sperm actin cytoskeleton, which consequently alters the cell or organelles volumes, as it was shown in our results corresponding to the sperm nucleus (Fig. 5).

Holt et al. Holt et al. [32], suggested that the reduction in sperm motility after cryopreservation is caused by decrease in ATP, effect expected when the mitochondrial sheath and matrix are damaged by cryopreservation. In this regard, measurement of ATP levels in frozen-thawed human sperm is not conclusive [4,42]. Concerning the actin cytoskeleton, it is well known that ATP depletion induces actin polymerization [2,16], therefore a depletion of ATP provoked by cryopreservation might cause actin polymerization in sperm and consequently a major damage on F-actin, since has been reported that F-actin is more sensitive to low temperatures than G-actin [33].

It is important to note, that the alteration in the F-actin network as well as its probable depolymerization may produce important damage to sperm, rendering them unable to fertilize, since F-actin is necessary for processes such as motility and fertilization [3,59]. However, it has been demonstrated that actin depolymerization and repolymerization take place during capacitation [28,6]; thereby we suggest that actin could be repolymerized during capacitation, specially in those sperm that have undergone little damage during cryopreservation, and therefore keep functional their protein machinery for actin polymerization, Rho proteins such as RhoA, RhoB and Cdc42 [14,17], and consequently are able to recover the fertilizing ability.

In this work we report that the localization pattern of β-dystrobrevin in the bovine sperm (Fig. 3) is similar to show by guinea pig sperm [29]. In addition, we observed that the cryopreservation process alters this pattern. Therefore, we suggest that since β-dystrobrevin is an actin binding protein, it might probably be dragged together with actin during the Brij 36-T treatment, furthermore, the dystrobrevin pattern could be recovered during capacitation when the actin is repolymerized.

Previous studies have demonstrated that the treatment with DTT–SDS solubilizes sperm membranes, flagellum and perinuclear theca, keeping the nuclear shape and size, as well as the structure of the nucleoskeleton, which includes sperm DNA and cytoskeletal proteins [30,46]. Our results show that DTT–SDS treatment drastically modified the sperm nuclear size after the cryopreservation process. Nuclear shape and size are kept by the so-called LINC complex which through the SUN and KASH proteins connects the nucleoskeleton with the cytoskeleton [56]. These proteins have been recently found associated to the nucleus of mammalian spermatides and mature sperm [23]. Altogether, the possible damages in the nuclear cytoskeleton and the LINC complex produced by cryopreservation could be responsible for the changes in nuclear size, although this hypothesis remains to be clarified.

In conclusion, the cryopreservation process alters important PT proteins like actin and dystrobrevin, as well as the nucleoskeleton; such damages could be impairing many crucial sperm functions, leading to fertilization failure. Therefore, it is important to be aware of the changes that are being produced by the freezing/thawing procedure, which affects important sperm physiological processes, such as motility and capacitation, this with the purpose to develop better cryopreservation protocols, whose main goal must be either to keep the actin cytoskeleton's integrity or to develop a treatment that may allow the proper actin repolymerization after the freezing/thawing procedure.

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