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Glycerol decreases the integrity of the perinuclear theca in boar sperm

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Summary

We evaluated the effect of glycerol on the perinuclear theca (PT) of boar sperm. Samples from six ejaculates obtained from three different boars were incubated in the detergent Brij 36-T. Spermatozoa were treated with a glycerol concentration of either 2 or 4%, and incubated for 10 or 30 min; two other samples were treated with protease inhibitors (PI; leupeptin or an inhibitor commercial cocktail), mixed with 4% glycerol, and incubated for 30 min. A third glycerol-free group was used as the control. The samples were processed for electron microscopy evaluation. The PT remained intact in 78% of the control samples while, after addition of glycerol for 30 min, the proportion of spermatozoa with disrupted or absent PT increased ($P < 0.05$). PT was preserved in PI samples, but PT changes increased ($P < 0.05$). Differences due to treatment with glycerol (2 or 4%) at 10 or 30 min were not observed. These results show, to our knowledge for the first time, the adverse effect of glycerol on the integrity of the PT.

Keywords: Boar spermatozoa, Glycerol, protease inhibitors, Perinuclear theca substructure

Introduction

Cryopreservation is a biotechnique used to preserve spermatozoa from different mammal species for an indefinite time. It has the advantage of being able to use the best breeders even after the animal is no longer available (Hafez, 1997).

Semen freezing was performed successfully for the first time in 1949 by Polge, Smith & Parkes, who discovered the cryoprotective effect of glycerol when used to freeze rooster semen (Polge *et al.*, 1949). However, today, semen cryopreservation continues to be a controversial approach, and freezing protocols are modified constantly in an effort to obtain better results in terms of survival and fertility of thawed semen. Indeed, approximately 50% of the initial spermatozoa population is lost after the process due to damage caused to the membranes, the cytoskeleton, the motor apparatus, and the nucleus of spermatozoa (Mazur, 1970; Parks & Graham, 1992; Watson, 1995). In bovines the use of glycerol has led to the commercial freezing of semen, but in the porcine species the technique is far from adequate, due to the greater susceptibility of pig spermatozoa to freezing–thawing-mediated damage

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(Polge *et al.*, 1970; Cerolini *et al.*, 2001; Córdova *et al.*, 2001).

It is known that the cryoprotective properties of glycerol are due to its ability to cross the plasma membrane and prevent the formation of intracellular ice crystals by lowering the water freezing point. However, the concentration at which it should be used as a cryoprotective agent is not well established. There are reports that a 2–6% glycerol concentration can produce good results, but that after freezing–thawing the fertilizing capacity of sperm depends on temperature and exposure time (Osinowo & Salamón, 1976; Fiser & Fairfull, 1990; Hammerstedt & Graham, 1992; Fiser *et al.*, 1993). It has been indicated (Hammerstedt & Graham, 1992; Meyers, 2005) that the osmotic stress that glycerol exerts on the sperm cell may be associated with its molar concentrations or with its ability to insert itself between the phospholipid bilayer or with its potential to enter as an intermediary in metabolic pathways. In addition, it was also found that glycerol interacts with microtubules and other cytoskeletal proteins (Petrunkina *et al.*, 2004).

In regard to the cytoskeleton, this structure is involved in mechanisms that are responsible for regulation of cell volume, e.g. activation or deactivation of ion conductance channels in various cell types. Particularly, it has been found that a microfilament-intact cytoskeleton is required to maintain the proper level of swelling and cell volume regulation in boar spermatozoa (Petrunkina *et al.*, 2004).

In relation to the loss of fertility associated with cryopreserved boar spermatozoa, damage to the acrosome has been attributed to the combined effect of the glycerol concentration, and the cooling rate (Wilmut & Polge, 1974; Fiser *et al.*, 1993; Meyers, 2005; Si *et al.*, 2006). However, recently it was demonstrated that alterations in the sperm head cytoskeleton (perinuclear theca, PT) of spermatozoa from bovine and boar are related to the loss of the acrosome (Martínez *et al.*, 2006; Barrientos-Morales *et al.*, 2009).

The perinuclear theca (PT) is the main cytoskeletal structure in the sperm head (Fouquet & Kann, 1994). It surrounds the nucleus, except at the base where the flagellum is inserted (Courtens *et al.*, 1976; Mújica *et al.*, 2003). The PT has been involved, among other things, in the assembly of the acrosome (Escalier, 2006; Arancibia-Salinas *et al.*, 2007) and is a possible reservoir for signalling molecules (Mújica *et al.*, 2003; Sutoksky *et al.*, 2003).

Juárez & Mújica (1999) reported the formation of a perinuclear theca substructure (PTS) in the apical region of the postacrosomal sheath of the PT in spermatozoa from several species, including boar (Juárez & Mújica, 1999; Juárez-Mosqueda, 2000); significantly, actin forms part of the PTS. Moreover, it has been reported that the PT of boar and bovine spermatozoa undergo alterations after the cryopreservation process (Martínez *et al.*, 2006; Orozco–Benítez *et al.*, 2008). Even though in other cell types it has been reported that cytoskeletal proteins may undergo changes because they are temperature sensitive (Noiles *et al.*, 1995), the idea that such changes may be induced primarily by the toxic effect of glycerol is not ruled out (Storey *et al.*, 1998).

Many investigators believe that the low fertility to cryopreserved semen can be attributed to membrane structure and function alterations (Juárez & Mújica, 1999). It was decided to determine whether this situation could be related to a toxic effect of glycerol on the PT, as recent work has indicated that this structure is damaged by freeze-thawing. Thus, the goal of this investigation was to determine whether there is a toxic effect of glycerol on the PT cytoskeletal proteins of boar spermatozoa, by assessing the integrity of PTS.

Materials and methods

Biological material

Ejaculates were obtained from three 2-year-old breeding male boars from the Teaching, Research and Extension Boar Production Center from the Veterinary Medicine and Zootechnics Faculty of the National University Autonomous of Mexico.

Semen was collected using the gloved hand technique. Only the sperm-rich fraction was collected, and was filtered through sterile gauze. After obtaining the ejaculate, the volume was measured based on weight (1 kg = 1 l) and diluted 1:1 with a commercial diluent (IMV USA 870 XT–R:St Louis 183 MO/USA). A monitored polyurethane thermal box was used to transport the collected semen at 23°C.

Semen evaluation

Sperm motility was evaluated in semen aliquots that were placed on slides at 37°C. Samples that did not comply with the established parameters of 80% motility and less than 15% morphological abnormalities were discarded. Percentages of morphological abnormalities (head alterations, tail alterations, and cytoplasmic drop) were evaluated during the cell count.

Percentages of live and dead spermatozoa were evaluated by staining with eosin-nigrosine, (supravital staining). A Neubauer chamber was used to count the number of sperm.

PT exposure

To expose the PT, sperm samples at a concentration of 35×10^6 cells/ml in NaCl 154 mM were treated

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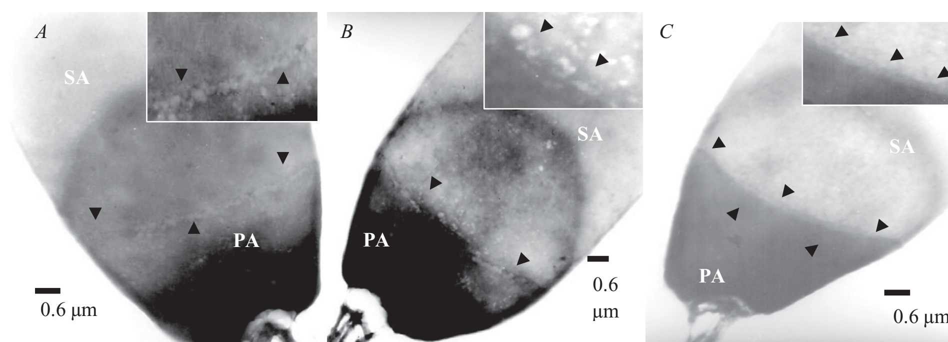

Figure 1 Electron micrograph of boar spermatozoa with exposed perinuclear theca (PT). (*A*) Sperm head with intact perinuclear theca substructure (PTS), in which one can see continuity in its morphology, which appears as a series of small, less electrondense zones with the appearance of chain links. The arrowheads indicate the links. A close-up of the same PTS is shown in the upper right-hand corner. (*B*) Sperm head with altered PTS. The black arrowheads indicate the different degrees of alteration in its morphology; these alterations are more apparent in the central zones and in the vertex on the right side of the PTS; the arrowheads in the upper right-hand box indicate the less electrondense zones with the appearance of chain links. (*C*) Sperm head with absent PTS. Note that in this micrograph the black arrowheads clearly indicate the groove running from vertex to vertex in the equatorial region of the sperm head, the position of the absent the PTS. The arrowheads in the upper right-hand box indicate more clearly the zones of the absent PTS. Also note that the groove is more defined. The three micrographs show the necks of the spermatozoa. PA, postacrosomal sheath; SA, subacrosomal sheath.

with the non-ionic Brij 36-T detergent (1.2% final concentration) and incubated for 10 min at room temperature. Then the samples were centrifuged (400 *g* for 3 min) and rinsed three times, by centrifugation–resuspension, with NaCl 154 mM and resuspension in the same solution at the initial concentration. An aliquot sample was fixed with Karnovsky fixative (Karnovsky & Dean, 1955) to evaluate the PT morphology by transmission electron microscopy (TEM).

Glycerol treatment

PT-exposed spermatozoa were divided into five sample groups: one was used as control (NaCl), two more were treated with glycerol (2% or 4% respectively) and two of them were treated with glycerol (4%) plus protease inhibitors (PI): 20 μl leupeptin (1 μM) or 80 μl of a protease inhibitor-cocktail tablets (Complete^{TM}, Roche, Germany).

The samples were incubated at 37°C and for 10 or 30 min; aliquots were taken and fixed in Karnovsky reagent (Karnovsky & Dean, 1955) to evaluate PT morphology by TEM.

Transmission electron microscope

The samples fixed in Karnovsky reagent were processed by the negative-staining electron microscopy technique. The samples were removed from the fixative by centrifugation and the pellet was rinsed twice and re-suspended in distilled water to the initial volume. Drops of the samples were placed on collodion-carbon-coated grids, 5–10 min was allowed for the cell adsorption to the grid. Excess sample was removed and samples were stained with an aqueous solution of phosphotungstic acid at 0.01% for 1.5–3 min or with 4% uranyl acetate in 70% ethylic alcohol for 12 s, samples were rinsed and one drop of distilled water was placed on the grid. To dry, the grids were placed on filter paper in Petri dishes and covered.

Samples were examined under a Zeiss E-M9 TEM. Fifty spermatozoa from each treatment were evaluated for PT integrity. The morphology of the substructure above the postacrosomal layer was evaluated in each sample and classified as PTS intact, PTS altered or PTS absent (Gutiérrez-Pérez, 2006).

Statistical analysis

The number of spermatozoa was analyzed by analysis of variance and Tukey test. Samples from the control ejaculates were compared with the ejaculates that had been subjected to the different treatments (2% and 4% of glycerol, PI: leupeptin, and commercial cocktail of inhibitors).

Results

The ultrastructural characteristics used to assess the PT are shown in Fig. 1. Samples were classified as: (1) intact when the PTS appeared in the form of chain links forming a belt around de equatorial region of the sperm head (Fig. 1*A*); (2) altered when the PTS had some damage, such as the lack of some chain link and/or disruption of continuity (Fig. 1*B*); or (3) absent, when the cells did not have PTS (Fig. 1*C*).

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Table 1 Evaluation of perinuclear theca substructure integrity

Treatment	Control	2% G30	4% G30	PI_1/4%G	PI_2/4%G
Intact	78.0 ± 4.2^a	3.33 ± 1.6^b	6.0 ± 4.2^b	6.67 ± 6.0^b	4.00 ± 3.5^b
Altered	21.33 ± 4.8^a	$36.0 \pm 12.9^{a,b}$	$46.67 \pm 21.7^{b,c}$	$62.0 \pm 15.7^{c,d}$	72.0 ± 11.0^d
Absent	0.67 ± 1.6^a	60.67 ± 13.9^b	$47.33 \pm 24.4^{b,c}$	31.33 ± 20.4^d	$24.0 \pm 11.8^{c,d}$

The perinuclear theca substructure (PTS) integrity (intact, altered or absent) in each treatment: glycerol (2 or 4%); glycerol plus protease inhibitors (PI_1: glycerol 4% and mixed with leupeptin; and PI_2/4% G: glycerol 4% and cocktail of protease inhibitors); and control. Means of percentage are shown ± the standard deviation for each group.
[a–d]Different values in rows indicate significant difference ($P < 0.05$).

The results obtained from the glycerol groups at 10 min presented no significant difference ($P < 0.05$) from all the groups at 30 min of incubation, for this reason these results were not included further.

Treatment with glycerol (2 or 4%) for 30 min reduced ($P < 0.05$) the percentage of spermatozoa with intact PTS compared with the control samples. Moreover, after glycerol 4% treatment, the proportion of spermatozoa with disrupted or absent PTS increased to 25.3 or 46.7% respectively. However the percentage of cells with a PTS absent was higher in the samples that had been treated with 2% glycerol (Table 1).

Although leupeptin or PI cocktail addition to the glycerol treated spermatozoa (4% glycerol for 30 min) did not allow the preservation of PTS integrity (Table 1), a significant decrease ($P < 0.05$) was observed in PTS absent-spermatozoa. In contrast, when leupeptin or protease inhibitors cocktail was added, the PT values presented a significant increase ($P < 0.05$) in spermatozoa with PTS alterations (Table 1).

Discussion

Although glycerol has always been the accepted cryoprotectant for freezing boar semen (Crabo & Einarsson, 1971; Pursel & Johnson, 1975), exposure of sperm to glycerol resulted in PT alterations and indicated that damage from glycerol addition is intracellular. In this study, PTS evaluation showed that sperm were damaged by use of this reagent (Table 1).

In the present study, the inclusion of glycerol in the incubation medium damaged the PT and the toxic effects of glycerol were directly proportional to the concentration used (2 or 4%) (Table 1). One explanation is that glycerol acts directly on tubulin and associated proteins, and addition may alter polymerization and depolymerization of microtubules, which was reflected in alterations in the cytoskeleton. Keates (1980) performed *in vitro* studies and reported this glycerol effect. Additionally, Marquez & Ogasawara (1977) and Hammerstedt & Graham (1992) have demonstrated that glycerol has harmful effects on the acrosome and middle piece. Previous reports from this laboratory (Gutiérrez-Pérez 2006; Martínez *et al.*, 2006; Arancibia-Salinas *et al.*, 2007; Barrientos–Morales *et al.*, 2009) have revealed a relationship between PTS and acrosome integrity. However, although there is evidence of alteration of the cytoskeleton there were no reports of the possible direct effects of glycerol on the PTS of the boar spermatozoid. To our knowledge, this work is the first report in relation to the possible direct effects of glycerol on the PTS from the boar sperm.

Dose–response studies of the addition of glycerol in the range of 0–7% have demonstrated that acrosomal integrity was affected negatively by increased glycerol concentration (Pursel *et al.*, 1978). In this report we observed toxic effects of glycerol at 2 and 4% on the PTS of the sperm head in relation to the morphological marker (PTS). Previously, Almlid & Johnson (1988) have evaluated the integrity of the acrosome, the membrane, and the percentage of motility in 2–6% glycerol, and reported greater damage to the acrosome and lower motility with concentrations of 5–6%, although with a certain degree of preservation of the integrity of the plasma membrane.

Previous studies have detected effects of glycerol on spermatozoa, independently of osmotic damage. One theory is that the damage results from the conversion of glycerol to a toxic compound called methylglyoxal, by means of a non-enzymatic and alternate mechanism of glycolysis (Riddle & Lorenz, 1973). However, the activity of glycerol kinase, an enzyme necessary for the spermatozoa to metabolize glycerol by the glycolytic route, has not been detected in boar spermatozoa (Mohri *et al.*, 1970). Consequently, we suggest that the damage observed in the PTS of the boar spermatozoa is due to the toxic effects of glycerol. In addition, in this study, we used spermatozoa without membranes and apparently without metabolic activity.

Glycerol may be deleterious to boar sperm fertility at higher concentrations (Fiser & Fairfull, 1990), and its toxicity in other species has been reported widely (Healey, 1969; Maxwell-Evans *et al.*, 1993; Fiser *et al.*, 1995; Meyers, 2005) If, as our results suggest,

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glycerol toxicity occurs at the level of the PT, then ultrastructural changes in the PTS can be detrimental to its functions such as the structural maintenance of the acrosome (Murdoch & Jones, 1978). This injury on the acrosome possibly affects the fertilization process.

The ultrastructural findings described in this paper are consistent with those of previous studies (Jones, 1973; Murdoch & Jones, 1978**)** on the effect of the temperature and glycerol on boar spermatozoa and confirm the occurrence of acrosome vesiculation in the presence of glycerol at 5°C.

It has been suggested that glycerol can be taken up and retained, probably as a phosphorylated derivative (Murdoch & Jones, 1978), within the cells during storage and washing to compete with exogenous glucose as a substrate for utilization and oxidation (Mann & White, 1957).

Clearly, the PI used in this study was not able to revert the toxic effect of glycerol. Nevertheless, these compounds diminished the percentage of spermatozoa with PT absence (Table 1). These results agree with other studies that indicated that proteolytic activity can cause degradation of elements of the cytoskeleton (Fulton, 1984).

The PI (with 4% glycerol for 30 min) used resulted in twice the percentage of spermatozoa with altered PT, as compared with glycerol at 2% (for 30 min) (Table 1). Our results showed that higher concentrations of glycerol, either alone or with protease inhibitor cocktail, promote PTS alteration in the sperm head. In other words, the toxic effects of glycerol were directly proportional to its concentration. These results may be related to prior findings by Fiser *et al.* (1993), who reported that diluents used to freeze boar spermatozoa, and that contained concentrations of glycerol at 2, 4, and 6%, had a negative influence on acrosomal integrity, most significantly at a concentration of 6%.

This study is the first to report that glycerol affects the cytoskeleton of the boar sperm head, specifically the PTS. Further experiments should be designed to explain this mechanism and thereby seek to minimize its effects on cryopreserved spermatozoa.

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