MEDWELL PUBLISHING
Journal of Animal and Veterinary Advances 8 (1): 155-164, 2009
ISSN: 1680-5593

Is There a Relationship Between the PT-Substructure Status and Acrosome Loss of Boar Spermatozoa Following Freezing-Thawing or Acrosomal Reaction?

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Abstract: The aim of this study was to determine whether *in vitro* boar sperm spontaneous acrosomal reaction is accompanied by changes on the Perinuclear Theca (PT) and to test the hypothesis that early changes in sperm PT-substructure integrity on frozen-thawed spermatozoa affects this process. Semen was collected thrice from six boars, making a total of 18 ejaculates; each ejaculate was divided into 2 samples, one for use in fresh and the other to be processed by freezing. Both samples were incubated to induce spontaneous acrosome reaction. At the end of the incubation period, capacitating and acrosome-reacted spermatozoa were isolated from one another by a density gradient. An evaluation of acrosome viability and integrity was conducted using the technique of triple stain. In order to expose the PT, both samples were treated with non-ionic detergent Brij 36-T and processed for electron microscopy observation after negative stain. The substructure was absent in capacitated cells with spontaneous acrosome reaction, while in capacitated sperm in which the acrosome still remained, the substructure persisted. Frozen-thawed sperm had a greater proportion ($p<0.05$) of acrosome-reacted sperm, as compared to fresh sperm; the integrity of the substructure was lower after freezing-thawing ($p<0.05$). In fresh semen, prior to the induction of spontaneous acrosome reaction, 6.6% of the sperm showed damages in the PT-substructure and after such process, the percentage increased to 51.6% ($p<0.001$), while in frozen-thawed sperm, such values were 70 and 82.6%, respectively ($p<0.005$). The correlation analysis showed that there was a relationship between ultrastructural changes in the PT-substructure and the loss of sperm acrosome, both in fresh ($r = 0.93$; $p<0.05$) and in frozen-thawed ($r = 0.71$; $p<0.05$) sperm. However, in thawed sperm incubated for spontaneous acrosome reaction, the percentage showing a damaged PT-substructure was higher than ($p<0.05$) sperm without acrosome. In conclusion, the acrosome reaction produces spontaneous loss of the substructure of PT and this damage on cryopreserved sperm affects the process.

Key words: Perinuclear theca, acrosome, freezing-thawing, spermatozoa, boar

INTRODUCTION

Several studies have shown that the fertilizing ability of porcine spermatozoa is reduced by cryostorage. This decrease has been associated with changes in the structure of the acrosomal and plasma membranes (Wilmut and Polge, 1977). We reported previously Arancibia-Salinas *et al.* (2007), that freezing-thawing causes ultrastructural changes in the sperm Perinuclear Theca (PT), which is associated with acrosome loss. PT is a unique cytoskeletal element, the anterior part of which is intercalated between the inner acrosomal membrane and the nuclear envelope of the mammalian sperm head and it plays an important role in spermiogenesis and the stabilization of sperm structures (Oko and Maravei, 1994, 1995). It has been shown that the PT of the equatorial segment may undergo structural changes during capacitaction and acrosome reaction, which may enhance the fusibility of the overlaying plasma membrane (Juárez-Mosqueda and Mújica, 1999). Interestingly, the

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cooled spermatozoa require no preliminary incubation to acquire fertilizing ability (Fuller and Whittingham, 1996) and are ready to undergo an acrosome reaction (Green and Watson, 2001). Thus, while spermatozoa are frozen-thawed for cryopreservation, some of them have been postulated to undergo alterations similar to those occurring during capacitation (Kaneto *et al*., 2002). The presence of such a "cryocapacitation" process has been considered merely to represent unspecific damage to the sperm plasma membrane (Peña *et al*., 2007). However, the possibility that the reduced fertilizing ability of cryostored sperm might also be associated with PT-substrucutre changes has not been explored.

To improve the fertility of cryopreserved sperm, either by reformulating protocols or devising new approaches to mitigate the detrimental effect of cryopreservation on sperm function, it is necessary to have an appreciation of the cryopreservation phenomenon and an evaluation of its effect on sperm function.

Therefore, the aims of the present study were: to investigate whether *in vitro* boar sperm spontaneous acrosomal reaction is accompanied by changes in the PT and to test the hypothesis that the presence of early changes in sperm PT-substructure integrity on frozen-thawed spermatozoa affects this process. Were such hypothesis true, using a triple-stain method and negative stain could suffice to test cryopreserved boar semen after undergoing spontaneous acrosomal reaction.

MATERIALS AND METHODS

Semen collection and evaluation: Semen was collected thrice from 6 mature fertile Duroc boars by the gloved-hand method at a weekly interval. Each ejaculate was processed separately. Ejaculates (only sperm-rich fractions) were collected in a pre-warmed thermo flask and stored at room temperature for 1 h. Then, the semen was diluted (1:1 (v:v)) with isothermal MR-A® (Kubus™, Madrid, Spain) extender and sperm motility, morphology and concentration were determined by standard laboratory techniques (Martín-Rillo *et al*., 1996). Only ejaculates with = 70% of progressive motility and <15% of sperm abnormalities were included in the study. Diluted semen was divided into two fractions: one fraction was frozen and the other was used in fresh (control semen).

Semen freezing and thawing protocol: Semen samples were processed according to the protocol by Thilmant (2001) modified by Martín-Rillo *et al*. (1996) and Córdova *et al*. (2001). Briefly, diluted semen was placed in a cooling machine (Coolmake®, Minitube™, Mexico City, Mexico) and set at 15°C for 3 h. Then, the semen was centrifuged at 800 x g for 10 min and the pellet (6×10^9 spermatozoa mL^{-1}) was resuspended with the cooling extender (11% dextrose and 20% egg yolk in distilled water, pre-cooled to 15°C) and cooled for 3 h at 5°C. This semen was further diluted (1:1 v/v) with the freezing extender (11% dextrose, 20% egg yolk and 4% glycerol in distilled water, pre-cooled to 5°C), to a concentration of 3×10^9 spermatozoa mL^{-}.^{1}Then, such diluted semen was loaded into 0.5 mL French straws (Minitube™, Mexico City, Mexico) sealed with polyvinyl alcohol (Elvanol®, Dupont™, Wilmington, DE). The straws were frozen by exposure to liquid nitrogen vapors (-130 to -150°C), approximately 3 cm above the level of liquid nitrogen, for 20 min and then were plunged into liquid nitrogen at -196°C and stored until thawing (about 2 weeks later).

For thawing, frozen straws were immersed in a circulating water bath at 37°C for 20 sec.

The techniques described below were applied to both sample treatments (fresh and frozen-thawed spermatozoa). Spontaneous acrosomal reaction assessment.

The samples were processed as follows: aliquots from both fresh and frozen-thawed semen (25×10^6 cells mL^{-1}) were washed (1:1 (v:v)) three times with saline solution pre-warmed at 37°C by centrifugation-resuspension (800× g for 3 min) to remove extenders. After washing, samples from both fresh and frozen-thawed spermatozoa were divided into 2 equal aliquots. The first aliquot, designated non-treated spermatozoa, was resuspended in saline solution to the initial volume and processed for evaluation of acrosomal and PT-substructure integrity. The second aliquot was resuspended in Hepes-Tyrode albumin lactate pyruvate (TALP, pH 7.4; TL (Modified Tyrode solution, Cell and Molecular Technologies, Lavallette, NJ), 3 mg mL^{-1} bovine serum albumin fraction V (Sigma, St. Louis, MO), 10 μL mL^{-1} sodium pyruvate (Sigma, St. Louis, MO), 1.5 μL mL^{-1} gentamicin (Sigma, St. Louis, MO)) at a concentration of 25×10^6 cells mL^{-1} and was incubated at 38°C and 5% CO_2 to achieve capacitation and spontaneous acrosomal reaction. In general, after a 4 h incubation, more than 25% of the sperm population showed a spontaneous acrosome reaction. At the end of the incubation time, capacitating and acrosome-reacted spermatozoa were isolated from one another by a density gradient. The sperm suspension (0.2 mL) was layered on top a Ficoll (2.5, 5 and 10%) step gradient made in saline solution. The gradient was centrifuged at 400× g for 10 min at room temperature. Acrosome-reacted spermatozoa were collected from the 2.5/5% Ficoll interface and capacitating spermatozoa

(cells with acrosome), from the 5/10% Ficoll interface. Isolated spermatozoa (with or without acrosome) were processed to evaluate sperm viability and acrosomal and PT-substructure integrity (see below).

Evaluation of sperm viability and acrosomal integrity: Sperm viability and acrosome integrity were evaluated using the triple stain protocol by Talbot and Chacon (1981). Aliquots from non-treated and spontaneous acrosome-reacted fresh and frozen-thawed spermatozoa were used. In brief, 2 mL test tubes were filled with 100 μL of sperm suspension (35×10^6 cells mL^{-1}) and 100 μL of 2% Trypan blue, dissolved in Phosphate-Buffered Saline (PBS), were added. The tubes were incubated at 37°C in a water bath for 15 min. After that, samples were centrifuged at 400 x g for 3 min and the supernatants were discarded. The pellets were resuspended in 2 mL PBS and centrifuged again twice. After three centrifugations, the coloration had vanished and clear or pale-blue suspensions were obtained. Then, samples were centrifuged again (400 x g for 8 min), the supernatants were removed and the spermatozoa pellets were fixed in 1 mL of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 30 min at 4°C. Then, spermatozoa were washed once in distilled water and centrifuged (400 × g for 3 min). The pellets were resuspended in 1 mL PBS and centrifuged at 400× g for 8 min, the supernatants were removed and the pellet was resuspended in PBS to the initial volume. Then, 10-20 μL of the spermatozoa suspensions were evenly spread on glass slides and air-dried. Slides were plunged into 8% Bismark brown solution in distilled water (pH 1.8) for 15 min at 37°C and then were rinsed with water, air-dried and stained with 0.8% rose Bengal solution in 0.1 M Tris-HCl buffer (pH 5.3) for 1 min at room temperature. Finally, slides were rinsed with water and examined (×1000) under a light microscope. A total of 100 spermatozoa per slide were evaluated to estimate sperm viability and acrosome integrity. The following stains were observed in the spermatozoa: rose stain over the acrosomal region and brown stain over the postacrosomal region in acrosome-intact live spermatozoa, no stain over the acrosomal region and brown stain over the postacrosomal region in acrosome-reacted live spermatozoa, rose stain over the acrosomal region and blue stain over the postacrosomal region in acrosome-intact dead spermatozoa and no stain over the acrosomal region and blue stain over the postacrosomal region in acrosome-reacted dead spermatozoa.

Evaluation of PT-substructure integrity: Aliquots from non-treated and spontaneous acrosome reacted fresh and frozen-thawed spermatozoa from the same ejaculates as above were processed to evaluate the PT morphology by negative staining electron microscopy (Ursitti and Wade, 1993). Spermatozoa were washed in saline solution (154 mM) and adjusted to a concentration of 35×10^6 cells mL^{-1}. To expose the PT surface, the plasma membrane, the nuclear membrane and the acrosome were solubilized by adding nonionic detergent Brij 36-T (Canamex, Nuevo León, Mexico) to a 1.2% final concentration and spermatozoa were incubated for 5 min at room temperature. Then, samples were centrifuged at 400× g for 3 min, washed in distilled water and fixed in Karnovsky fixative (Toshio *et al*., 2004), for 20 min at room temperature. After that, samples were centrifuged at 400× g for 3 min, washed twice in PBS and twice in distilled water and resuspended in distilled water to the initial volume. A drop of each of the sperm suspensions was placed on collodion-carbon coated grids, allowing 5-10 min for the spermatozoa to adhere to the grid; the excess of each sample was removed and the grids were air-dried. Samples were stained with an aqueous 0.02% phosphotungstic acid (Merck, Darmstadt, Germany) for 3 min and were rinsed placing one drop of distilled water on the grid. The grids were placed on filter paper in Petri dishes and covered for drying. Samples were examined on a Zeis EM-9 transmission electron microscope (Oberkochen, Germany) at 50 kV. For evaluation of PT-sustructure integrity, 100 spermatozoa were assessed and classified and the characteristic considered to assess the PT was the morphological appearance of the substructure above the postacrosomal layer. Samples were classified as: intact or normal, when the substructure appeared as a continuous row of papillae and altered or absent, when the substructure had some damage, or the cells did not have this substructure, respectively (Martínez *et al*., 2006).

Statistical analyses: An ANOVA test was used to evaluate differences between treatments (non-treated and spontaneous acrosome-reacted fresh spermatozoa, non-treated and spontaneous acrosome-reacted frozen-thawed spermatozoa) for the dependent variables AILS (acrosome-intact live spermatozoa), ARLS (acrosome-reacted live spermatozoa), AIDS (acrosome-intact dead spermatozoa), ARDS (acrosome-reacted dead spermatozoa), IPTS (intact PT-substructure) and APTS (altered or absent PT-substructure). A test of difference in means was used by the Scheffé test and a simple linear correlation was carried out. The test used was from SAS (Cody and Smith, 1997).

RESULTS

***In vitro* spontaneous acrosomal reaction in boar sperm is accompanied by changes in the PT-substructure:** To determine whether the PT-substructure loss was an event

related to sperm acrosomal reaction, *in vitro* spontaneous acrosomal-reacted boar sperm samples were assayed for the presence of the substructure. The substructure was absent in 81% of capacitated cells with spontaneous acrosome reaction (a sperm population enriched at 79%, by Ficoll gradient) (Table 1). On the other hand, in capacitated sperm which still had the acrosome (enriched at 80%, by Ficoll gradient), the substructure persisted in 83% of the cells (Table 1). Only in the last samples, the proportion of acrosome-reacted or intact acrosome spermatozoa was correlated, respectively, with the proportion of absent or normal PT-substructure spermatozoa ($r = 0.94$; $p<0.005$).

Evaluation of PT: early changes in sperm PT-substructure integrity on frozen-thawed spermatozoa affect *in vitro* acrosomal reaction: The APTS proportion increased in non-treated spermatozoa and those incubated for acrosome reaction after freezing-thawing ($p<0.05$), as compared to fresh semen (Table 2). However, whereas in fresh semen the percentage of spermatozoa with APTS was lower ($p<0.05$) for those non-treated, the frozen-thawed semen showed no difference ($p>0.05$) between non-treated and incubated spermatozoa with APTS (Table 2).

The percentage of ARLS was higher ($p<0.05$) for frozen-thawed semen, either non-treated or incubated for spontaneous acrosome reaction, as compared to fresh semen (Table 2). However, while the fresh semen showed a difference ($p<0.05$) in the proportion of ARLS between both samples (non-treated and incubated for spontaneous acrosome reaction), the frozen-thawed semen showed no difference ($p>0.05$; Table 2).

Non-treated spermatozoa, frozen-thawed spermatozoa incubated for spontaneous acrosome reaction and fresh spermatozoa incubated for spontaneous acrosome reaction, showed a lower proportion ($p<0.05$) of AILS, as compared to the non-treated fresh semen (Table 2). There was a difference ($p<0.05$) in the percentage of AILS between non-treated and incubated for spontaneous acrosome reaction fresh semen, but not ($p>0.05$) for the frozen-thawed semen (Table 2).

There was no difference ($p>0.05$) in the proportion of AIDS or ARDS, either non-treated or incubated for acrosome reaction, for fresh and frozen-thawed semen samples (Table 2).

A connection was found between acrosome integrity and PT-substructure integrity. The proportion of acrosome-reacted spermatozoa was correlated with the proportion of sperm with altered PT-substructure, in both fresh ($r = 0.93$; $p<0.05$) and frozen-thawed ($r = 0.85$; $p<0.05$) sperm. In fresh sperm, the proportion of intact PT-substructure was correlated with the proportion of LSAI ($r = 0.88$; $p<0.05$) and with the proportion of acrosome-intact spermatozoa ($r = 0.91$; $p<0.05$), whereas the proportion of spermatozoa with altered PT-substructure had a very high correlation with the proportion of LSAR ($r = 0.95$; $p<0.05$) and a correlation with the acrosome-reacted sperm ($r = 0.91$; $p<0.05$). In frozen-thawed sperm, the proportion of intact PT-substructure was correlated with the proportion of SLAI ($r = 0.86$; $p<0.05$) and tended to correlate with the proportion of acrosome-intact spermatozoa ($r = 0.60$; $p<0.05$); whereas the proportion of spermatozoa with altered PT-substructure tended to correlate with the proportion of acrosome-reacted spermatozoa ($r = 0.69$; $p<0.05$) and LSAR ($r = 0.61$; $p<0.05$), conversely in the thawed sperm incubated for spontaneous acrosome reaction, the percentage with damaged PT-substructure was higher than that ($p<0.05$) of sperm without acrosome.

Table 1: Evaluation of the capacitating and acrosome-reacted boar sperm isolated by a Ficoll density gradient

	2.5-5% Ficoll interface	5-10% Ficoll interface
Intact PTS (%)	19[a]	83[b]
Altered PTS (%)	81[a]	17[c]
With Acrosome (%)	21[a]	80[b]
Without Acrosome (%)	79[a]	20[c]

Acrosome-reacted spermatozoa were collected from the 2.5-5% interface and capacitating spermatozoa, from the 5-10% interface. [a]there is no correlation; PTS: Perinuclear Theca-Substructure. [b]$r = 0.9462$| $p = 0.0043$, [c]$r = 0.9462$| $p = 0.0043$

Table 2: Mean (±SD) end points for fresh and frozen-thawed boar spermatozoa (n = 18)

	Fresh semen		Frozen-thawed semen	
Spermatozoa (%)	NT	AR	NT	AR
AILS[d]	88.3±8.0[a]	45.6±12.8[b]	34.9±9.4[c]	30.2±8.4[c]
ARLS[d]	6.7±4.3[a]	48.1±9.4[b]	46.4±14.7[b]	41.5±15.1[b]
AIDS[d]	2.5±4.2[a]	1.8±2.9[a]	6.1±3.8[a]	9.0±3.8[a]
ARDS[d]	2.5±2.7[a]	4.5±3.0[a]	12.6±6.4[a]	19.3±10.7[a]
IPTS	93.3±3.1[a]	48.3±7.6[b]	30.0±4.6[c]	17.3±2.5[c]
APTS	6.7±3.1[a]	51.7±7.6[b]	70.0±4.6[c]	82.7±2.5[c]

NT: Non-Treated; AR: Acrosome Reacted; AILS: Acrosome-Intact Live Spermatozoa; ARLS: Acrosome-Reacted Live Spermatozoa; AIDS: Acrosome-Intact Dead Spermatozoa; ARDS: Acrosome-Reacted Dead Spermatozoa; IPTS: Intact PT-Substructure; APTS: Altered or Absent PT-Substructure. [a,b,c]Different superscript within rows are different ($p<0.05$), [d]Determined with trypan blue, Bismark brown and rose Bengal

DISCUSSION

Being aware of the sperm response to the cryopreservation process is of the essence to understand what factors affect the survival of the cell. In this study, the results showed that there was a relationship between the PT-substructure status and acrosome loss in boar spermatozoa following a freezing-thawing or an acrosomal

reaction process. The data also showed that the presence of early changes in PT-substructure integrity on frozen-thawed boar spermatozoa affect the latter. This supports the idea that the decrease in the viability of cryopreserved semen can be attributed not only to a primary damage to the plasma membrane (McGann *et al*., 1988; Jager *et al*., 1990; Holt, 2000), but also to damage to internal elements, such as the cytoskeleton (Watson, 2000), as observed by Martínez *et al*. (2006) in bull sperm and Arancibia-Salinas *et al*. (2007) in boar sperm.

In early publications, it was reported that after the freezing-thawing process, the percentage of absent acrosome was similar to the percentage of PT damage (Martínez *et al*., 2006; Arancibia-Salinas *et al*., 2007). In addition, in guinea pig sperm, a loss of the PT-substructure was detected in spermatozoa that underwent an Acrosomal Reaction (AR) (Juárez-Mosqueda and Mújica, 1999). Nevertheless, the former authors did not isolate sperm with or without acrosome from one another and to the best of our knowledge, studies on PT-ultrastructural changes during AR have not been performed in fresh and frozen-thawed boar sperm.

For this study, in order to learn if the acrosomal status was already related to the PT-substructure integrity, we made sure that sperm with and without acrosome were distinguishable as separate subpopulations, confirming that there was a relationship between the stability of both structures. Thus, if the substructure of the PT remains intact, the sperm keeps its acrosome, whereas if it is damaged, there is acrosome loss.

Sperm capacitation and AR are two key steps in the fertilization process (Gadea *et al*., 2005). Only if the sperm has undergone capacitation, AR will occur (Spungin *et al*., 1995). AR is essential for fertilization because only acrosome-reacted sperm are capable of fusing with the oocyte plasma membrane (Harvey and Ducibella, 2006; Breitbart and Spungin, 1997; Breitbart and Naor, 1999; Brewis and Wong, 1999; Wasserman, 1999; Wasserman *et al*., 2001).

It is well documented that *in vitro* capacitation of sperm cells not only leads to their activation, but also causes an increased occurrence of "spontaneous" acrosome reaction (Harrison *et al*., 1993). We found that boar sperm incubation in a capacitating medium (TALP), to induce the spontaneous AR, increased the percentage of spermatozoa with structural changes in the PT-substructure. Therefore, this situation might be general among mammalian spermatozoa. Sutovsky *et al*. (2003) discussed the possibility that individual parts of the PT may serve different purposes during fertilization and may be removed at different time points during this process. For instance, such changes in the PT-substructure may enhance the fusibility of the overlying plasma membrane (Ellis *et al*., 2002).

In boar spermatozoa, there is evidence of capacitation-like changes induced by cooling and rewarming (Kaneto *et al*., 2002), which have been suggested as partially responsible for the reduced fertility of cryopreserved semen (Watson, 2000; Green and Watson, 2001). In this manuscript we report, for the first time, that the capacitation process was not responsible for an increase in the percentage of spermatozoa with AR in thawing semen; however, the percentage of sperm with damaged PT-substructure was higher. Several investigations have shown that alterations in the spermatic cell that lead to the "cryocapacitation" or false capacitation involve a loss of membrane selective permeability caused by protein and phospholipid migration or their release from the plasma membrane and subsequently, the uptake of calcium and sodium experiences a dramatic increase (Green and Watson, 2001; Maxwell and Johnson, 1997; Kaneto *et al*., 2002). Moreover, other investigators have shown that alterations in boar sperm cytoskeleton led to a disturbance of the volume regulatory function and to a rapid decrease in the proportion of acrosome-reacted sperm (Petrunkina *et al*., 2004). We suggested that PT-alteration may contribute to the sub-lethal cell damage that occurs during the cryopreservation process and may be the basis for the phenomenon of capacitation-like changes observed in frozen-thawed sperm. Even though there is no direct evidence of this process, damages to the fine structure of the PT of boar sperm could interfere with capacitation, the AR and/or viability of the sperm. Accordingly, tyrosine kinase c-yes is a signaling molecule detected in the sperm head PT and is involved in sperm capacitation (Leclerc and Goupil, 2002) and/or acrosome reaction (Sutovsky *et al*., 2003). On the other hand, the actin cytoskeleton has been shown to undergo polymerization and depolymerization during capacitation and AR in the sperm of several mammalian species (Castellani-Ceresa *et al*., 1992). Additionally, in guinea pig, the sperm disruption of F-actin leads to a PT-substructure loss (Juárez-Mosqueda and Mújica, 1999), as cooling in other cells results in a premature depolymerization of F-actin. PT is a unique cytoskeletal extranuclear structural element in the sperm head; the PT-substructure is located in the apical region of its postacrosomal region, which is thought to be the site for actin in the spermatozoa of certain mammalian species (Yagi and Paranko, 1995; Mújica *et al*., 2003). Perhaps

the remodeling of the actin cytoskeleton during cryopreservation prematurely activates sperm for fertilization, rendering them incapable of "re-activating" when necessary (Correa *et al*., 2007); the results obtained from our frozen-thawing experiment support this proposal.

However, other investigators have reported that sperm did not lose the physiological property of acrosome reaction; for instance, Córdova *et al*. (1997) showed that after 2.5 h of incubation in TALP medium, boar spermatozoa exhibited a 40% induced AR.

On the other hand, spontaneous AR is generally attributed to a degenerative process of sperm that are fully capacitated, ultimately leading to cell death (Cremades *et al*., 2005). Optimal conditions observed to promote *in vitro* boar sperm capacitation were incubation in a capacitating medium for up to 120 min, conditions that showed an increase in the number of capacitated sperm and acrosome-reacted sperm, without affecting the viability of the sperm cells (Dapino *et al*., 2006). Only in living sperm does the AR occur after they have been capacitated. Sperm do undergo a "false AR" upon death that requires no capacitation (Green and Watson, 2001). In our study of over 4 h incubation, up to one-half of the sperm in fresh treated samples were acrosome-reacted. The rate and extent of AR were similar to those reported for porcine spermatozoa (Flesch *et al*., 1999; Dapino *et al*., 2006). Moreover, sperm incubation in a capacitating medium showed an increase in the number of acrosome-reacted sperm, without affecting their viability (93.7%, live sperm). By contrast, the rate of AR did not vary significantly after 4 h of incubation in frozen-thawed sperm and they also preserved their initial percentage of viability.

Diverse studies indicate that the percentage of spermatozoa without acrosome increased after a thawing process (Fiser *et al*., 1993; Green and Watson, 2001; Petrunkina *et al*., 2004; Martinez *et al*., 2006; Hernández *et al*., 2007), concordant with our observations, since after the cryopreservation process, the relevant percentage increased from 9.1-59.1. On the other hand, our data show that almost half of the cells that were alive (81.3%) after thawing, failed to preserve their fertilizing potential, since spermatozoa with AR (46.4%) were not able to interact with the ovum.

In our study, viability was assessed using the triple stain method. Eosine-nigrosine staining is one of the most frequently used methods for viability evaluation (Rodríguez-Martínez, 2003). However, the triple stain technique not only allows us to obtain more reliable results, but also provides more useful information, such as details about viability and at the same time, the acrosomal status of spermatozoa (Garde *et al*., 1997). Nevertheless, some research works point out that classic supravital stain is not appropriate for predicting the viability of thawed spermatozoa, because the presence of glycerol interferes with the differential stain between live and dead cells (Tartaglione and Ritta, 2004). We do not discard the idea that the very high post-thaw viability reported in this study may be due to problems related to the technique used.

Finally, several authors have reported the role of PT in other important functions, such as the maintenance and formation of the functional plasma membrane domains of the sperm head, the stability of the nucleus and the activation of the egg (Paranko *et al*., 1988; Longo and Cook, 1991; Sutovsky *et al*., 2003). Therefore, damages to the PT integrity could affect other physiological functions of sperm, as it has also a major role in the decreased viability of frozen-thawed semen (Martínez *et al*., 2006).

CONCLUSION

In conclusion, this study showed that *in vitro* acrosomal reaction produces the destabilization of the PT-substructure of boar spermatozoa and that cryocapacitation induces these same changes in frozen-thawed spermatozoa. Additionally, this study also showed that changes in the PT-substructure of frozen-thawed boar spermatozoa affect the acrosomal reaction process, which might be in part responsible for the low fertility rates of frozen-thawed boar spermatozoa. Since, there are no previous reports of studies concerning the effect of cryopreservation on the AR and on the integrity of the PTS of boar spermatozoa, this study provides important information that can be useful for further research.

Further studies will be necessary to elucidate the specific PT-biochemical changes resulting from the sperm cryopreservation process.

ACKNOWLEDGEMENTS

This investigation was supported by DGAPA-UNAM (PAPIIT IN-206506). We are indebted to the staff of the Unidad de Microscopia Electrónica de la Facultad de Medicina Veterinaria y Zootecnia of the National Autonomous University of Mexico (UNAM), for providing transmission electron microscopy facilities. We also thank Sirenia González and Lourdes Rojas of Unidad de Microscopia Electrónica del CINVESTAV-IPN for their technical assistance.

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