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Morphological Perinuclear Theca Alterations Are Related to Acrosome Loss in Cryopreserved Boar Spermatozoa

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Abstract: The aim of this study was to determine the effect of cryopreservation of boar spermatozoa on the integrity of the Perinuclear Theca (PT) substructure and the integrity of the acrosome. Semen from 12 boars were divided in two fractions; one was cryopreserved and thawed the other was used fresh as a control sample. In order to expose the PT structure both samples were treated with the nonionic detergent Brij 36-T and processed for electron microscopy observation after negative staining. Before freezing, 16.7% of spermatozoa showed PT-substructure alterations, that increased to 53% after freezing and thawing ($p<0.001$). Similarly, acrosome damage increased from 19.5-54.9% after cryopreservation. Furthermore, significant correlations were found between PT-substructure alterations and acrosome defeat. In addition, whilst no difference in PT substructure damage was observed between breeds in control sample, the Mexican hairless breed had significantly more spermatozoa with damage PT substructure after freezing-thawing. The acrosome loss of boar spermatozoa increased with an increased proportion of PT-altered spermatozoa.

Key words: Perinuclear theca, acrosome, cryopreservation, spermatozoa, boar

INTRODUCTION

The use of cryopreserved semen in pigs has been hidered by the low sperm viability and fertility observed after the freezing-thawing process (Roca *et al*., 2006). Freezing and thawing causes damages to the cells and the spermatozoa is particularly sensitive to low temperature (Purdy, 2006; Hernández *et al*., 2007). It has been long recognized that fertility of frozen boar semen is lower than the refrigerated semen (Bwanga *et al*., 1991). This is possibly due to physiological and/or morphological injuries suffered by the spermatozoa during the freezing and thawing process (Cerolini *et al*., 2001). A key aspect for increasing the fertility of frozen-thawing semen would be to maintain the integrity of the Plasma Membrane (PM) during cryopreservation since only spermatozoa with intact membranes can undergo capacitation (Tienthai *et al*., 2004; Córdova *et al*., 2001) and Acrosome Reaction (AR) (Yanagimachi, 1981; Abou-Haila and Tulsiani, 2000). It is well known that freezing and thawing cause alterations to the PM (Muldrew and McGann, 1990; Curry, 2000; Nield *et al*., 2003). Several studies explored the relationship between sperm survival and the chemical/physical features of the PM. However, none has been able to establish a significant relationship between the low viability observed in cryopreserved semen and the degree of damage on the PM (Ostermeier *et al*., 2001). Recent studies have revealed that cryopre-servation process induces substantial damage to the PT of bull spermatozoa (Martínez *et al*., 2006). Moreover, it was shown that in boar spermatozoa the cytoskeleton appears to be important for volume regulation (Petrunkina *et al*., 2004, 2005).

The Perinuclear Theca (PT) is the main cytoskeleton structure of mammalian spermatozoa head. The PT surrounds the nucleus completely except for the basal region where the tail attaches to the head (Longo *et al*., 1987; Oko and Clermont, 1988; Longo and Cook, 1991;

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Bellvé *et al*., 1992) and this cytoskeletal element has been subdivided in two morphologically distinct areas, the subacrosomal and the postacrosomal layer (Mújica *et al*., 2003). From a purely structural point of view, the PT seems to connect the membranes of the spermatozoa head. The subacrosomal layer is located between the inner acrosomal membrane and the nuclear envelope, while the postacrosomal layer is located between the PM and the nuclear envelope. The PT continues with another layer called inner periacrosomal layer, located between the PM and the outer acrosomal membrane. Because of the location of this specialized cytoskeleton it has been suggested that the PT ensure stability of the acrosome and the structural design of the sperm head (Oko and Morales, 1994). In addition, the PT is involved in other important sperm functions, such as the formation of functional PM domains (Gadella *et al*., 1995) egg activation (Kimura *et al*., 1998) and mammalian fertilization where it is required for the formation of the male pronucleus. Surrounding the apical region of the postacrosomal layer the PT has a substructure (Koehler, 1970; Olson *et al*., 1983; Oko and Maravei, 1994; Watson *et al*., 1995; Juárez-Mosqueda and Mújica, 1999; Lecuyer *et al*., 2000; Martínez *et al*., 2006) that disappears early during fertilization (Juárez-Mosqueda and Mújica, 1999) and that could be used as an early marker of theca disruption. Therefore, the evaluation of the PT integrity in others species as the boar could provide important information of the functional capacity of the spermatozoa. Further, it will facilitate the evaluation of the effects of sperm processing on spermatozoa cytoskeleton damage.

The objective of this study, was used the PT substructure as a morphological marker of PT integrity and to evaluate the changes caused by cryopreservation on the PT substructure and their relationship with acrosomal status in boar spermatozoa.

MATERIALS AND METHODS

Semen collection and evaluation: Ejaculates were obtained from 12 fertile boars belonging to four different breeds: Duroc (n = 3), Landrace (n = 3), Yorkshire (n = 3) and Mexican hairless (n = 3). The ejaculates were obtained by the gloved hand method. Then, the semen was extended (1:1 [v/v]) with MR-A^{R} (Kubus^{TM}, Madrid, Spain) extender and taken to the laboratory at 18-20°C, where it was evaluated macroscopically and microscopically. Only samples with at least 80% of progressive motility, less than 15% of abnormalities and a minimum sperm concentration of 150×10^{6} cells mL^{-1} were included in the study. Motility was assessed in a drop of semen placed on a glass slide at a 37°C temperature and observed under a light microscope by a single observer. The percentage of abnormalities was determined with eosin-nigrosin staining assessing 200 cells. Sperm concentration was determined using a Neubauer chamber.

After collection, the semen was divided in two fractions. One fraction was frozen using the Thilman (1997) method and the other was used in fresh (control semen).

Semen freezing and thawing: Extended semen samples containing 6×10^{9} spermatozoa were centrifuged at 800×g for 10 min and pellets were resuspended with the cooling extender (11% dextrose and 20% egg yolk in distilled water) set at 15 °C for 3 h. Then, the semen was diluted (1:1) with the second extender (11% dextrose, 20% egg yolk and 4% glycerol). Final sperm concentration was adjusted at 6×10^{8} cells mL^{-1} and samples were packaged in 0.5 mL plastic straws and sealed. The straws were immediately placed in contact with nitrogen vapor about 3 cm above the nitrogen liquid level (-130-150°C) for 20 min and finally plunged into the liquid nitrogen (-196°C) and stored for at least two weeks until used.

For thawing the semen straws were taken out of the liquid nitrogen and quickly placed in a water bath at 56°C for 12 sec.

The following techniques described, were applied to both sample of treatments (fresh and frozen-thawed spermatozoa).

Assessment of the perinuclear theca substructure integrity: To expose the PT surface, the PM and acrosome were solubilized by treating the samples with the nonionic detergent Brij 36-T (1.2% final concentration) for 5 min at room temperature. Then the samples were washed in 154 mM NaCl by centrifugation/resuspention at 300×g for 3 min, placed in Karnowsky (1965) fixative (and processed for examination of the PT morphology by electron microscopy after negative staining.

Acrosome evaluation: Acrosome reaction was assessed by triple stain technique (Talbot and Chacon, 1981). Briefly, a 100 μL aliquot from each ejaculate (35×10^{6} cells mL^{-1}) was added to 100 μL of 2% Trypan blue in phosphate-buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 9.6 mM Na_2HPO_4, pH 7.4) and incubated at 37°C for 15 min. Then, the aliquot washed several times by centrifugation/resuspention at 300×g for 3 min, until the supernatant fraction appeared clear. Next the sperm cells were resuspended in 1 mL 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and fixed for 30 min at 4°C. Afterwards, cells were centrifuged

at 300×g for 8 min, the supernatant fraction was eliminated and the cells pellet was resuspended and washed in PBS. One drop was used to make a smear that was left to dry. The smears were incubated with 0.8% Birsmack brown solution in distilled water (pH 1.8) at 37°C for 15 min and rinsed with distilled water. Thereafter ,smears were stained with 0.8% rose Bengal solution in 0.1 M Tris-HCl buffer at room temperature for one min, rinsed once with distilled water, left to dry and examined under a light microscope. Spermatozoa were classified as: Live with intact acrosome (LAI; pink stain over the acrosome region and brown over the postacrosomal region); live with acrosome reaction (LAR; no stain over the acrosomal region and brown stain over the postacrosomal region); dead with or without acrosoma reaction (pink stain over the acrosomal region and blue stain on the postacrosomal region, as well as dead with acrosome reaction no stain over the acrosomal region and blue stain on the postacrosomal region).

Electron microscopy: Negative staining of whole spermatozoa.- The samples treated with Brij 36-T were placed in Karnowsky (1965) fixative for 2 h at 4°C, washed by centrifugation/resuspention twice in PBS and twice in distilled water and resuspended in their initial volume with distilled water. One drop of each sample was placed on collodion-carbon coated grids. The excess sample was removed with filter paper and the spermatozoa were stained with aqueous 0.2% phosphotungstic acid for 3 minutes and rinsed in distilled water. The samples were examined in a Zeiss EM-900 Transmission Electron Microscope (TEM) at 50 kV. Fifty cells on each sample were observed. During the TEM evaluation, the PT substructure was classified as: Normal damaged and absent.

In addition, sperm samples not treated with Brij detergent were in a similar way processed. This was to show that the PT-substructure is not visible because the plasma membrane and the acrosome remain covering it (Fig. 1a and b).

Thin sections.- Spermatozoa treated with Brij 36-T, were fixed with Karnowvsky solution for 20 min. After post fixation with 1% (w/v) OsO_4 in PBS buffer (ph 7.4) for 1 hour at room temperature, the samples were dehydrated through a graded ethanol series and embedded in Spurr's resin, thin-sectioned and stained with uranyl acetate and lead citrate, prior to TEM examination (Fig. 1c).

Statistical analysis: The effect of freezing-thawing on the PT-integrity was evaluated by a logistic regression analysis for ordinal variables. The experimental unit was the ejaculate. The dependent variable was the integrity of the PT substructure with three levels; normal damaged and absent. The independent variables were the freezing-thawing treatment, breed and the treatment by breed interaction. Spearman's correlation coefficient was used to estimate the relationship between the percentages of sperm with damaged PT substructure with the percentage of live acrosome reaction spermatozoa, within each ejaculate of both treatments.

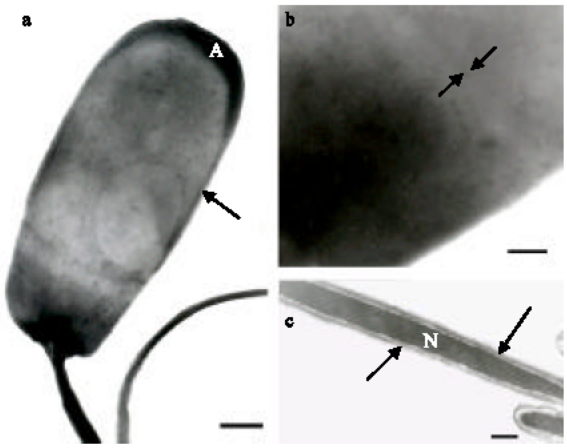

Fig. 1: Microphotographs of fresh and post-thaw pig spermatozoa heads. (a) A non-Brij-treated spermatozoa head negatively stained. The plasma membrane (arrow) as well as the acrosome (A) can be seen. Bar 1.1 μm. (b) A high-magnification view of the sperm head in (a) to show that when the plasma membrane remains the PT-substructure is not seen (arrows). Bar 0.4 μm. (c) A thin section of a Brij-treated spermatozoa showing that the plasma membrane, acrosome and the nuclear membrane have been solubilized, leaving a hull, the PT (arrows) covering the sperm nucleus (n). Bar 0.25 μm

RESULTS

Morphology of the PT-substructure: The ultrastructural morphology of the PT-substructure of boar spermatozoa was considered as normal when this appeared scalloped because of numerous papillae directed toward the equatorial segment of the PT layer (Fig. 2a). Damage substructure was considered when it had a discontinuous scalloped (Fig. 2b) or when eyelashes-like substructures were observed (Fig. 2c only observed after cryopreserved). Substructure was considered absent when it was not observed (Fig. 2d).

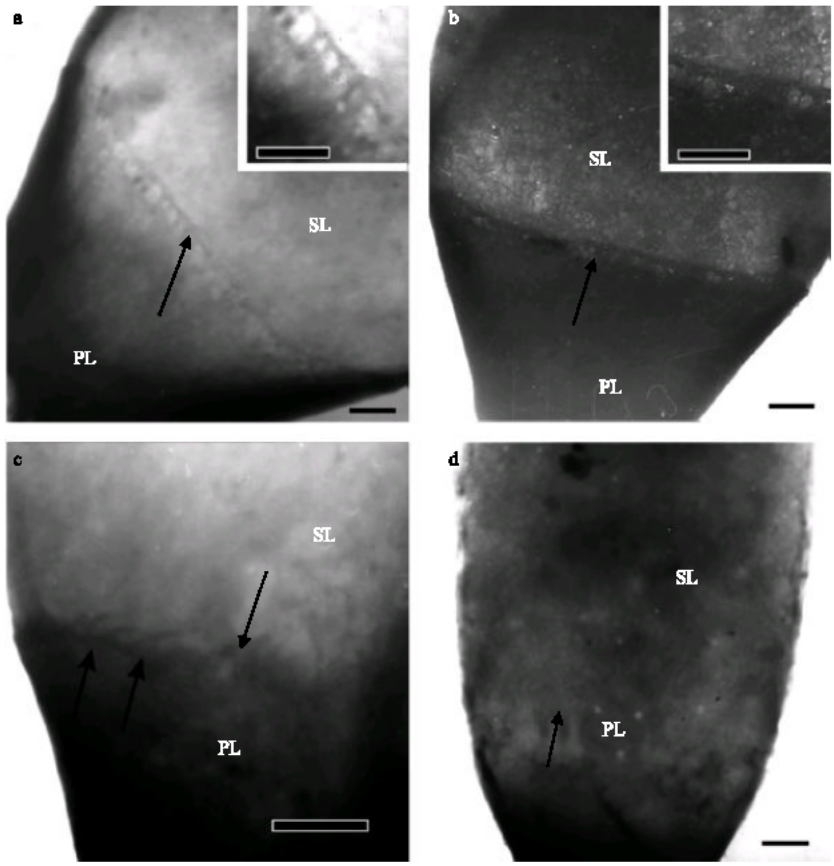

Fig. 2: Electron microphotographs of negatively stain preparations showing the PT substructure fresh and post-thaw pig spermatozoa. To expose the PT the spermatozoa were treated with Brij. (a) Whole-mount sperm heads with PT exposed. Over the PT the substructure is apparent (arrow) between the Subacrosomal Layer (SL) and the Postacrosomal Layer (PL) of the PT; it consists of a row of appears scalloped pattern circling the sperm head. The inset shows a high-magnification view of the substructure. Note that the substructure, which characterize the apical margin of the postacrosomal layer, remains intact. Bar 0.4 μm. (b) Whole-mount post-thaw sperm head showing a damage substructure. Note a discontinuity on the scalloped pattern (arrow). Inset shows a high magnification of the substructure where some frets are lacking. Bar 0.4 μm. (c) Whole-mount post-thaw sperm head showing a substructure with a "eyelashes" appearance (arrows). Bar, 0.4 μm. (d) Whole-mount post-thaw sperm head without substructure (arrows). Note that the apical portion of the posacrosomal layer does not show PT substructure appearance. Bar 0.6 μm

Integrity of the PT and the acrosome: The results obtained for the PT and acrosome integrity evaluation on fresh and frozen-thawed semen are presented on Table 1. The freezing-thawing process damaged the PT substructure integrity. The percentage of spermatozoa with normal substructure decreased after freezing-thawing (83 vs. 47%) ($p<0.001$). The percentage of live spermatozoa with intact acrosome before freezing was 78.7% and decreased to 27.6% after the process ($p<0.001$). The percentage of live spermatozoa with acrosome reaction increased from 19.5-54.9 after freezing-thawing process ($p<0.001$). The percentage of dead spermatozoa increased after the cryopreservation (2.1 vs. 17.5) ($p<0.001$).

Table 1: The percentage of PT substructure; normal, damage and absent and the percentage of acrosome integrity in fresh and frozen-thawed boar spermatozoa

	PT integrity %			Acrosome integrity %		
Treatment	PTsN	PTsD	PTsAb	LIA*	LAR*	Dead*
Fresh	83.3	11.9	4.8	78.7	19.5	2.1
Frozen-thawed	47.0	30.8	22.2	27.6	54.9	17.5

PT: Perinuclear Theca; (*)determine with tripan blue, Bismark brown and rose Bengal; PTsN: PT substructure Normal; PTsD: PT substructure Damage; PTsA: PT substructure Absent; LIA: Live Intac Acrosome; LAR: Live Acrosome Reaction, $p<(0.001)$

The Spearmm's coefficient of correlation between the percentage of sperm with damaged PT substructure and the percentage of live acrosome reaction spermatozoa, for all data was 0.68 ($p = 0.0026$).

Table 2: The percentage of PT integrity and marginal effect in fresh and frozen-thawed spermatozoa from four boar breed. The marginal effect it is the difference between fresh and frozen-thawing

	Treatment								
	Fresh semen (%)			Frozen-thawed semen (%)			Marginal effect (%)		
Breed	PTsN	PTsD	PTsAb	PTsN	PTsD	PTsAb	PTsN	PTsD	PtsAb
Duroc	87.5	9.5	3.0	55.0	27.5	17.5	71.3	18.5	10.3
Mexican hairless	82.7	16.0	1.3	33.3	34.0	32.7	58.0	25.0	17.0
Landrace	78.0	14.0	8.0	48.7	26.7	24.7	63.4	20.4	16.4
Yorkshire	83.3	10.7	6.0	49.3	34.0	16.7	66.3	22.4	11.4

Pts: perinuclear theca-substructure; PTsN: PT substructure Normal; PTsD: PT substructure Damage; PTsA: PT substructure Absent; LIA: Live Intac Acrosome; LAR: Live Acrosome Reaction, Effect of the treatment p = 0.0001, race p = 0.0034, race interaction treatment p = 0.0736

Perinuclear theca integrity by breed: The effect of breed was significant (p = 0.0034) over the integrity of the PT substructure. All breeds evaluated in this study had an increase on PT substructure alteration (damaged substructure+absent substructure) and therefore, a reduction in the spermatozoa with normal PT substructure percentage resulting from the freezing-thawing process (p = 0.0001). No breed by treatment interaction effect was observed (p = 0.0736) (Table 2). There was high variability between boars in their response to cryopreservation. This variability is shown by a significant (p = 0.0057) boar by treatment interaction.

DISCUSSION

The use of cryopreserved sperm for artificial insemination of sows is limited (Gilian *et al*., 2004; Wongtawan *et al*., 2006) due to the low viability of the sperm after cryopreservation (Watson, 2000; Meyers, 2005) It is well known that cryopreservation causes damage to the spermatozoa (Watson, 2000; Meyers, 2005; Holt *et al*., 2005). The results of this investigation indicate that the freezing-thawing process causes alterations to the PT substructure. The damage of PT substructure and acrosome caused by freezing coincides with the reported by Martinez *et al.* (2006) who found that the cryopreservation of bull semen causes significant damage to the PT substructure. Both findings support the proposal by Jager *et al.* (1990) who points out that the freezing-thawing process causes alteration at the interior of human spermatozoa which allow external agents, such as heparin to act on the genetic material of the cell. Sutovsky *et al.* (1997) found that in the bovine spermatozoa the cytoskeleton may prevent access of cytoplasmic factors to the sperm DNA and thus prevents decondensation of the sperm nucleus. Therefore, one may assume that are the alterations on the PT integrity in the frozen-thawed spermatozoa that decreased the sperm nuclear stability, becoming sensible to foreign material or agents, such as decondensing factors.

It had been reported that the process of freezing and thawing may result in a significant deterioration of boar spermatozoa acrosomal integrity (Yagi and Paranko, 1995). Acrosome integrity is important considering that it contains enzymes with a crucial role in the penetration of the zona pellucida (Kjoestad *et al*., 1993; Kumar *et al*., 2003). On the other hand, it has been proposed that the PT could play a roll on stabilizing to the acrosome (Fiser *et al.,* 1991) since PT appears early in the spermiogenesis closely with the acrosomal and nuclear membranes (Longo and Cook, 1991; Oko and Maravei, 1994). Furthermore, failure in PT differentiation has been associated with acrosome deficient rounded head spermatozoa (Escalier, 1990). Longo *et al.* (1987) have suggested that PT proteins may contribute to the nucleus-acrosome association by providing some sort of intermembranous cement. The results in this study show that after the freezing-thawing process the percentage of absent acrosome is similar to the percentages PT damage. However, while Martínez *et al.* (2006) found a correlation between the number of sperm with absent PT substructure and the number of spermatozoa with an acrosome reaction in the bull, the present study found that acrosome reaction correlated to slighter alteration to the PT substructure. Thus suggesting that for bull semen the PT substructure is less sensitive to cryopreservation process. This could be possibly due to differences in the composition of the PT substructure, since the substructure morphology is species-specific (Koehler, 1970; Olson *et al*., 1983; Watson *et al*., 1995; Juárez-Mosqueda and Mújica, 1999; Martínez *et al*., 2006).

In addition, since the PT is involved in events such as maintenance of spermatic head integrity (Sutovsky *et al*., 1997) maintenance of the functional plasma membrane domains (Gadella *et al*., 1995), spermatozoa penetration of the egg (Yagi and Paranko, 1995), protection of spermatic DNA (Von Bölow *et al*., 1997; Lecuyer *et al*., 2000), activation of the oocyte (Kimura *et al*., 1998) and decondensation of sperm genetic material (Sutovsky *et al*., 2003), the damage observed to the PT substructure could relate to more extensive damage to the PT, thus affecting the fertilization process.

In the present study, only in the cryopreserved boar spermatozoa was observed the eyelash-like morphology of the PT substructure, which was reported in the boar spermatozoa (Juárez-Mosqueda and Mújica, 1999). However, Juárez-Mosqueda and Mújica (1999) did not mentioned whether the samples they used had been cryopreserved, thus is possible that the eyelash-like morphology associates with alterations suffered by the PT in the cryopreservation process. Nevertheless, it appears that the morphology of the PT substructure is species specific, as it morphology differs for the rabbit (Koehler, 1970), bovine (Olson *et al*., 1983; Ostermeier *et al*., 2001), ram (Watson *et al*., 1995) and guinea pig (Juárez-Mosqueda and Mújica, 1999).

CONCLUSION

Freezing-thawing caused ultrastructural changes to the PT-substructure which was associated with acrosome loss. Our data reinforce the hypothesis that PT stabilizes the acrosome, which may partially explain the decrease fertility observed in cryopreserved boar semen. At the moment, investigations are being made to define if the PT alterations affect the ability of the spermatozoa to acquire fertility capacity.

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