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Alterations of Domains in the Plasmatic Membrane Due to Damages of the Perinuclear Theca of Pig Preserved Spermatozoa

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Abstract: Samples of semen from 12 pigs, three from Yorkshire, Landrace, Duroc and Mexican Hairless each where obtained to study cryopreservation methods. Three stages of boar semen cryopreservation were evaluated: none (fresh stage), cooling at 5°C and freezing at -196°C then thawing to 56°C for 12 sec. Perinuclear theca damage and domain alterations were selected as indices of seminal quality, as measured by electronic and fluorescence microcopy, respectively according to two lineal models considering by separately the effect of semen preservation and breed. Integrity and absence of perinuclear theca significantly ($p<0.001$) decreased and increased, respectively according to a decrease in temperature of cryopreservation, from 87.4 to 58.8% and from 0.8 to 26.2%, respectively. This same significant ($p<0.001$) effect was found for acrosomal and post-acrosomal membrane distribution of domains, from 92.1 to 76.8% and from 3.1 to 13.1% in this same order. Slight but highly significant ($p<0.001$) differences were observed when theca integrity was evaluated as affected by breed, with highest and lowest values for Yorkshire and Pelón Mexicano pigs, respectively. No breed effect was encountered for presence of acrosomal domains. A strong interdependence was found between perinuclear theca damage and domain distribution. In this connection, a highly significant ($p<0.001$) positive, interdependence was observed between the theca damage and acrosomal domain ($r = 0.87$), while this same relationship was although highly significant ($p<0.001$), negative in nature for equatorial and post-acrosonal domains ($r = -0.77$ and -0.85, respectively). This experiment confirmed that cryopreservation methods may severely affect semen quality of pigs and that genotype may further influence these same indices. More research is needed for improving methods of preservation of pig semen quality, from the point of view of perinuclear theca and domain characteristics of spermatozoa.

Key words: Pig, semen quality, perinuclear theca, spermatozoa, breed

INTRODUCTION

The use of frozen pig semen is far from being placed in a relevant position (Gilian *et al*., 2004; Wongtawan *et al*., 2006) and it is at least partially due to the spermatozoid susceptibility to damage provoked by the freezing-thawing process (Wevar *et al*., 1997; Johnson *et al*., 2000; Guthrie and Welch, 2005; Roca *et al*., 2006). This fact leads to the use of higher cell concentrations per dosage during insemination (Cerolini *et al*., 2001). However, freezing of pig semen shows interesting possibilities for applying not only in breeding or commercial pig herd improving, but in programs of local breed conservation and research in reproduction (Curry, 2000; Holt, 2000).

The real problem that cryopreservation experiences is not the ability degree of the spermatic cell to be kept viable during storage at -196°C, but it is in fact the combination of negative effects that freezing-thawing process determine on sperm physiology and morphology (Watson, 2000; Córdova and Gutierrez, 2002). Overall, cell damage is reflected in a decrease in motility and ultrastructural membrane damage (Johnson *et al*., 2000; Tienthai *et al*., 2004). In this connection, membranes of pig spermatozoid are more sensible to damage due to freezing than those of other domestic species, due to its lipid composition (Cerolini *et al*., 2001). This membrane is considered as a puzzle of domains where every one does have specific characteristics. The domains are established

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during spermiogenesis and they experiment changes during cell life (Mújica *et al*., 2003).

The domains are organized as mitochondrial leaf, flagellum, acrosoma and perinuclear theca (Watson, 2000).

Among these domains, the perinuclear theca exerts diverse functions such as protection of the spermatozoid nucleus and other processes involved in fecundation, by favouring the penetration of structure of ovocites during fusion, activation of this cell and decondensation of the genetic material after fertilization (Sutovsky *et al*., 1997; Sutovsky and Schatten, 2000; Juárez, 2000; Mújica *et al*., 2003).

The aim of the present investigation was to study the effect of different cryopreservation processes of pig semen on alterations of domains in the plasmatic membrane, due to damages of the perinuclear theca.

MATERIALS AND METHODS

This investigation was undertaken in 2007, at the Laboratory of Histology, Faculty of Veterinary Medicine of the National Autonomous University of Mexico (UNAM in Spanish) and at the Laboratory of Reproduction, Faculty of Veterinary Medicine of the Autonomous University of Nayarit (UAN in Spanish).

This investigation was part of a Mexican, national project concerning the study, rescue and conservation the Pelón Mexicano pig through cryopreservation of boar spermatozoa for futurein animal improvement.

Three boars of approximately two years old of three commercial breeds, Landrace, Yorkshire and Duroc and the creole breed of Mexican Hairless (Pelón Mexicano) pigs were used. The animals were trained for semen collection by the method of the gloved hand. Two ejaculates per boar were evaluated. Following sample collection, the techniques of volume, temperature and color estimation were conducted in the pig sperm immediately for quality evaluation, together with other microscopic procedures: motility, concentration and alive to dead cell ratio in the pig semen. Only those ejaculates exhibiting a motility of 80% and morphoanomalies lower than 15% and a cell concentration of 300 millions mL^{-1} were packed in 0.5 mL plastic straws and sealed. The modified procedure of Westendorf for semen freezing was used (Bwanga, 1990). All determinations were conducted in triplicate.

Semen samples at either fresh state, at 35°C in cooling curves at 5°C or those at -196°C, then thawed in water bath at 56°C during 12 sec, were processed for evaluating semen quality. The status of perinuclear theca was established by transmission electronic and fluorescent microscopy, using two types of lectins, agglutinin ex-*Triticum vulgare* and concanavamine ex-*Canavalia ensiformis*, conjugated with flourescein isothiocyanate. Agglutinin identifies the N-acetyl glucosamine residues and sialic acid and whereas concanavamine A binds mannose and glucose residues.

Surface domains of protoplasmatic membrane localization were observed in the spermatozoa. For both above referred techniques, a concentration of 3×10^6 cells mL^{-1} was employed. In order to expose the perinuclear theca surface, the spermatozoids were suspended in aqueous solution of Brij 36-T at 10% and then fixed by the Karnovsky technique. After this, the samples were adsorbed in copper meshes of 200 mesh, covered with membranes of collodion-carbon support, dyed with 0.02% phosphotungstic acid and placed in a grid support for its observation in an electronic microscope.

The identification of surface domains of spermatozoids was conducted by cell fixation in 3% formaldehyde (in volume) for 20 min. afterwards centrifuged and then discarding the supernatant, for re-suspension of the sediment in NH_4Cl 50 mM for 10 min.

Thereafter, the new supernatant was discarded again and the sediment was suspended again in PBS at pH 7.4 for preparation of frotis in a defatted glass slide. After air drying, reagents containing the two types of lectins (*Vide supra*), were added in parallel, at a concentration of 10 μL lectin mL^{-1} PBS sample solution. Control samples were only treated with the PBS reagent, then incubated during 30 min in a humid chamber at 37°C in the darkness and finally washed four times with the PBS reagent and dried. A glycerol/PBS (9:1) mixture was applied and thereafter protected from light until its examination by fluorescence microscopy provided of a video camera.

Damages of perinuclear theca were identified by electronic microscopy, using as morphologic marker the appearance of the sub-structure of the post-acrosomal leaf. The status of this structure was classified as intact, altered or absent. Surface domain distribution was identified by fluorescence microscopy, according to florescence patterns of the acrosomal, aequatorial and post-acrosomal regions of spermatozoa, corresponding to the main, central and post-head regions. In both types of examination, counting at random of 50 sperm cells per sample was carried out.

Perinuclear theca damage and domain alterations were selected as indices of seminal quality, as measured by electronic and fluorescence microcopy, respectively according to two lineal models considering by separately the effect of semen preservation and breed. For measurements carried out by electronic microscopy of the perinuclear theca, three samples per animals were observed by duplicate (n = 72) and for membrane domain

distribution, the same procedure was adopted, except that no duplicates were evaluated (n = 36). When pig genotypes were examined, 24 and 12 observations were conducted for theca integrity and domain distribution, respectively. All indices were expressed in percentage.

When the analysis of variance revealed significant differences ($p<0.05$), means were separated by the Tukey test. In addition correlation analysis was conducted in the necessary cases (SAS, 1995).

RESULTS AND DISCUSSION

The electronic microphotography evidence clearly shows different status of the perinuclear theca of pig spermatozoa heads, either intact, altered or absent. These identifications were clearly delimited and there were no difficulties for identification of the different degrees of perinuclear theca modifications, caused by the cryopreservation methods assayed in the current investigation.

The Inmunofluorescence microscopy evidence clearly shows of different distribution of domains, acrosomal, aequatorial and post-acrosomal. In this connection, fluorescence determined with the selected lectins in this experiment did not revealed breed differences. This fluorescence as determined with WGA mainly marked the surface surrounding the head and tail of spermatozoa, whereas concanavamine A slightly limited head membrane and the middle of spermatozoa in fresh spermatozoa of the examined samples.

Integrity and absence of perinuclear theca significantly ($p<0.001$) decreased and increased, respectively according to a decrease in temperature of cryopreservation, from 87.4 to 58.8% and from 0.8 to 26.2%, respectively (Table 1). This same significant ($p<0.001$) effect was found for acrosomal and post-acrosomal membrane distribution of domains, from 92.1 to 76.8% and from 3.1 to 13.1% in this same order. Overall, a notorious deterioration in integrity of the perinuclear theca of pig spermatozoa was evident.

Slight but highly significant ($p<0.001$) differences were observed when theca integrity was evaluated as affected by breed, with highest and lowest values for Yorkshire and Pelón Mexicano pigs, respectively (Table 2). In fact these two breeds showed extreme values for all the examined indices determined in the current investigation. On the other hand, no breed effect was encountered for presence of acrosomal domains.

The interdependence found between theca damage and domain distribution. A highly significant ($p<0.001$) positive, interdependence was observed between the perinuclear theca damage and acrosomal domain (r, 0.87), while this same relationship was although highly significant ($p<0.001$), negative in nature for aequatorial and post-acrosonal domains (r, -0.77 and -0.85, respectively).

Table 1: Changes in pig spermatozoa status as affect by method of semen cryopreservation

	Temperature of cryopreservation (°C)			
	35[1]	5	-196[2]	SEM ±
Perinuclear theca status (%)				
N	72.00	72.00	72.00	-
Intact	86.40^{a}	77.82^{b}	58.90^{c}	1.58***
Altered	11.76	14.50	14.76	0.58
Absent	0.82^{c}	7.58^{b}	26.34^{a}	1.46***
Membrane domain distribution (%)				
N	36.00	36.00	36.00	-
Acrosomal	92.16^{a}	87.83^{b}	76.83^{c}	1.12***
Aequatorial	4.66^{c}	7.33^{b}	9.66^{a}	0.40***
Post-acrosomal	3.16^{c}	4.83^{b}	13.50^{a}	0.79**

[1]Fresh state (environmental temperature): [2]Samples examined after thawing at 56°C for 16 sec. *** $p<0.001$, ^{abc}Means without letter in common in the same row differ significantly ($p<0.05$) according to the Tukey test

Table 2: Perinuclear theca integrity and presence of membrane domains in pig spermatozoa as affected by breed according to method of processing

	Temperature of cryopreservation (°C)		
	35[1]	5	-196[2]
N	12.00	12.00	12.00
Theca integrity (%)			
Landrace	85.66^{ab}	71.66^{b}	62.00^{ab}
Yorkshire	90.32^{a}	86.00^{a}	67.00^{a}
Duroc	89.32^{ab}	82.66^{a}	56.00^{b}
Pelón Mexicano	84.32^{b}	71.32^{b}	50.66^{c}
SEM ±	0.82***	1.46***	1.56***
Membrane domain distribution (%)			
Landrace	93.00	90.00	78.66
Yorkshire	91.66	86.66	76.66
Duroc	92.66	86.66	76.00
Pelón Mexicano	91.33	88.00	76.00
SEM ±	0.38	0.57	0.50

[1]Fresh state (environmental temperature), [2]Samples examined after thawing at 56°C for 12 sec.*** $p<0.001$, ^{abc}Means without letter in common in the same column differ significantly ($p<0.05$) according to the Tukey test

Results of the present experiment indicate that the freezing-thawing process originates alterations in the perinuclear theca of the several studied breeds. These damages are in agreement with those of others (Tovich *et al*., 2004; Martínez *et al*., 2006), who reported a similar effect after freezing bovine spermatozoids and even more, suggested that an increase in the percentage of sperm cell containing no perinuclear theca determined a decrease in live cells. On the other hand, Arancibia *et al.* (2007) found damages of importance in the perinuclear theca during freezing pig spermatozoids, possibly due to a destabilization of actin, which would provoke an alteration or even a total lost of this cell sub-structure. In this connection, it is worthy to note that in other types of cells, during the freezing-thawing process actin F can experience polymerization and depolymerization, depending on temperature (Delgado *et al*., 1999; Watson, 2000).

The observed changes in the present investigation concerning the perinuclear theca are of importance, since this theca is the main cytoskeletal structure in the spermatic head, to what it has been attributed functions of protection, keeping of membrane domains and participation in the processes of fusion and decondensation of spermatic cells after fertilization (Juárez and Mújica, 1999). In this connection, it has been shown that this structure also suffers alterations after thawing (Hernández *et al*., 2007; Martínez *et al*., 2006). On the other hand Petrunkina *et al*. (2004, 2005) reported that cytoskeleton proteins which support plasmatic and acrosomal membranes have a depolymerization and repolymerization depending on temperature, which is reflected in the accelerated changes in volume occurring in the spermatic cells during the freezing process.

It has been shown that the plasmatic membrane is affected by cryopreservation (Holt, 2000; Watson, 2000; Roca *et al*., 2006) and that there is a re-distribution of components in the domains of this membrane. In the current investigation, it was observed that fresh semen contained the highest number of spermatozoid with the fluorescence pattern in the acrosomal region and that when the temperature decreased during freezing up to 5°C, there was an increase in the percentage of cell sperms with a fluorescence pattern in the aequatorial region. This same shift of fluorescence pattern, but towards the post-acrosomal region, was observed in thawed samples (Frits *et al*., 1998; Jiménez *et al*., 2003).

In this study, both agglutinin and concanavanine A linked to glycoconjugates present in the acrosomal region of the plasmatic membrane of fresh spermatozoa, in accordance to that reported by others (Frits *et al*., 1998; Jiménez *et al*., 2003; Marini and Cabada, 2003), who employed the same types of lectins in pig spermatozoids.

In fact, it has been observed (Frits *et al*., 1998; Jiménez *et al*., 2003; Marini and Cabada, 2003) that N-acetyl glucosamine, sialic acid, mannose and glucose are widely distributed in the spermatozoid membrane of the pig, including tail and acrosomal membrane.

Furthermore, these authors found that after agglutinin treatment of capacitated spermatozoa, there was a decrease in the site of linkage and this compound was concentrated in the acrosomal region. These results confirm that the presence of N-acetyl glucosamine and sialic acid in the plasmatic membrane of pig spermatozoa, in fact indicate that this cells were not capacitated yet (Jiménez *et al*., 2003).

After the freezing-thawing process, it was observed a major frequency of fluorescence in the domain of the aequatorial region of spermatozoids marked with agglutinine, whereas this phenomenum occurred in the post-acrosomal domain when concanavamine A was the marker substance. There are several investigations where changes in carbohydrates of the plasmatic membrane surface are described during capacitation and acrosomal reaction (Frits *et al*., 1998; Jiménez *et al*., 2003; Marini and Cabada, 2003). According to these authors, there is an increase in fluorescence of the acrosomal portion of the plasmatic membrane in capacitated pig spermatozoa, after treatment with concanavamine A. It has also been reported that the perinuclear theca experiment changes during acrosomal reaction (Juárez and Mújica, 1999) where actin F is involved too. As it is well known, spermatozoa capacitation implies changes in the plasmatic membrane.

CONCLUSION

This experiment confirmed that cryopreservation methods may severely affect semen quality of pigs and that genotype may further influence these same indices. More research is needed for improving methods of preservation of pig semen quality, from the point of view of perinuclear theca characteristics of spermatozoa.

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