DESCRIPTION OF ULTRASTRUCTURAL DAMAGES IN FROZEN-THAWED CANINE SPERMATOZOA

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ABSTRACT

In spite of the advances in transmission electronic microscopy (TEM), there are few studies presenting a systematic description of the canine spermatozoa and they are only focused on sperm cell heads. The goal of the present study was to describe ultrastructural appearance of canine fresh and frozen-thawed spermatozoa, focusing on damages induced by freezing-thawing in different sperm regions. Ten ejaculates from five proven stud dogs (two ejaculates/dog) were collected, evaluated, extended in Tris-egg yolk-glycerol, frozen and stored in liquid nitrogen, and thawed six weeks later. Samples were evaluated for progressive motility, morphology, and for ultrastructural analysis by TEM. Concerning to TEM, the most striking differences between fresh and frozen-thawed samples were observed over the mid-piece since the fresh spermatozoa showed a well preserved mid-piece. However, the frozen-thawed spermatozoa mid-piece showed signs of damage such as mitochondrial vacuolization. In conclusion, freezing-thawing processes using a Tris-egg yolk extender induce ultrastructural damages in head and mid-piece of canine sperm, affecting the mitochondrial ultrastructure besides.

KEY WORDS: Cryopreservation, dog, semen, ultrastructure.

RESUMO

DESCRIÇÃO DE DANOS ULTRAESTRUTURAIS EM ESPERMATOZOIDES CANINOS CONGELADOS-DESCONGELADOS

Apesar dos avanços na microscopia eletrônica de transmissão (MET), existem poucos estudos apresentando uma descrição sistemática do espermatozoide canino, os quais estão focados apenas na cabeça espermática. Objetivou-se, com o presente estudo, descrever a aparência ultraestrutural do espermatozoide canino fresco e congelado-descongelado, enfocando os danos induzidos pela congelação e descongelação em diferentes regiões espermáticas. Dez ejaculados obtidos de cinco cães (dois ejaculados por cão) foram coletados, avaliados e diluídos em Tris-gema-glicerol, congelados e armazenados em nitrogênio líquido, e descongelados seis semanas após. Avaliaram-se as amostras quanto à motilidade progressiva, morfologia espermática e análise ultraestrutural por MET. Em relação à MET, as principais diferenças entre o espermatozoide fresco e descongelado foram observadas na peça intermediária, visto que o espermatozoide fresco apresentava uma peça intermediária bem preservada. Entretanto, a peça intermediária dos espermatozoides descongelados mostrava sinais de danos como a vacuolização das mitocôndrias. Em conclusão, os pro-

cessos de congelação e descongelação utilizando o diluente Tris-gema induzem danos ultraestruturais na cabeça e peça intermediária de células espermáticas caninas, afetando, inclusive, a ultraestrutura das mitocôndrias.

PALAVRAS-CHAVES: Cão, criopreservação, sêmen, ultraestrutura.

INTRODUCTION

Although the basic cryogenic damages could be morphological and often result in dysfunction, the physical stress to the sperm membranes during the freezing process is seen as the most limiting factor (PESCH & BERGMAN, 2006). Even if the sperm motility and morphology evaluations are used as a fast analysis of a semen sample, these tests do not evidence alterations when the morphological defects are expressed on a nanometric scale (CRESPILHO et al., 2006).

Various regions of the plasma membrane of the sperm cell play different roles in sperm function and survival. Structural components of the head, mid-piece and tail respond differently to factors such as thermal and osmotic shock during preservation in the liquid or frozen state (NAGY et al., 1999). ROCHA et al. (2006) studied the alterations in the ultrastructure of the mid-piece of bull spermatozoa, identifying a series of defects with unknown origin. These authors support the idea that many alterations exist in mid-piece and they can express a variable effect on the fertility.

In spite of the advances in transmission electronic microscopy (TEM), there are few studies presenting a systematic description of the canine spermatozoa and most of them are only focused in sperm cells heads (ENGLAND & PLUMMER, 1993; RODRIGUEZ-MARTINEZ et al., 1993; STROM-HOLST et al., 1998; BURGESS et al., 2001; CHIRINÉA et al., 2006). There is a lack of information regarding cryo-induced damage in the ultrastructure of other canine sperm regions, such as the mid-piece (NIZANSKI et al., 2005). It was aimed with the present study to describe the ultrastructural appearance of canine fresh and frozen-thawed spermatozoa, focusing on damages induced by freezing-thawing in different sperm regions.

MATERIAL AND METHODS

Animals

Five privately-owned, proven stud dogs (one Brazilian Mastiff, one American Pit Bull Terrier and three Boxers) were selected for this experiment. The dogs, aged from 1 to 6 years, were maintained in individual pens and fed dry food once daily, with free access to water.

Semen collection and initial evaluation

Each dog was submitted to two semen collections by manual stimulation, and the sperm-rich fraction was evaluated. Semen volume and color were grossly evaluated. Progressive sperm motility was subjectively analyzed by light microscopy (100x). Sperm concentration was determined using a Neubauer counting chamber. Sperm morphology was evaluated by phase-contrast microscopy, under 1000x magnification, analyzing a slide stained with Bengal Rose, counting 200 cells per slide. Sperm morphologic alterations were classified according to their origin as primary and secondary (JOHNSTON et al., 2001).

Semen freezing-thawing

An extender consisting of 3.028g Tris-hydroxymethyl-aminomethane, 1.78g monohydrated citric acid and 1.25g D-fructose, dissolved in 100mL distilled water (SILVA et al., 2002) was used. The osmolarity of this solution was 295mOsm/L and the pH 6.6. Twenty percent of this solution was replaced by egg-yolk (vol:vol). During initial analysis, semen remained at room temperature (27°C) for 10 min and further extended in Tris-egg yolk. Semen was kept in a thermal box for 40 min and cooled to 15ºC at a rate of -0.30°C/min. Samples were then transferred to a refrigerator for 30 min and cooled to 4ºC at -0.37°C/min. After cooling, semen was added to Tris-egg yolk plus 12% glycerol also at 4ºC, which

provided a final concentration of 6% glycerol in the extender. Final sperm concentration was 200 x 10^6 spermatozoa/mL. Samples were packaged in 0.25mL plastic straws, which were placed horizontally on a metal rack, and plunged in liquid nitrogen vapor. After reaching -70ºC, straws were plunged liquid N_2 for storage (SILVA et al., 2006a) for until six weeks. Then, samples were thawed in a water bath at 38ºC for 1 min and reevaluated.

Transmission electronic microscopy - TEM

After collection and thawing, semen aliquots from each dog were centrifuged at 800G for 5 min. Pellets were fixed using a KARNOVSKY (1965) solution slighted modified and containing 2% paraformaldehyde and 2% glutaraldehyde in phosphate buffer 0.1M pH7.4 (PB - Sigma Co, St. Louis, USA) and held at 4ºC. Fresh samples were kept at 4ºC for until six weeks, in order to be processed at the same moment as frozen-thawed samples. Fixed samples were washed in phosphate buffer, then post-fixed with osmium tetroxide 1%, dehydrated in propylene oxide and embedded in epon resin (Embed 812, Electron Microscopy Sciences, Hatfield, USA). Ultra fine sections (80nm) were manually stained with uranyl acetate and lead citrate. Randomly fields were examined by a transmission electronic microscope (JEOL 1010, Japan) and photographed for further analysis and description.

Statistical analysis

Data were expressed as mean and standard deviation, Arcsin transformed and analyzed by the ANOVA (Statview 5.0, SAS Institute Inc., Cary, USA). Comparison between sperm progressive motility and morphology for fresh and frozen semen were performed by Student's t test ($p < 0.05$).

RESULTS

Fresh dog semen was milky-white in color. The mean (± SD) volume of the sperm-rich fraction was 0.9 ± 0.4 mL, with a concentration of 1030.3 ± 600.2 x 10^6 sperm/mL. Progressive sperm motility was 97.5 ± 3.5% and 57.3 ± 8.1% for fresh and frozen semen, respectively. A significant reduction on sperm motility was verified after thawing ($P<0.05$).

Concerning sperm morphology, 93.2 ± 3.5% and 87.1 ± 5.9% of sperm cells were normal in fresh and frozen semen, respectively ($p>0.05$). The percentage of primary sperm defects was 0.9 ± 0.8% in fresh semen compared to 1.6 ± 1.8% in frozen-thawed semen ($p>0.05$). The percentage of secondary sperm defects were 6.0 ± 3.9% in fresh and 11.1 ± 4.5% in frozen-thawed semen ($p>0.05$).

In TEM, fresh sperm cell showed the typical morphology for canine spermatozoa. The outer acrosomal membrane was slightly undulating (Figure 1A) but the acrosomal content was preserved and the limit between nucleus and acrosome were preserved (Figure 1B). In contrast, the frozen-thawed spermatozoa head showed more alterations such as ballooning of the plasma membrane and loss of the acrosomal content (Figure 1C; Figure 1D).

The most striking differences between the two samples were observed over the mid-piece. The fresh spermatozoa showed a well preserved mid-piece exhibiting a well defined axoneme surrounded by outer dense fibers and the mitochondrial sheath (Figure 2A). The mitochondria are well preserved showing several internal cristae immersed in a dense matrix (Figure 2A; Figure 2C). The frozen-thawed spermatozoa mid-piece showed the same structure as observed for the fresh spermatozoa, however the mitochondrial sheath showed signs of damage (Figure 2B). The mitochondria were vacuolated as the matrix has been washed out, few cristae were visible (Figure 2B), nevertheless it was also possible to find some well preserved mid-piece spermatozoa in the frozen-thawed sample (Figure 2D).

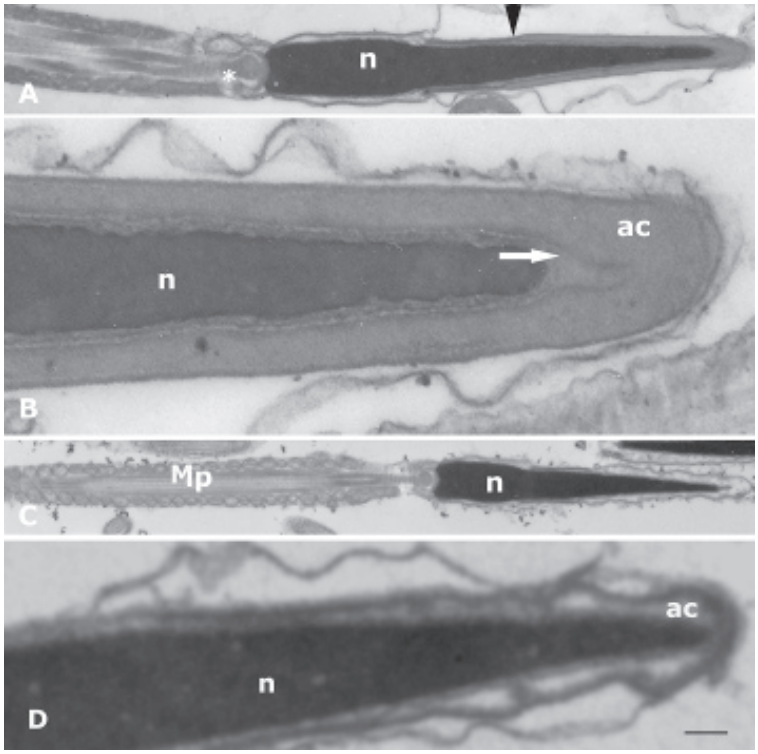

FIGURE 1. Electron microscopy images of sagital sections through fresh and frozen thawed canine sperm cells. (A): Low magnification of a fresh canine sperm showing a well defined head and preserved acrosome covering partially the nucleus (n), the arrow indicates the beginning of the acrosome. The head is connected to the mid-piece (mp) through the neck (*). (B): Higher magnification of the head of a fresh sperm cell. The acrosomal content is moderately electron-dense and the limits between nuclear and acrosome membrane (arrow) are observed. (C): Low magnification of a frozen-thawed canine sperm showing that the acrosomal content is washed out (arrow). (D): Higher magnification of the head of a frozen-thawed canine sperm showing a washed out and swollen acrosome. Nucleus (n); mid piece (mp). Scale bar: (A) 440nm; (B) 66nm; (C) 835nm; (D) 264nm.

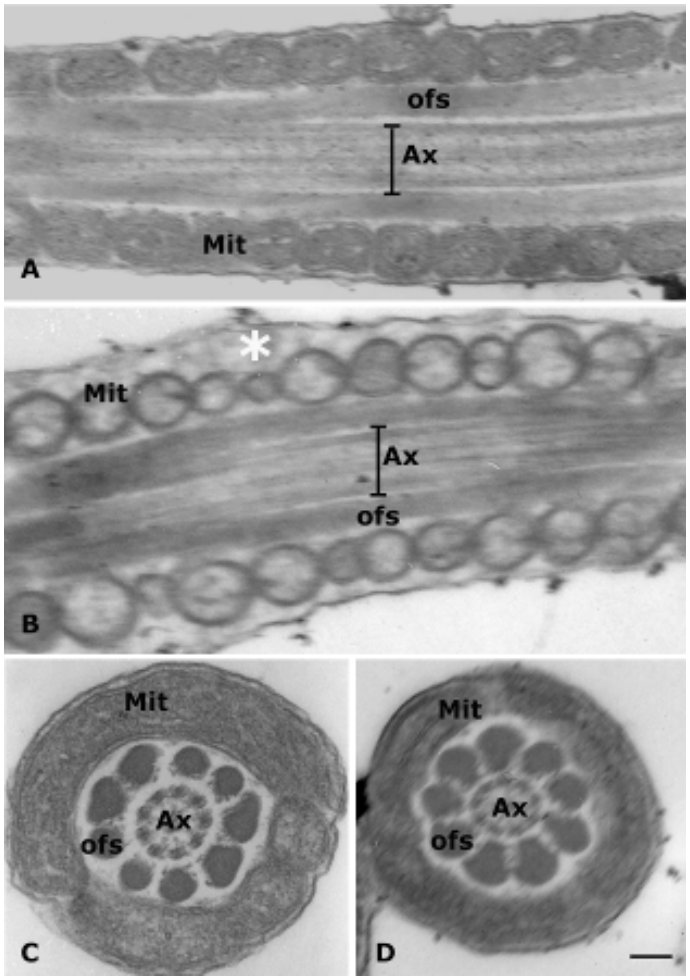

FIGURE 2. Electron microscopy sections through the mid piece from either fresh or frozen-thawed canine sperm cells. (A): A longitudinal section through the mid-piece of a fresh sperm cell with the mitochondrial sheath well preserved showing rows of mitochondria (Mit). The axoneme (Ax) is surrounded by outer fibrous sheath (ofs). (B): A longitudinal section of a frozen-thawed sperm mid piece showing the same features as the fresh one besides the appearance of mitochondria that seems vacuolated. A slight distension of the plasma membrane can be observed suggesting edema (*). (C) and (D) are transversal sections of a mid-piece from a fresh and frozen spermatozoon, respectively. Both exhibit the same features and showed normal mitochondrial organization. Mitochondria (Mit); Outer fibrous sheath (ofs); Axoneme (Ax). Scale bar: (A) and (B) 120nm; (C) 100nm; (D) 106nm.

DISCUSSION

Although the relationship between motility and fertilizing capacity of canine spermatozoa has not been fully elucidated, most researchers use sperm motility as the main parameter to evaluate extenders, cryoprotectors and freezing techniques. Reduction in sperm motility could be associated to the stress factors induced by freezing, such as oxidation of sperm membrane lipids and damage to the selective permeability mechanisms of the membrane (PESCH & BERGMANN, 2006).

The main morphological abnormalities observed in frozen semen were secondary defects (SEAGER, 1986), probably originated from the thermal shock and osmotic stress caused by the freezing-thawing procedures. However, since not all damage would be apparent with phase-contrast microscopy, ideally other techniques (e.g. electron microscopy) should be used for sperm analysis.

While light microscopy is suitable to give a general survey of sperm configuration consisting of head, neck and tail, TEM delivers insights in the inner and outer fine structures of spermatozoa (PESCH & BERGMANN, 2006). According to CHIRINÉA et al. (2006), TEM could be used as a descriptive test for semen evaluation. However, fine structure is not a very useful approach to evaluate semen routinely for a number of reasons, such as technically challenging, limited observations and costs. Furthermore, quantitative analysis by TEM is difficult to be performed due to the costs of the procedure and the fragility of the tissue under prolonged exposition to electron bunch.

The occurrence of slightly swollen plasmalemma above the acrosomal region observed in fresh canine spermatozoa in the present study was previously reported by CHIRINÉA et al. (2006). These authors considered this to be a consequence of the semen sample being exposed to glutaraldehyde during the fixation procedure for TEM. Moreover, canine spermatozoa undergo several alterations in the head region after cryopreservation, but the plasmalemma was not ruptured. According to AISEN et al. (2005), freezing and thawing cause gross alterations in the plasma membrane of the ram spermatozoa including detachment at the head and tail, as verified for canine semen. Similar results were reported by RODRIGUEZ-MARTINEZ et al. (1993) in dogs and they suggested that the plasmalemma remaining intact after cryopreservation might account for the low percentage of acrosomal defects usually reported when using phase contrast microscopy.

Undulating acrosomal membrane seems to be a common consequence of cryopreservation in dog spermatozoa (BURGESS et al., 2001). Vesiculation in acrosomal membranes and loss of acrosomal contents observed in our study with frozen-thawed semen could be a result of acrosomal reaction by the fusion of the plasma membrane with the outer acrosomal membrane (EILTS, 2005). In fact, alterations found in the fine structural appearance of canine frozen-thawed semen could be interpreted as a false acrosomal reaction (RODRIGUEZ-MARTINEZ et al., 1993).

We verified that mid-piece axoneme seemed to remain intact after cryopreservation. Similar results were described by NIZANSKI et al. (2005) that reported that the normal axial 9+2 fiber pattern in cross sections of sperm tails is maintained after cryopreservation of canine semen. The same authors reported the presence of a firmly granulated material within mitochondria after freezing.

In the present research, mitochondrial sheath of some sperm cells was vacuolated and unorganized after thawing. These damages may suggest that those spermatozoa may undergo a degenerative process or, more probably, an apoptotic phenomenon. According to WYLLIE et al. (1980), necrosis and apoptosis are two forms of cell death. Necrosis results from injury and affects large numbers of cells, causing cell swelling and rupture. On the other hand, apoptosis is programmed cell death that affects single cells. In fact, BLANC-LAYRAC et al. (2000) worked with electron microscopy and found that human spermatozoa exhibited certain characteristics of apoptotic somatic cells, such as mitochondrial distension. The presence of apoptotic signaling in spermatozoa may be partially responsible for the low fertilization and implantation rates seen with reproductive assisted techniques (GRUNEWALD et al., 2007).

In humans, it is known that abnormalities in the mitochondrial sheath can directly affect the sperm motility (RAO et al., 1989). Ultrastructural damage can be observed in mitochondrial sheath of mammal spermatozoa after freezing-thawing and is always accompanied by biochemical changes or even loss of their vital contents (PESCH & BERGMANN, 2006). A positive correlation between survivability and the number of intact mitochondria was also reported, since mammalian sperm with many mitochondria have a higher survivability than for example spermatozoa of aquatic vertebrates with only two mitochondria (ROVAN, 2001).

Mitochondria are thought to provide the mid-piece and the sperm head with ATP required for housekeeping processes (SILVA & GADELLA, 2006). The functional integrity of mitochondria may thus be important for sperm survival in the female genital tract. Addition of glycerol during freezing affects reactions that require ATP and thus may cause an ATP deficit in mitochondrial membrane potential in bull sperm (GARNER et al., 1999). The altered sperm motility found after thawing may be a result from mitochondrial damage that leads to a less efficient ATP production or even to the sperm death.

Once that recent studies relate mitochondrial damage to the occurrence of apoptosis, several tests have been conducted by using fluorescent probes such as lipofilic cationic carbocyanine (JC-1), in order to measure the mitochondrial membrane potential of sperm cells from different species (BOLLWEIN et al., 2008). These tests can confirm the apoptotic induction by the sperm cells cryopreservation. However, there is still a lack of information concerning such studies in canine species.

In a previous work, we demonstrated that canine semen is rather distributed in sperm subpopulations, but none of these subpopulations present relations to frozen sperm-oocyte interactions in this species (SILVA et al., 2006b). The mechanism of formation of sperm subpopulations and their physiological role are not very clear. Interestingly the suggestion that different sperm subpopulations can be found in canine ejaculates provides an answer to why not all sperm undergo ultrastructural damage due to freezing-thawing procedure. The reason for this different response of the cells to the freezing remains to be elucidated as well as the amount of cell death that occurs during the cryopreservation or thawing procedures.

CONCLUSIONS

In conclusion, freezing-thawing processes using a Tris-egg yolk extender induce ultrastructural damages in head and mid-piece of canine sperm, affecting the mitochondrial sheath besides. Further studies must be conducted in order to evaluate the possibility for the occurrence of degenerative or apoptotic phenomena induced by cryopreservation in canine semen.

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