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DNA integrity of fresh and frozen canine epididymal spermatozoa

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ABSTRACT

The aims of this study were to evaluate: (1) the effect of cryopreservation on DNA fragmentation of canine epididymal spermatozoa, and (2) the potential protective effect of melatonin on post-thaw sperm quality (motility, morphology, acrosomal and DNA integrity). Epididymal spermatozoa were collected after orchiectomy of ten dogs. Sperm samples were frozen in the presence or absence of melatonin (1 mM). DNA fragmentation index (percentage of spermatozoa with fragmented DNA) was similar in fresh samples (3.3 ± 3.6) and samples frozen with (4.2 ± 3.8) or without (3.6 ± 3.7) melatonin. Sperm motility was significantly ($p < 0.0001$) higher in fresh compared to frozen samples. The presence of melatonin in the freezing extender did not affect the sperm motility. Proportions of spermatozoa with normal morphology were similar in fresh and frozen samples, irrespective of the presence of melatonin in the extender. Acrosome integrity was significantly decreased ($p < 0.01$) by cryopreservation, and melatonin did not exert any beneficial effects. In conclusion, DNA fragmentation of canine epididymal spermatozoa was not affected by the freezing procedure, and the presence of melatonin did not preserve motility and acrosome integrity which were adversely affected by cryopreservation. The evaluation of DNA status of thawed gametes is particularly relevant for epididymal spermatozoa since these spermatozoa are usually stored and used in assisted reproductive techniques.

1. Introduction

The cryopreservation of epididymal spermatozoa is aimed at maintaining long-term availability of male germplasm for future use. This is particularly crucial for the conservation of endangered species and for the generation of offspring from individuals of high genetic value that die accidentally or undergo orchiectomy for medical purposes. In dogs, artificial insemination with frozen epididymal spermatozoa resulted in

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the birth of offspring with a low conception rate [1–3]. The effects of cryopreservation on motility, as well as membrane and acrosomal integrity of canine epididymal spermatozoa have been previously investigated [4–6], but no information is available on its potential effect on DNA integrity.

Sperm DNA integrity has been evaluated in fresh ejaculated [7–10] and epididymal canine semen [11]. Few reports have compared fresh and post-thaw chromatin integrity of canine ejaculated spermatozoa, obtaining variable results [12–16], but the post-thaw DNA stability of canine epididymal spermatozoa has not been investigated. The integrity of the paternal DNA is of crucial importance for embryo development [17], and a relationship between DNA damage and infertility has been demonstrated in humans. Spermatozoa with severe DNA damage remain functionally intact, with normal fertilizing ability, but a high index of DNA fragmentation results in a significant decrease in pregnancy rates [18,19]. Nevertheless, there is no agreement neither on whether cryopreservation induces DNA fragmentation, nor on the mechanism which actually induces this damage [20,21]. It has been hypothesized that the increase of reactive oxygen species (ROS) during cryopreservation and the decrease of antioxidant activity of the spermatozoa cause the peroxidative damage to the sperm plasma membrane and affect DNA integrity [13,14,20].

The role of antioxidant supplementation in protecting the sperm DNA from oxidative damage is still under investigation. Among antioxidants, it has been shown that melatonin (1–2 mM), has an effective action in protecting ram and bull spermatozoa from the freezing injuries as evidenced by post-thaw DNA integrity, viability, motility, morphology and fertilizing ability [22,23]. The aims of this study were to evaluate the effect of cryopreservation on DNA fragmentation of canine epididymal spermatozoa and the potential protective effect of melatonin on post-thaw sperm quality (motility, morphology, acrosomal and DNA integrity).

2. Materials and methods

2.1. Animals and epididymal spermatozoa retrieval

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated. Ten healthy and pubertal, privately owned stud dogs, aged between 1 and 10 years (6–30 kg body weight) presented to the Department for routine orchiectomy were included in this study. Canine gonads were transported to the laboratory within 10 min after surgical removal. Each epididymis was dissected from the testis and pampiniform plexus using a scalpel blade. The small vessels were removed with scissors to reduce blood contamination, and each cauda epididymis was isolated and placed in a Petri dish containing 4 mL of Ham's F-10 medium supplemented with 2 mM glutamine, 100 IU/mL Na-benzyl penicillin, 0.1 mg/mL streptomycin sulphate, and 5% fetal bovine serum (mOsm 285). The caudae were minced with a scalpel blade, and after 30 min of incubation (37 °C), the suspension was collected from each dish and divided into three aliquots.

2.2. Semen freezing procedure

One aliquot was used as fresh control, and the others were frozen with or without melatonin (1 mM) in a freezing extender. After centrifugation (700 × *g* for 5 min) and removal of the supernatant, the second aliquot was diluted (200×10^6 sperm/mL) with the following freezing extender: TRIS buffer with 5% glycerol, 1% Equex and 20% egg yolk, and the third aliquot was diluted with the extender supplemented with melatonin. Both aliquots were frozen according to the Uppsala system [24]. The 0.5 mL straws placed in a styrofoam box were submerged in liquid nitrogen vapors (10 min, 4.5 cm above liquid nitrogen) and subsequently immersed into liquid nitrogen. The straws were thawed in a water bath at 37 °C for 30 s.

2.3. Spermatozoa evaluation

Sperm concentration in fresh semen was determined with a Bürker chamber. Sperm motility, morphology and acrosomal integrity were evaluated in fresh and thawed samples. Motility was subjectively assessed under a light microscope with a heated stage (38 °C). The spermatozoa were considered to be motile only if they exhibited progressive motility of a score of at least 3 or 4 on a scale of 0–4 (0, absent; 1, weak or sluggish; 2, definite; 3, good; 4, vigorous) [25]. Morphology of spermatozoa was assessed following smear staining with Bengal Rose and Victoria Blue B. At least 100 spermatozoa were evaluated under light microscopy with oil immersion objective (1000× magnification). Normal spermatozoa and defect sites (head, neck/midpiece, tail) in abnormal spermatozoa were recorded. Abnormal sperm heads included those that were pear-shaped, narrow at the base or detached. Alterations of the neck/midpiece included bent neck and proximal or distal cytoplasmic droplet, and abnormal tail included single bent, coiled or broken tail.

The acrosome integrity was evaluated by staining spermatozoa with Peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI). Acrosome status was evaluated under fluorescent microscope (Axiovert 100, Zeiss, Oberkochen, Germany) in at least 100 spermatozoa per group by FITC-PNA/PI staining according to the procedure described for stallion spermatozoa [26]. The observed fluorescence images of the stained spermatozoa were classified as: (1) intact acrosome – spermatozoa displaying intensively bright fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane; (2) vesiculated acrosome – spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane; and (3) acrosome residues or loss – spermatozoa displaying a fluorescent band at the equatorial segment indicated residues of the outer acrosomal membrane or displaying no fluorescence indicated a complete loss of the outer acrosomal membrane [26].

2.4. Assessment of sperm DNA fragmentation

The sperm DNA fragmentation was assessed using the Sperm-Halomax® commercial kit specifically developed for canine