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Canine epididymal spermatozoa: characteristics, collection and cryopreservation.

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CHAPTER 1

Introduction

1. Introduction

In veterinary medicine the Artificial Reproductive Technologies (ART) were at first aimed at improving genetic progress in cattle. More recently the application of these techniques has been extended to Carnivores for the maintenance of biodiversity and the improvement of reproductive performances.

The maintenance of biodiversity in endangered wild species can be achieved both with the protection of their natural environment and with the collection and preservation of genetic material. The improvement of reproductive performances in case of infertility problems or when mating cannot occur is achieved with ART and reproductive biotechnologies as artificial insemination or *in vitro* fertilization.

In individuals of high genetic or emotional value that cannot mate or ejaculate semen, epididymal spermatozoa represent the only source of genetic material. Isolated epididymides are available when the male dies unexpectedly or undergoes orchiectomy for medical reasons, but in case of neuropathic conditions or obstructive problems of the reproductive tract that cause erectile dysfunction or ejaculation problems, the collection of spermatozoa from *in situ* epididymides might be an option to obtain progeny.

The fertilizing ability of epididymal spermatozoa has been demonstrated in several mammalian species including dogs (Dacheux and Paquignon, 1980; Hewitt et al., 2001). Intravaginal and intrauterine artificial insemination with

fresh and frozen spermatozoa retrieved from isolated epididymis *caudae* resulted in pregnancy and in the delivery of viable puppies.

Marks and co-workers (1994) collected epididymal spermatozoa postmortem and after freezing and surgical intrauterine insemination obtained one pup. Better results were achieved with fresh epididymal semen collected after orchiectomy of a dog affected by prostatic hyperplasia. The intravaginal insemination of a bitch resulted in pregnancy and in the delivery of 8 puppies, (Klinc et al., 2005). Other studies compared the conception rates after insemination of frozen epididymal semen in different experimental conditions. Hori et al., 2004 obtained a low conception rate (6.3% and16.7%, respectively) after intrauterine and intratubal insemination of frozen epididymal spermatozoa.

The conception rate was significantly improved (80%) with the sensitization of the epididymal frozen semen with prostatic fluid (Hori et al., 2005).

1.1 The epididymis

The epididymis consists in a long duct tightly folded and it is divided into three gross anatomical regions: head (*caput*), body (*corpus*), and tail (*cauda)*. The *caput* involves the terminal parts of the efferent ducts and the first tract of the

epididymal duct. The *corpus* connects the *caput* with the *cauda*, which continues distally in the *vas deferens* and represents the site of storage of mature spermatozoa (Robaire et al., 2006).

In the mammalian epididymis, substantial changes of spermatozoa occur. During epididymal transit from *caput* to *cauda*, functional and structural modifications leading to full maturation enable male gametes to reach, recognize and fertilize the oocytes.

Maturational changes of spermatozoa have been described in different species including humans. Gradual modifications in the motility and morphology have been observed in spermatozoa collected from the different regions of the epididymis (Bedford, 1963; Briz et al., 1996; Axner et al., 1999; Robaire et al., 2006; Tajik et al., 2007; Cooper, 2011; Contri et al.,2012).

Previous studies proposed a further role of the epididymis in the recognition and removal of abnormal spermatozoa (Sutovsky et al., 2001; Axner, 2006; Robaire et al., 2006). In addition, some authors hypothesized that the epididymis might be a site where sperm abnormalities originate (Hirao and Kubota, 1980; Bonet et al., 1992; Briz et al., 1996; Axner et al., 1999).

In dogs, little is known on the post-testicular modifications of spermatozoa during the passage in the epididymis. Examination of canine spermatozoa obtained from different regions of the epididymis has been done only in one study (Hori et al., 2004). In that study, the organ was divided only in

two portions (*caput/corpus* and *cauda*) and the samples collected from the *caput* and the *corpus* were not differentiated. Furthermore, a detailed description of site and type of morphological abnormalities and of acrosomal patterns was not reported.

Additional information on the morphological and acrosomal changes of epididymal spermatozoa would contribute to clarify some aspects of the maturational process and of the potential above mentioned roles of the epididymis.

1.2 Retrieval techniques of epididymal spermatozoa

1.2.1 Veterinary medicine

In veterinary medicine the retrieval of epididymal spermatozoa is usually performed on isolated organs and represents the last option to collect male gametes when ejaculate semen cannot be obtained by other techniques (e.g.: digital manipulation, electroejaculation, etc..).

Isolated epididymides, dissected from the testis and after removal of the blood vessels, can be processed by mincing the epididymal tissue, squeezing the *vasa deferentia*, or flushing the epididymis *cauda*.

In the first technique the *cauda* of epididymis is isolated and minced by scissors or scalpel blade in a Petri dish containing a specific medium (Axner et al., 1999; Hori et al., 2004). A period of incubation is advisable to improve spermatozoa release from the tissue.

Since mincing allows processing the whole epididymis *cauda* a high sperm concentration can be obtained, but a high grade of contamination with blood cells and tissue fragments can be present.

The squeezing of the *vas deferens* with forceps from the cranial to the caudal part results in the collection of a sample. Although the sample might be less contaminated, the volume and the concentration are usually lower than those obtained by mincing.

The third procedure consists in the collection of spermatozoa by retrograde flushing of the *cauda* epididymis throughout a needle inserted into the *vas deferens* (Ponlgowhapan et al., 2006) The applicability of this technique is conditioned by the size of the testis, but the samples obtained are not contaminated by other tissues.

1.2.2 *Human medicine*

In humans, when the aetiology of infertility is obstructive azoospermia or when a previous surgical vasectomy was performed (Collins et al., 1996; Glina et al., 2003), epididymal spermatozoa are collected from *in situ* epididymides.

The advent of intracytoplasmic sperm injection (ICSI), which involves the use of a single spermatozoon directly injected into the oocyte, has further increased the interest toward collection techniques progressively less invasive for the patients microsurgical epididymal sperm aspiration (MESA), open fine needle aspiration (OFNA) and percutaneous epididymal sperm aspiration (PESA).

Microsurgical retrieval (MESA) involves the scrotal incision and the exteriorization of the testis. Then, the incision of the epididymal tunica and the dissection and opening of epididymal tubules are performed under an operating microscope to allow the aspiration of spermatic fluid. A microsuture of the ductule is required at the end of the procedure. Due to the direct visualization of the ductule, a blood-free sample is obtained. However, MESA is an open surgical procedure that needs an operating microscope and a trained andrological microsurgeon (Shah, 2011).

The OFNA is similar to the previous technique because involves the scrotal incision and the exteriorization of the testis, but the tunica is not

dissected. Different ductules can be directly aspirated with a 26-G needle through the tunica and this procedure is easy and quick and it does not need special equipment or training. However, it is an open surgical procedure as MESA (Shah, 2011).

The PESA consists in the needle aspiration of epididymal spermatozoa without scrotal incision. Epididymis is not directly visualized and the site of aspiration is guided by palpation

The procedure is simple, quick, avoids open surgery and it is a less-invasive technique with a lower rate of postoperative morbidity compared to MESA (Saade et al. 2008; Shah, 2011).

The disadvantages, due to the lack of direct visualization of epididymis, include the missing of sperm-containing ductules and the possibility of puncturing blood vessels with the contamination of the sample (Shah, 2011).

1.3 Cryopreservation of canine epididymal spermatozoa

Cryopreservation of spermatozoa is an important tool for preserving genetic material and for maintaining genetic diversity. The use of epididymal spermatozoa is strictly related to their cryostorage. When a valuable animal dies unexpectedly or is gonadectomized, the opportunity to use the semen sample at

the time of collection is very rare and the postponing of its use at a proper time can only be obtained with cryopreservation.

As previously mentioned, in dogs artificial insemination with frozen epididymal spermatozoa resulted in the birth of offspring, but the conception rates were low (Marks et al., 1994; Hori et al., 2004; 2011). One of the reasons is the negative impact of freezing on sperm quality. The effects of cryopreservation on motility, membrane and acrosomal integrity of canine epididymal spermatozoa have been previously investigated (Hewitt et al., 2001; Ponglowhapan et al., 2006, Hori et al., 2009; Farstad, 2012; Martins et al., 2012), but no information are available on its potential effect on DNA integrity.

Sperm DNA integrity has been evaluated in fresh ejaculated (Nunez-Martinez et al., 2005; Hidalgo et al., 2009; Lange Consiglio et al., 2010) and epididymal canine semen (Garcia-Masias et al., 2006). Only few reports have compared fresh and post-thaw chromatin integrity of canine ejaculated spermatozoa obtaining variable results (Rota et al., 2005; Koderle et al., 2009; Kim et al., 2010; Prinosilova et al., 2012).

The integrity of the paternal DNA is of crucial importance for the embryo development (Andrabi, 2007) 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 (DFI) results in a significant decrease in pregnancy rates (Virro et al., 2004; Silva and Gardella, 2006).

Nevertheless, there is no agreement neither on whether cryopreservation induces DNA fragmentation, nor on the mechanism which actually induces this damage (Di Santo et al., 2012). 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 (Koderle et al., 2009; Kim et al., 2010; Di Santo et al., 2012).

The role of antioxidant supplementations in protecting the sperm DNA from oxidative damage is still under investigation. Among antioxidants, it has been shown that melatonin (1mM), that directly neutralizes a high number of free radicals, has an effective action in protecting ram spermatozoa from the freezing injuries as evidenced by post-thaw DNA integrity, viability, motility, and fertilizing ability (Succu et al., 2011).

CHAPTER 2

Objectives

2. Objectives

To describe the characteristics of spermatozoa retrieved from the different regions of canine epididymis. For this purpose, motility, morphology and acrosomal patterns of spermatozoa obtained from epididymis *caput*, *corpus* and *cauda* were compared **(Paper I)**.

To investigate the feasibility of PESA in dogs and whether it might provide a population of epididymal spermatozoa similar to the population that can be obtained by processing isolated epididymis *caudae*. For this purpose, concentration, motility, morphology and acrosomal integrity of spermatozoa retrieved by PESA, *in vitro* aspiration and mincing of the *cauda* of the epididymis were compared **(Paper II).**

To evaluate the preservation of DNA integrity in canine epididymal frozen spermatozoa and the potential protective effect of the antioxidant melatonin on post-thaw sperm quality (motility, morphology, acrosomal and DNA integrity) **(Paper III).**

CHAPTER 3

Materials and methods

3. Materials and methods

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

3.1 Animals and experimental designs

Epididymal spermatozoa were collected from a total of 43 private owned stud dogs presented to the Department of Health, Animal Science and Food Safety of this University for routine orchiectomy. All dogs were pubertal and considered healthy at the clinical examination.

In the first study (**Paper I**) the epididymides were collected from 13 dogs, aged between 1 and 2.5 years (8-33 kg body weight). Spermatozoa from the tree epididymal portions (*caput*, *corpus* and *cauda*) were collected by mincing and evaluated for motility, morphology and acrosomal integrity.

Twenty dogs were used in the second study (**Paper II)**. The age and body weight ranged between 1 to 7 years and 9.5 to 52 kg, respectively. Twelve dogs were included in the Experiment 1 where PESA of the right epididymis *cauda* and *in vitro* aspiration of the left isolated epididymis *cauda* were performed.
In Experiment 2, PESA of the right epididymis *cauda* and mincing of the left isolated *cauda* of the epididymis were carried out in eight dogs. All epididymal

samples were examined for concentration, motility, morphology and acrosomal integrity.

In the third study (**Paper III**) ten dogs aged between1 and 10 years (6-30 kg body weight) were included.

Samples obtained by mincing of the epididymal *cauda* were divided into three aliquots, one was used as fresh control and the others were frozen with or without 1mM melatonin (+M/-M) in the freezing extender. Fresh and thawed spermatozoa were evaluated for motility, morphology, acrosomal and DNA integrity.

3.2 Retrieval of epididymal spermatozoa

3.2.1 In vitro mincing (Paper I, II and III).

The epididymis was dissected from the testis. The blood vessels on the surface of epididymis were removed and the *cauda* (plus *caput* and *corpus* in paper **II**) were isolated and placed in a Petri dish containing 4 ml of Ham's F-10 (HF10) medium supplemented with 2 mmol glutamine, 100 IU/ml Na-benzyl penicillin, 0.1 mg/ml streptomycin sulphate, and 5% Fetal Bovine Serum (mOsm 285). The organ was minced with a scalpel blade and after 30 min of incubation at 37°C, 1 ml **(Paper I-II)** or 4 ml **(Paper III)** of the suspension were collected and processed for spermatozoa evaluation.

3.2.2 PESA (Percutaneous Epididymal Sperm Aspiration) (Paper II).

In all cases PESA was performed under general anesthesia and analgesia before orchiectomy. The procedure was performed according to Shah (2011) with some modifications.

Briefly, the scrotum was cleaned with antiseptic solution and the *cauda* of the epididymis was palpated and stabilized between thumb and forefinger. A 26-G needle connected to a 2.5 ml syringe containing 0.2 ml of HF10 was inserted through the scrotal skin into the epididymis *cauda*. Aspiration was performed and a negative pressure was maintained while the tip of the needle was partially withdrawn and gently moved in and out within the epididymis in different directions for 360°. Collected samples were transferred into a tube and then processed for spermatozoa evaluation.

3.2.3 In vitro aspiration of the cauda of the epididymis (Paper II).

Fifteen minutes after surgical excision of the testis the same procedure of PESA was adopted, with the only difference that epididymal spermatozoa were retrieved by aspiration of the *cauda* of the epididymis under direct visualization of the isolated organ.

3.3 Freezing procedure

In the third study (**Paper III**) after centrifugation (700 x g for 5 min) and removal of the surnatant, one aliquot (-M) was diluted (200 x 10^6spz/ml) with the following freezing extender: TRIS buffer with 5% glycerol, 1% Equex and 20% egg yolk and the other with the same extender supplemented with 1mM melatonin (+M). Both aliquots were frozen according to the Uppsala system described by Linde-Forsberg (2002).

Briefly, this method consists of two extension steps before freezing in 0.5 ml straws. Straws were submerged in liquid nitrogen vapors in a Styrofoam box (10 min at −120°C) and subsequently immersed into liquid nitrogen. The straws were thawed in a water bath at 37°C for 30 sec

3.4 Spermatozoa evaluation

3.4.1 Sperm concentration, motility and morphology (Paper I, II and III).

Sperm concentration was determined with a Bürker chamber, motility was subjectively assessed with a light microscope with a heated stage at 38°C and 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) (Mortimer, 1994).

Morphology of spermatozoa was assessed following staining with Bengal Rose and Victoria Blue B on at least 100 spermatozoa. Normal spermatozoa and site of defects in abnormal spermatozoa (head, neck/midpiece, tail) 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.

3.4.2 Acrosome integrity (Paper I, II and III).

Acrosome integrity was evaluated by staining spermatozoa with Peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI).

Evaluation of the acrosome patterns by FITC-PNA/PI was performed according to the procedure described by Cheng and others (1996) for stallion spermatozoa. Staining solution was prepared with 90 μl of FITC-PNA (40 μg/ml in PBS) added with 10 μl of PI (340 μM in PBS).

An amount of 10 μl of sperm suspension was smeared on a microscope slide and fixed in ethanol 96% for 30 seconds. Slide was dried in the dark, and then a droplet of 20 μl of FITC-PNA/PI was added to the slide. Slide was

incubated in a moist chamber at +4°C and after 30 min it was rinsed with +4°C distilled water and air dried at +4°C in the dark over-night. At least 100 spermatozoa were evaluated under fluorescent microscope (Axiovert 100, Zeiss, Germany).

The observed fluorescence images of ethanol-permeabilized spermatozoa, stained with FITC-PNA/PI, were classified into three patterns: 1) spermatozoa displaying intensively bright fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane (intact acrosome); 2) spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane or swollen acrosomal cap (vesiculated or abnormal acrosome); 3) 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 (acrosome residues or loss) (Cheng et al., 1996).

3.4.3 Sperm DNA fragmentation (Paper III).

The sperm DNA fragmentation was assessed using the Sperm-Halomax® commercial kit specifically developed for canine semen (Halotech DNA SL, Madrid, Spain) and based on the sperm chromatin dispersion (SCD) test.

Sperm DNA fragmentation was evaluated in fresh and frozen samples (+M/-M) at the concentration of 50 x 10^6 spz/ml and processed following the manufacturer's instructions. Briefly, twenty-five microliters of diluted samples were added to a vial with fifty microliters of low melting agarose and mixed. Provided pre-treated slides were placed onto a metallic plate which has been previously cooled at 4°C. A drop of the cell suspension (2 □ l) was spread onto the treated face of the cooled slide, covered with a glass coverslip and maintained at 4°C for 5 min. The coverslip was smoothly removed, and the layered sample was covered with the lysing solution provided in the kit. Finally, slides were washed for 5 min, dehydrated in sequential 70 and 100% ethanol baths and stained for 35 min in 1:1 Wright solution (Merck, Whitehouse Station, NJ, USA) and phosphate buffer (pH 6.88, Merck). When the slide was perfectly dried, it was mounted with Eukitt® and observed under bright-field microscopy (40x).

A minimum of five hundred spermatozoa was evaluated in each sample. Unfragmented sperm showed a small and compact halo, intensely coloured, around the spermatozoa head. Spermatozoa with fragmented DNA presented a widespread and soft halo of chromatin dispersion. Proportions of spermatozoa showing a halo of dispersion were considered positive for high DNA fragmentation index (DFI) as previously suggested (De Ambrogi et al., 2006).

3.4 Statistical analysis

In **Paper I** data are resumed as mean ± standard deviation. Mean concentration, motility, normal morphology, type of abnormalities, acrosomal patterns were analyzed by a mixed linear model by a GLM procedure, taking into account the region as fixed factor and the subject as random factor, in order to reduce the error variability due to the animal. The overall morphological abnormalities on spermatozoa site (head, neck/midpiece, tail) and the overall immature spermatozoa were analyzed by one-way ANOVA, followed by the Tukey-Kramer test for multiple comparisons.

In **Paper II** the data were not normally distributed and are summarized as median and range. Differences between the characteristics of epididymal spermatozoa retrieved using different techniques were evaluated with a Wilcoxon Signed Rank test for paired samples.

In **Paper III** values are presented as mean ± standard deviation (SD). Significant differences ($p<0.05$) were determined by Student's t-test .

In all cases P-values <0.05 were considered to be significant. All statistical procedures were performed by the software SAS release 9.13 for Windows platform.

CHAPTER 4

Results

4. Results

Paper I

Sperm concentration (spz. x 10^6/ml) was significantly higher (P=0.002) in the samples collected from the *cauda* (138.1 ± 161.5) compared to those collected from the *caput* (11.4 ± 16.7), while no differences were observed among *corpus* (61.4 ± 43.7) and the other regions.

Sperm motility (%) after dilution increased progressively in samples collected from *caput* to *cauda*. Proportions of motile cells were significantly higher in the distal region (53.1 ± 25.9) compared to *corpus* (16.2 ± 11.6; P<0.0001) and *caput* (1.3 ± 2.1; P<0.0001). In the *caput* most of the cells that did not progress showed a flagellating tail.

Proportions of spermatozoa (%) with normal morphology were significantly higher in *corpus* (39.0 ± 13.8; P=0.02) and *cauda* (50.5 ± 13.3; P<0.0001) compared to *caput* (24.7 ± 11.9).

Overall morphological abnormalities (%) of the head and neck/midpiece were similar in the three different epididymal regions *caput*, *corpus* and *cauda* respectively (head: 2.5 ± 6.8 vs. 3.5 ± 9.5 vs. 3.8 ± 12.7; neck/midpiece: 7.1 ± 5.0 vs. 6.3 ± 4.0 vs. 8.5 ± 4.7).

A significant prevalence of tail defects, mainly represented by single bent tails, was observed in the *corpus* (40.1 ± 11.1) compared to the *caput* (26.2 ± 12.5; P<0.0001) and to the *cauda* (29.0 ± 13.6; P=0.006).

Immature sperms with cytoplasmic droplets decreased from the proximal to the distal region of the epididymis. Particularly, proximal cytoplasmic droplets were more frequently found in spermatozoa collected from the epididymis *caput* (44.8 ± 19.5) than in the *corpus* (8.9 ±6.8; P<0.0001) and in the *cauda* (3.6 ± 3.9; P<0.0001), whereas the occurrence of distal cytoplasmic droplets was higher in the *corpus* (20.0 ± 10.4) than in the *caput* (4.7 ± 6.8; P=0.0003) and in the *cauda* (11.4 ± 9.0; P<0.05).

Significantly higher proportions (%) of spermatozoa with intact acrosomes were retrieved from the epididymis *cauda* (49.5 ± 19.9) than from the *caput* (35.4 ± 22.4; P=0.03) and the *corpus* (31.6 ± 17.4; P=0.008).

Paper II

In the present study PESA was performed in 20 epididymis *caudae* and semen samples were always collected. In the Experiment 1 the median values of the sperm concentration and total number of spermatozoa in the samples collected by PESA were similar to that of samples obtained with *in vitro* aspiration of the contralateral epididymis *cauda* (concentration: 111.2 vs. 93 spz. x

10^6/ml; total number: 34.6 vs. 25.8 spz. x 10^6). In the Experiment 2, *in vitro* mincing provided a higher concentration and total number of spermatozoa compared to PESA method (concentration: 104.2 vs. 27.8 spz. x 10^6/ml; $P<0.05$. Total number: 104.2 vs. 6.0 spz. x 10^6/ml; $P<0.05$). Highly variable sperm concentrations among animals were observed within the same collection method.

Sperm motility (%) and morphology (%) did not differ in samples retrieved by the different methods, although individual variability was observed (Motility: (Exp 1) 52.5 vs. 50; (Exp 2) 40 vs. 65; $P<0.05$. Normal morphology: (Exp 1) 66.3 vs. 72.2; (Exp 2) 54.3 vs. 48.9; $P<0.05$).

In the samples retrieved with PESA, proportions (%) of spermatozoa with intact acrosomes were slightly lower to those retrieved by *in vitro* aspiration, but higher compared to those obtained by mincing of the epididymis *caudae* (Experiment 1: 68.5 vs. 76; $P<0.05$. Experiment 2: 72.3 vs. 54.5; $P<0.05$).

Paper III

Spermatozoa concentration averaged 252.7±161.8 spz. x 10^6/ml.

No effect of the cryopreservation procedure was observed on DNA status of canine epididymal spermatozoa as demonstrated by a similar DNA

fragmentation index (%) in fresh and frozen samples (Fresh 3.3 ± 3.6 vs. +M 4.2 ± 3.8 vs. -M 3.6 ± 3.7).

Motility (%) was significantly higher in fresh compared to frozen spermatozoa. Motility (%) was significantly higher ($P<0.0005$) in fresh (74.5 ± 9.6) compared to frozen spermatozoa and the presence of melatonin in the freezing extender did not enhance the result (+M 36 ± 9.7 and -M 37.5 ± 15.3).

Proportions (%) of spermatozoa with normal morphology were similar in fresh (47.9 ± 24.9) and frozen samples irrespective of the presence of melatonin in the extender (+M 52.7 ±12.9 and -M 49.9 ± 17.3).

Acrosome integrity was significantly affected by cryopreservation. Proportions (%) of spermatozoa showing intact acrosome were significantly higher in fresh samples (66.6 ± 25.5) compared to frozen samples with melatonin (36.3 ± 15; $P=0.005$) and without (37.2 ± 11.6; $P=0.004$).

CHAPTER 5

General discussion

5. General discussion

The collection and preservation of epididymal spermatozoa is an option for the conservation of germplasm of genetically superior animals that need to be orchiectomized or have died. The collection from *in situ* epididymides allows the retrieval of gametes from males that cannot mate or ejaculated semen for reproductive or neurological disorders.

An in-depth study of epididymal spermatozoa characteristics, methods of collection and possibilities of storage might contribute to extend their use in ART.

The **Paper I** described the characteristics of canine spermatozoa collected from the epididymal *caput*, *corpus* and *cauda* with the aim of highlighting the functional and structural changes of spermatozoa occurring during the transit.
It is well known that some modifications of mammalian spermatozoa occurring into the epididymis are related to the maturational process that involves functional and structural changes of the gametes.

Among functional changes, the capacity for sperm motility is gradually acquired from *caput* to *cauda* with a quantitative and qualitative modification of its patterns from only a faint twitch of the flagellum to a progressive and vigorous forward movement (Amann et al., 1993; Robaire et al., 2006).

Canine spermatozoa collected from the *cauda* of the epididymis (**Paper I)** showed a higher motility compared to those retrieved from *caput* and *corpus* in addition to a high concentration due to the storage role of this area. Spermatozoa in the *caput* often displayed a flagellating movement of the tail instead of being immotile, as also observed in cats (Axner et al., 1999).

Concomitant with these functional changes, spermatozoa undergo structural modifications during epididymal transit such as migration of the cytoplasmic droplet and acrosomal reshaping in order to achieve the normal morphology of mature spermatozoa (Robaire et al., 2006).

Cytoplasmic droplets develop during normal spermatogenesis and represent a residue of the cytoplasm after Sertoli cells have phagocytized most spermatic cytoplasm (Cooper, 2011). The migration from the proximal to the distal end of the midpiece takes place in a specific region of the epididymis, which slightly varies among species. In cats, the migration occurs in the terminal part of the *corpus* (Axner et al., 1999), whereas in donkeys from the first to the second half of the *corpus* (Contri et al., 2012). In other species as rabbit, bull and boar, this change occurs earlier either in the *caput* or in the passage from *caput* to *corpus* (Briz et al., 1995; Perez-sanchez et al.,1997; Tajik et al., 2007).

The results of **Paper I** showed that the highest proportion of spermatozoa with proximal cytoplasmic droplets was in the *caput*. In the *corpus* their abrupt decrease concomitant with the significant increase in the number of

the spermatozoa showing the distal droplet, suggests that this region is the site of migration in this species.

Concerning acrosomal reshaping, it has been demonstrated in the rabbit that the acrosome dimensions of spermatozoa collected from the *caput* are greater than those of spermatozoa from the *cauda*. During epididymal passage these swollen acrosomes contract and localize adjacent to the nuclear surface of the sperm head (Bedford, 1963). In the present work the occurrence of abnormal acrosomes, mainly represented by swollen acrosomes, was higher in the *caput* and in the *corpus* compared to the *cauda* where a higher proportion of spermatozoa had normal acrosomes (**Paper I)**.

This gradual change toward a normal shape of the acrosome might be due to the reshaping during maturation from the proximal to the distal epididymal region as observed in the rabbit (Bedford, 1963).

However, Axner and co-workers (1999) suggested that the decrease of feline spermatozoa with abnormal acrosomes in the *cauda* might be also due to the epididymal recognition of these spermatozoa as abnormal. Besides the well-known function of the epididymis in sperm maturation, an additional role in "sperm quality control" through the removal of abnormal spermatozoa by different mechanisms (i.e. phagocytosis, dissolution by ubiquitination and degradation by other proteins) has been proposed (Sutovsky et al., 2001; Axner,

2006). However, the elimination of abnormal spermatozoa in the reproductive tract is still controversial (Robaire et al., 2006).

In the present study, the proportion of canine spermatozoa with normal morphology increased significantly from *caput* to *cauda*. This increase was mainly due to the reduction of immature spermatozoa. It remains to be elucidated whether the decrease of immature spermatozoa is due to the effective maturation or to other mechanisms of removal of abnormal gametes. Frequency of anomalies of the head, neck /midpiece and tail were not different between *caput* and *cauda*.

In the cat, a significant decrease of the spermatozoa with abnormalities of testicular origin (i.e. head defects) has been described from the efferent ducts to the *cauda* (Axner et al., 1999). A decrease frequency of anomalies of the midpiece, including cytoplasmic droplets, was observed among testicular and epididymal spermatozoa in the rabbit, whereas the comparison between epididymal *caput* and *cauda* failed to detect significant decrease in the frequencies of all the defects (Perez-sanchez et al., 1997), as observed in this study.

On the other hand the epididymis has been considered a site where some peculiar sperm anomalies develop (Hirao and Kubota, 1980; Bonet et al., 1992; Briz et al., 1996; Axner et al., 1999).

A significant increase in sperm tail abnormalities between the proximal to the distal regions of the epididymis was reported in the cat (Axner et al., 1999).

In the boar, some types of sperm malformations of the tail were observed more frequently in the *cauda*, whereas other sperm defects were more uniformly distributed along the epididymis (Briz et al., 1996).

In the present work, although the proportions of abnormal canine spermatozoa between *caput* and *cauda* were not different, the frequency of appearance of the single bent tail in the *corpus* was significantly higher than in the other epididymal tracts, and it was often associated with the distal droplet. In some cases, the distal droplet was localized along the flagellum, rather than at the distal end of the midpiece, and the tail was bent on the droplet. A possible explanation of this association is the premature release of hydrolytic enzymes by the droplet. This might produce the digestion and disorganization of structural components of the tail with consequent weakness of the structure and folding of the flagellum, as hypothesized in boar spermatozoa (Briz et al., 1996).

The single bent tail may indeed be considered as an abnormality originating in the epididymis, but as it correlates with the presence of the droplet, it is also linked to the maturational process.

Epididymal spermatozoa represent an important source of germplasm. It would be interesting to evaluate whether the immaturity traits of spermatozoa (i.e. cytoplasmic droplet or swollen acrosome) recovered from different tracts negatively influence the fertilization.

It has been reported that ejaculated spermatozoa with proximal droplet have poor adherence to the zona pellucida in different mammalian species (Cooper, 2011) including dogs (Peña et al., 2007). However, the presence of the proximal droplet in the ejaculated spermatozoa is a sign of a defective sperm maturation that could be responsible for other biochemical alterations interfering with the normal progress of capacitation (Peña et al., 2007), whereas in the epididymis the presence of the droplet represents a physiological condition of the gametes. For this reason, an in-depth study of fertilizing ability of epididymal spermatozoa retrieved from the entire organ would contribute to extend their use in assisted reproductive techniques.

The results of the **Paper II** indicate that PESA is a feasible procedure to retrieve a population of spermatozoa in dogs. The quality is similar to that of spermatozoa collected *in vitro*, although a wide variation among animals was observed.

Hori et al., (2004) demonstrated that epididymal semen quality, although variable amongst animals, is almost the same in the bilateral epididymides of the same animal. According to this finding, a comparison between different collection techniques performed either on the right (PESA) or on the left (*in vitro*

aspiration or mincing) *cauda* of the epididymis of the same subject supports PESA efficiency.

A similar concentration and total number of spermatozoa retrieved by PESA and *in vitro* aspiration demonstrates that the blind aspiration of the *cauda* of the epididymis in PESA ensures a retrieval of gametes comparable to that of the *in vitro* aspiration that was performed under direct visualization.

On the other hand, sperm concentration obtained by mincing was higher than that obtained by PESA. Mincing results in the release of cells in a Petri dish after processing the whole epididymis *cauda*, therefore a high concentration was expected (104.2 sp. x 10^6/ml) and consistent with that previously reported (100.8 ± 115.8 x 10^6/ml; range 4.6-400 x 10^6/ml; Hewitt et al., 2001).

Furthermore, because of the different volumes of suspension obtained with PESA (about 0.2 ml) and *in vitro* mincing (1 ml) a higher total number of spermatozoa was expected in the latter method.

However, within the same collection method, a wide variability in the sperm concentrations and in the total number of spermatozoa was observed amongst animals.

Several factors of individual variability might affect the number of retrieved spermatozoa. A strong association between the degree of epididymal distension and the success of PESA has been documented, as spermatozoa are largely unobtainable form non-distended tubules (Collins et al., 1996).

Furthermore, the number of spermatozoa in extragonadal site of storage is related to the period of sexual rest, unknown in these dogs, and to testicular size and body weight (Johnston et al., 2001). The latter relation could not be assessed in this study due to the limited number of dogs with different bodyweights.

Median values of sperm motility were similar in samples collected by PESA and by *in vitro* techniques. However, the wide range of results further confirms the variability in animals previously reported, where the mean values of motility of fresh epididymal semen varied between 50 to 89.4% (Marks et al., 1994; Yu and Leibo, 2002; Hishinuma and Sekine , 2004; Hori et al., 2004; 2005; 2009; Klinc et al., 2005; Ponglowhapan et al., 2006).

In the samples collected with the different methods, no differences were observed in the proportions of morphologically normal spermatozoa (median values from 49% to 72%) and of abnormal spermatozoa with head, neck/midpiece or tail defects. Similar sperm morphology was expected because samples had the same origin (epididymis *cauda*), but it excludes that the negative pressure of aspiration (PESA or *in vitro*) might have exerted a damaging effect on the cells.

Aspiration techniques (PESA and *in vitro*) better preserve acrosomal integrity compared to mincing. There are no reliable data to explain the increased presence of acrosomal defects in the mincing procedure. It can be hypothesized

that in these experimental conditions, the autolysis of tissue fragments and blood cells, during incubation of minced tissue for the release of spermatozoa into the medium, might have affected the environmental/osmotic conditions to which spermatozoa were exposed with consequent membrane deterioration. In a previous study no significant effect of blood and tissue fragments on semen quality (motility, viability and morphology) was observed, but the status of the membranes was not evaluated (Hori et al., 2004).

As mentioned before, artificial insemination with canine epididymal spermatozoa resulted in pregnancy and delivery of puppies. In most of these studies intrauterine insemination of frozen spermatozoa was adopted (Hori et al., 2004, 2005, 2011). Total number of spermatozoa used for insemination ranged between 2-3 x 10^8 and the motility after thawing was between 20% and 40%.

Thus, pregnancies were obtained with the insemination of a total number of motile spermatozoa of approximately 50-60 x 10^6. In this study, PESA allows the retrieval of a similar total number of motile spermatozoa in some individuals, but only one epididymis was aspirated as the contralateral was used for *in vitro* retrieval.. For potential application in ART, PESA could be accomplished in both epididymis *caudae* to obtain a sufficient number of gametes.

In humans epididymal spermatozoa are used for ICSI; hence the aim of PESA is the retrieval of only few good quality spermatozoa and if the first retrieval is unsuccessful, the aspiration can be repeated either on the same or the

other epididymis until ad adequate specimen of spermatozoa is obtained (Glina et al., 2003; Esteves, 2011). From a technical point of view, PESA was a quick technique, easy to perform and no bleeding was noticed during or immediately after the procedure. The epididymis was well identified by palpation and the *cauda* was easily localized.

Epididymal aspiration has been used in dogs as a diagnostic tool to evaluate azoospermia and only rarely required sedation. However, the need of local anaesthesia, routinely used in humans, has not been investigated. In this study, discomfort or pain were not evaluated because PESA was performed in dogs under general anaesthesia and intravenous analgesia before orchiectomy.

Moreover, due to the removal of testes, side effects could not be assessed.

Previous reports on humans have established that PESA is a safe technique; the trauma to the epididymis is minimal (Rosenlund et al., 1998) as revealed by the absence of a developing haematoma or focal lesions at serial ultrasonographic examinations at variable time intervals after PESA (Ron-El et al., 1998). However, the histological examination of the rat epididymides evidenced inflammatory alterations after repeated PESA (Saade et al*.,* 2008).

Among consequences of epididymal aspiration, there is also the potential loss of the integrity of blood-testis barrier with the resulting formation of anti-sperm antibodies. Attia and co-workers (2000) found that one of three dogs that underwent epididymal aspiration developed a transient formation of anti-sperm

IgG on spermatozoa with no negative effect on total spermatozoa output or motility. Although this study concluded that epididymal aspiration is a safe procedure, side effects of PESA require further investigations.

The results of **Paper III** showed that the freezing procedure did not affect DNA integrity of canine epididymal spermatozoa. The presence of melatonin did not preserve motility and acrosome integrity that were severely affected by cryopreservation. Since sperm morphology and DNA integrity were not compromised by the freezing procedure, the potential protective effect of melatonin on their preservation could not have been proved.

The impact of cryopreservation on sperm DNA integrity is still a controversial matter in mammals including dogs. Some authors assessed the chromatin status of canine ejaculated spermatozoa and showed that the freezing/thawing procedure does not produce significant adverse effect on sperm DNA (Rota et al., 2005; Prinosilova et al., 2012). Conversely, Kim and co-workers (2010) found a higher level of DNA fragmentation in thawed compared to fresh spermatozoa.

Notably, in the present study, DNA fragmentation has been evaluated with the commercial kit Halomax® based on the sperm chromatin dispersion (SCD) test, and previously used for ejaculated canine semen (Hidalgo et al.,

2009), whereas other authors used different techniques as the Sperm Chromatin Structure Assay (SCSA) or the Acridine orange assay. However, it has been demonstrated in human spermatozoa that the results obtained with SCD, that is a simple, highly reproducible and inexpensive technique, are highly correlated to those obtained with SCSA (Fernandez et al., 2003).

Present results indicated that the DNA fragmentation index was similar in fresh and frozen canine epididymal spermatozoa and this finding confirms the resilience of canine sperm DNA to cold stress.

The sperm DNA stability after freeze/thawing can be a species-specific characteristic as recently suggested by Gosalvez and co-authors (2011). In a comparative study of sperm DNA fragmentation rate in eleven different mammalian species, a correlation between the structure of basic proteins, protamines 1 (P1) and 2 (P2), in the sperm head and the DNA status after thawing has been found. Particularly, the spermatozoa of those species lacking P2 resisted fragmentation more effectively during freeze/thawing than those that contained both P1 and P2. Unfortunately, the dog was not included in the aforementioned study, but the lack of P2 in the canine spermatozoa (Lee and Cho, 1999) might explain the resilience of DNA to the cold damages.

Another factor that may preserve DNA stability during cryopreservation is the presence of seminal plasma in the sample. It has been demonstrated that

post-thaw DNA integrity was improved when human spermatozoa were frozen with seminal plasma (Di Santo et al., 2012).

In dogs, the removal of plasma from the second fraction of the ejaculate before cryopreservation is not recommended, because the samples frozen with prostatic fluid showed a higher DNA stability (Koderle et al., 2009).

The beneficial effect of seminal plasma on DNA could be related to the presence of antioxidants (Koderle et al., 2009).

As previously mentioned, one possible mechanism which induces DNA fragmentation in cryopreserved spermatozoa is the oxidative damage due to the imbalance between the concentrations of ROS and antioxidant compounds (Koderle et al., 2009; Kim et al., 2010; Di Santo et al., 2012).

Epididymal semen that does not benefit of the antioxidant effect of seminal plasma, might be more vulnerable to the oxidative stress occurring during cryopreservation.

In this study the supplementation with melatonin aimed at compensating for the lack of antioxidants was not able to demonstrate a protective effect on sperm DNA as cryopreservation did not damage it.

Sperm morphology was also not affected by cryopreservation. Kim and co-workers (2010) observed an increased DNA fragmentation index and a higher proportion of head abnormalities in thawed compared to fresh spermatozoa. It would have been interesting to evaluate the possible correlation between head

anomalies and DNA fragmentation because spermatozoon head consists almost entirely of DNA and a correlation among the head shape and the chromatin status has been previously demonstrated in dogs (Nunez-Martinez et al., 2005; Lange Consiglio et al., 2010).

A potential protective effect of melatonin has also been evaluated on other sperm parameters. The results showed that sperm motility and acrosomal integrity were significantly affected by cryopreservation, but no effect of melatonin has been observed.

The presence of 1 mM melatonin in the freezing extender of ram ejaculated semen, other than preserve sperm DNA integrity, had a protective effect on post-thaw motility (Succu et al., 2011). The incubation of thawed epididymal spermatozoa of red deer with the same concentration of melatonin showed instead only a limited protection in terms of different sperm parameters including motility and acrosomal integrity (Dominguez-Rebolledo et al., 2010).

It remains to be elucidated whether the lack of ameliorative effect of melatonin in canine cryopreserved semen was due to an inappropriate melatonin concentration or to an iatrogenic non-oxidative damage.

CHAPTER 6

Conclusions

6. Conclusions

Canine spermatozoa undergo several modifications in the epididymal environment. The acquisition of progressive motility, migration of cytoplasmic droplet and acrosomal reshaping lead to mature spermatozoa which are then stored in the epididymal cauda. From this site, spermatozoa can be retrieved and used in assisted reproductive techniques as a valuable tool for propagating genetic traits of high value individuals that dies accidentally or undergoes orchiectomy for medical purposes. Further investigations should be also focused on the potential use of spermatozoa recovered from other epididymal regions. **(Paper I).**

PESA is a feasible alternative to *in vitro* collection methods for spermatozoa retrieval. It can be applied in dogs with compromised reproductive performances, in which orchiectomy cannot be performed for medical or owner reasons. Although an accurate evaluation of side effect is needed, PESA technique might ensure the possibility of repeated semen collections **(Paper II).**

The DNA integrity of canine epididymal spermatozoa is well preserved after cryopreservation and a protective effect of melatonin on post-thaw sperm quality has not been demonstrated.

The DNA stability after thawing is particularly relevant for epididymal spermatozoa which potential use in assisted reproductive techniques is mainly after storage. However, other sperm characteristics as motility and acrosomal integrity are compromised by freezing and further investigations should be focused on their preservation **(Paper III).**

CHAPTER 7

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CHAPTER 8

Summary

8. Summary

Epididymal spermatozoa represent the only source of genetic material when the male dies unexpectedly or undergoes orchiectomy for medical reasons. Yet, in individuals of high genetic or emotional value that cannot mate or ejaculate semen, the collection of spermatozoa from *in situ* epididymides might be an option to obtain progeny.

The aims of the studies presented here were to describe the characteristics of spermatozoa collected from the different regions of the epididymis (*caput, corpus*, and *cauda*) and to investigate the feasibility of percutaneous epididymal sperm aspiration (PESA) in dogs. Furthermore, the preservation of DNA integrity in canine epididymal frozen spermatozoa and the potential protective effect of the antioxidant melatonin on post-thaw sperm quality were evaluated.

Overall results showed that canine epididymal spermatozoa undergo several modifications in the epididymis, leading to mature cells which are stored in the epididymal cauda. From this site, spermatozoa can be retrieved by PESA and the quality of the sperm population is similar to that of spermatozoa collected *in vitro*, although a wide variation amongst animals was observed.

The cryopreservation of epididymal spermatozoa showed that the DNA integrity is well preserved, but a protective effect of melatonin on post-thaw sperm quality has not been demonstrated.

The DNA stability after thawing is particularly relevant for epididymal spermatozoa which potential use in assisted reproductive techniques is mainly after storage. However, other sperm characteristics as motility and acrosomal integrity are compromised by freezing and further investigations should be focused on their preservation.

CHAPTER 9

Paper I

Morphological and acrosomal changes of canine spermatozoa during epididymal transit.

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Morphological and acrosomal changes of canine spermatozoa during epididymal transit

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Abstract

Background

During epididymal transit, functional and structural modifications leading to full maturation enable male gametes to reach, recognize and fertilize the oocytes. In dogs, little is known on the modifications of spermatozoa during the passage in the epididymis. The aim of this study was to describe the motility, morphology and acrosomal patterns of canine spermatozoa retrieved from the epididymis caput, corpus and cauda.

Results

After the dilution required for the collection of epididymal content, sperm motility was significantly higher (P <0.0001) in the cauda compared to corpus and caput.

Proportions of spermatozoa with normal morphology were significantly higher in corpus (P =0.02) and cauda (P <0.0001) compared to caput. Overall morphological abnormalities of the head and neck/midpiece were similar in the three different epididymal regions. A significantly increased prevalence of tail defects, mainly represented by single bent tails, was observed in the corpus compared to caput (P <0.0001) and cauda (P =0.006).

Numbers of immature sperm with cytoplasmic droplets decreased from the proximal to the distal region of the epididymis. Particularly, proximal cytoplasmic droplets were more frequently found in spermatozoa collected from the caput epididymis than in the corpus (P

<0.0001) and in the cauda (P <0.0001), whereas the occurrence of distal cytoplasmic droplets was higher in the corpus than in the caput (P =0.0003) and in the cauda (P <0.05).

Significantly higher proportions of spermatozoa with intact acrosomes were retrieved from the cauda epididymis than from the caput (P =0.03) and the corpus (P =0.008). This difference was mainly due to a lower proportion of spermatozoa with abnormal acrosomes (mainly swollen acrosomes) rather than with absent acrosomes.

Conclusions

Canine spermatozoa undergo several modifications in the epididymis. The acquisition of progressive motility, migration of the cytoplasmic droplet and acrosomal reshaping lead to mature spermatozoa which are then stored in the cauda epididymis. From this site, spermatozoa can be retrieved and used in assisted reproductive techniques as a valuable tool for propagating genetic traits of high value individuals that dies accidentally or undergoes orchiectomy for medical purposes. Further investigations should be also focused on the potential use of spermatozoa recovered from other epididymal regions.

Keywords

Dog, Spermatozoa, Epididymis.

Background

In the mammalian epididymis, substantial changes of spermatozoa occur. During epididymal transit from caput to cauda, functional and structural modifications leading to full maturation enable male gametes to reach, recognize and fertilize the oocytes.

Maturational changes of spermatozoa have been described in different species including humans. Gradual modifications in motility and morphology have been observed in spermatozoa collected from different regions of the epididymis [1-7].

Previous studies proposed a further role of the epididymis in the recognition and removal of abnormal spermatozoa [4,8,9]. In addition, some authors hypothesized that the epididymis might be a site where sperm abnormalities develop [2,3,10,11].

In dogs, little is known on the post-testicular modifications of spermatozoa during the passage of the epididymis, whereas their fertilizing ability has been demonstrated by birth of offspring following artificial insemination with gametes retrieved from the cauda epididymis [12-16].

Examination of canine spermatozoa obtained from different regions of the epididymis has been done only in one study [13]. In that study, the organ was divided only in two portions (caput/corpus and cauda) and the samples collected from the caput and the corpus were not differentiated. Furthermore, a detailed description of site and type of morphological abnormalities and of acrosomal patterns were not reported.

Additional information on the morphological and acrosomal changes of epididymal spermatozoa would contribute to clarify some aspects of the maturational process and of the potential above mentioned roles of the epididymis.

The aim of this study was to describe the characteristics of spermatozoa retrieved from the different regions of the canine epididymis. For this purpose, motility, morphology and acrosomal patterns of spermatozoa obtained from caput, corpus and cauda epididymis were compared.

Methods

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Animals

Thirteen healthy and pubertal privately owned stud dogs, aged between 1 and 2.5 years (8 to 33 kg body weight), presented to the Department for routine orchiectomy were included in this study.

Epididymal spermatozoa retrieval

Thirteen pairs of canine gonads were transported to the laboratory within 10 min after surgical removal. The epididymis was dissected from each testis and pampiniform plexus using a scalpel blade. The small vessels were removed with scissors to reduce contamination by blood, and then each epididymis was macroscopically divided into three portions, caput, corpus and cauda, according to Schimming et al. [17,18].

Each portion was placed in a Petri dish containing 4 ml of Ham's F-10 medium supplemented with 2 mmol glutamine, 100 IU/ml Na-benzyl penicillin, 0.1 mg/ml streptomycin sulphate, and 5 % fetal bovine serum (mOsm 285). The different tracts were minced with a scalpel blade, and after 30 min of incubation at 37 °C, 1 ml of suspension was collected from each dish and processed for spermatozoa evaluation.

Spermatozoa evaluation

Sperm concentration was determined with a Bürker chamber. After the dilution required for the collection of epididymal content, motility was subjectively assessed by the same investigator with a light microscope (40x) with a heated stage at 38 °C. 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) [19].

Morphology of spermatozoa was assessed following staining of the smear with Bengal Rose and Victoria Blue B. A total of 100 spermatozoa was evaluated under light microscope with oil immersion objective at 100x magnification. Normal spermatozoa and site of defects in abnormal spermatozoa (head, neck/midpiece, tail) were recorded [20]. For each abnormal sperm all the anomalies of different sites were considered [21]. Abnormal sperm heads included those that were pear-shaped, large, small, or amorphous. Alterations of the neck/midpiece included bent neck, bent and thick midpiece; abnormal tail included single

bent and coiled tail. Immature sperm with proximal and distal cytoplasmic droplet were recorded separately.

The acrosome integrity was evaluated by staining spermatozoa with Peanut agglutinin (PNA) conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) according to the procedure described previously for stallion spermatozoa [22]. Staining solution was prepared with 90 µl of FITC-PNA (40 µg/ml in Phosphate Buffered Saline - PBS) added with 10 µl of PI (340 µM in PBS).

An amount of 10 µl of sperm suspension was smeared on a microscope slide and fixed in 96% ethanol for 30 seconds. The slide was dried in dark, and then a droplet of 20 µl of FITC-PNA/PI was added to the slide. The slide was incubated in a moist chamber at 4 °C and after 30 min it was rinsed with 4 °C distilled water and air dried at 4 °C in dark overnight. At least 100 spermatozoa were evaluated under fluorescent microscope (Axiovert 100, Zeiss, Germany). The intact acrosome was stained green, whereas the head of the sperm was stained red.

The observed fluorescence images of ethanol-permeabilized spermatozoa, stained with FITC-PNA/PI, were classified into three patterns: 1) spermatozoa displaying intensively bright fluorescence of the acrosomal cap as "intact acrosome"; 2) spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap or swollen acrosomal cap as "abnormal acrosome"; 3) spermatozoa displaying a fluorescent band at the equatorial segment or displaying no fluorescence as "absent acrosome".

Statistical analysis

Data were resumed as mean ± standard deviation. Mean concentration, motility, normal morphology, type of abnormalities, acrosomal patterns were analyzed by a mixed linear model by a GLM procedure, taking into account the region as fixed factor and the subject as random factor, in order to reduce the error variability due to the animal. The overall morphological abnormalities on spermatozoa site (head, neck/midpiece, tail) and the overall immature spermatozoa were analyzed by one-way ANOVA, followed by the Tukey-Kramer test for multiple comparisons. *P* -values <0.05 were considered to be significant. All statistical procedures were performed by the software SAS release 9.13 for Windows platform.

The variables motility, site of abnormalities (head, neck/midpiece, tail), cytoplasmic droplets (proximal and distal) and acrosomal patterns (abnormal and absent acrosome) were processed by principal component analysis (PCA) in order to evaluate the behavior of these variables in the multivariate space.

Results

Sperm concentration (spz x 10^6/ml) was significantly higher (*P* =0.002) in the samples collected from the cauda (138.1 ± 161.5) compared to those collected from the caput (11.4 ± 16.7), while no differences were observed among corpus (61.4 ± 43.7) and the other regions.

Sperm motility (%) after dilution increased progressively in samples collected from caput to cauda. Proportions of motile cells were significantly higher in the distal region (53.1 ± 25.9)

compared to corpus (16.2 ± 11.6; P <0.0001) and caput (1.3 ± 2.1; P <0.0001). In the caput most of the cells that did not progress showed a flagellating tail.

Morphology of spermatozoa retrieved from different epididymal regions are summarized in table 1.

Table 1 Morphology of canine spermatozoa retrieved from different epididymal regions

SPERMATOZOA			Caput	Corpus	Cauda
Normal			24.7 ± 11.9[a]	39.0 ± 13.8[b]	50.5 ± 13.3[b]
	HEAD	pear-shaped	2.0 ± 6.9	3.2 ± 9.6	3.6 ± 12.7
		small	0.2 ± 0.6	0.2 ± 0.6	0.2 ± 0.6
		large	0.1 ± 0.3	0	0
		amorphous	0.2 ± 0.4	0.2 ± 0.4	0
		Total abnormalities	2.5 ± 6.8	3.5 ± 9.5	3.8 ± 12.7
Abnormal	NECK/MIDPIECE	bent neck	4.8 ± 4.0	4.7 ± 2.5	6.2 ± 3.5
		bent midpiece	1.9 ± 1.5	1.4 ± 2.1	2.2 ± 1.7
		thick midpiece	0.4 ± 0.8	0.2 ± 0.6	0.2 ± 0.4
		Total abnormalities	7.1 ± 5.0	6.3 ± 4.0	8.5 ± 4.7
	TAIL	single bent	15.1 ± 7.5[a]	33.8 ± 11.8[b]	21.6 ± 8.6[a]
		coiled	11.0 ± 11.0	6.2 ± 5.9	7.4 ± 7.3
		Total abnormalities	26.2 ± 12.5[a]	40.1 ± 11.1[b]	29.0 ± 13.6[a]
Immature	CYTOPLASMIC DROPLET	proximal	44.8 ± 19.5[a]	8.9 ±6.8[b]	3.6 ± 3.9[b]
		distal	4.7 ± 6.8[a]	20.0 ± 10.4[b]	11.4 ± 9.0[a]
		Total immature	49.6 ± 20.1[a]	28.9 ± 11.9[b]	15.0 ± 11.0[c]

Data are percentages, presented as mean ± SD.
Different superscripts within rows (abc) indicate significant differences (P<0.05).

Proportions of spermatozoa with normal morphology were significantly higher in corpus (P =0.02) and cauda (P <0.0001) compared to caput. Overall morphological abnormalities of the head and neck/midpiece were similar in the three different epididymal regions. A significantly increased prevalence of tail defects, mainly represented by single bent tails, was observed in the corpus compared to caput (P <0.0001) and cauda (P =0.006).

Numbers of immature sperm with cytoplasmic droplets decreased from the proximal to the distal region of the epididymis. Particularly, proximal cytoplasmic droplets were more frequently found in spermatozoa collected from the caput epididymis than in the corpus (P <0.0001) and in the cauda (P P<0.0001), whereas the occurrence of distal cytoplasmic droplets was higher in the corpus than in the caput (P =0.0003) and in the cauda (P <0.05).

Significantly higher proportions of spermatozoa with intact acrosomes were retrieved from the cauda epididymis than from the caput (P =0.03) and the corpus (P =0.008). This difference was mainly due to a lower proportion of spermatozoa with abnormal acrosomes (mainly swollen acrosomes) rather than with absent acrosomes (Table 2).

Table 2 Acrosomal patterns of canine spermatozoa retrieved from different epididymal regions

Acrosome patterns	Caput	Corpus	Cauda
Intact (%)	35.4 ± 22.4a	31.6 ± 17.4a	49.5 ± 19.9b
Abnormal (%)	61.7 ± 22.2a	62.1± 16.7a	41.5 ± 18.9b
Absent (%)	2.9 ± 2.6a	6.3± 4.0b	9.0± 3.2c

Data are percentages, presented as mean ± SD.
Different superscripts within rows (abc) indicate significant differences (P<0.05).

The results for PCA analysis are reported in figure 1.

Figure 1 Principal component biplot of variables in the space of the first two principal components (PC). The length of each vector represents the weight of the variable in the plane, and the angle between variables quantifies the correlations among the variables. The objects (i.e. the samples) dispose themselves in the space of the PC relating to the variables: an object close to the end of a vector is highly correlated to the variable. Convex hulls include the spermatozoa samples from caput (cross), corpus (hollow square), and cauda (full square).

The space of the first two principal components shows that motility, abnormal acrosomes, and proximal cytoplasmic droplets are the most representative variables, and that the multivariate characteristics of the three regions are quite distinct from each other. Motility is negatively correlated with abnormal acrosomes and proximal cytoplasmic droplet in the multivariate space (univariate correlations: motility-abnormal acrosomes, r=−0.37, P <0.05; motility-proximal cytoplasmic droplet, r=−0.51, P <0.01).

Discussion

This study describes the characteristics of canine spermatozoa collected from the caput, corpus and cauda epididymis with the aim of highlighting the modifications occurring during transit.

It is well known that some modifications of mammalian spermatozoa occurring in the epididymis are related to the maturational process that involves functional and structural changes of the gametes. Among functional changes, the capacity for sperm motility is gradually acquired from caput to cauda with a quantitative and qualitative modification of its patterns from only a faint twitch of the flagellum to a progressive and vigorous forward movement [4,23].

In the present study, canine spermatozoa collected from the cauda of the epididymis showed a higher motility compared to those retrieved from caput and corpus in addition to a high concentration due to the storage role of this area. Spermatozoa in the caput often displayed a flagellating movement of the tail instead of being immotile, as also observed in cats [3].

Concomitant with these functional changes, spermatozoa undergo structural modifications during epididymal transit such as migration of the cytoplasmic droplet and acrosomal reshaping in order to achieve the normal morphology of mature spermatozoa [4].

Cytoplasmic droplets develop during normal spermatogenesis and represent a residue of the cytoplasm after Sertoli cells have phagocytized most spermatidic cytoplasm [6]. The

migration from the proximal to the distal end of the midpiece takes place in a specific region of the epididymis, which varies slightly among species. In cats, the migration occurs in the terminal part of the corpus [3], whereas in donkeys occurs from the first to the second half of the corpus [7]. In other species such as rabbit, bull and boar, this change occurs earlier either in the caput or in the passage from caput to corpus [5,24,25].

In dogs, the present results showed that the highest proportion of spermatozoa with proximal cytoplasmic droplets was in the caput. In the corpus, an abrupt decrease in frequency of proximal cytoplasmic droplets concomitant with a significant increase in the number of spermatozoa showing the distal droplet, suggests that this region is the site of migration in this species.

Concerning acrosomal reshaping, it has been demonstrated in the rabbit that the acrosome dimensions of spermatozoa collected from the caput are greater than those of spermatozoa from the cauda. During epididymal passage, these swollen acrosomes contract and localize adjacent to the nuclear surface of the sperm head [1]. In the present work, the occurrence of abnormal acrosomes, mainly represented by swollen acrosomes, was higher in the caput and in the corpus compared to the cauda where a higher proportion of spermatozoa had normal acrosomes. This gradual change toward a normal shape of the acrosome might be due to the reshaping during maturation from the proximal to the distal epididymal region as observed in the rabbit [1].

However, Axnér and co-workers [3] suggested that the decrease of feline spermatozoa with abnormal acrosomes in the cauda might also be due to the epididymal recognition of these spermatozoa as being abnormal. Besides the well-known function of the epididymis in sperm maturation, an additional role in “sperm quality control” through the removal of abnormal spermatozoa by different mechanisms (i.e. phagocytosis, dissolution by ubiquitination and degradation by other proteins) has been proposed [8,9]. However, the elimination of abnormal spermatozoa in the reproductive tract is still controversial [4].

In the present study, the proportion of canine spermatozoa with normal morphology increased significantly from caput to cauda epididymis. This increase was mainly due to the reduction of immature spermatozoa. It remains to be elucidated whether the decrease of immature spermatozoa is due to the effective maturation or to other mechanisms for removal of abnormal gametes. Frequency of anomalies of the head, neck/midpiece and tail did not differ between caput and cauda. In the cat, a significant decrease of the spermatozoa with abnormalities of testicular origin (i.e. head defects) has been described from the efferent ducts to the cauda [3]. A decreased frequency of anomalies of the midpiece, including cytoplasmic droplets, was observed among testicular and epididymal spermatozoa in the rabbit, whereas the comparison between caput and cauda epididymis failed to detect significant decrease in the frequencies of all the defects [25], as observed in this study.

On the other hand, the epididymis has been considered a site where some peculiar sperm anomalies develop [2,3,10,11]. A significant increase in sperm tail abnormalities between the proximal to the distal regions of the epididymis was reported in the cat [3]. In the boar, some types of sperm malformations of the tail were observed more frequently in the cauda, whereas other sperm defects were more uniformly distributed along the epididymis [2].

Although the proportions of abnormal canine spermatozoa between caput and cauda did not differ, the frequency of single bent tail in the corpus was significantly higher than in the other

epididymal compartments, and it was often associated with presence of a distal droplet. In some cases, the distal droplet was localized along the flagellum, rather than at the distal end of the midpiece, and the tail was bent on the droplet. A possible explanation of this association is the premature release of hydrolytic enzymes by the droplet. This might produce digestion and disorganization of structural components of the tail with consequent weakness of the structure and folding of the flagellum, as hypothesized for boar spermatozoa [2]. The single bent tail may indeed be considered as an abnormality originating in the epididymis, but as it correlates with the presence of the droplet, it is also linked to the maturational process.

The analysis of the variables in a multivariate space underlines that the traits of immaturity (i.e. low motility, proximal cytoplasmic droplet, and abnormal acrosomes) show the greatest variability in the epididymal spermatozoa, confirming that the epididymis has a crucial role in sperm maturation also in dogs as previously reported in other species.

Epididymal spermatozoa represent an important source of germplasm. It would be interesting to evaluate whether the immaturity traits of spermatozoa (i.e. cytoplasmic droplet or swollen acrosome) recovered from different compartments negatively influence the fertilization.

It has been reported that ejaculated spermatozoa with proximal droplet have poor adherence to the zona pellucida in different mammalian species [6] including dogs [26]. However, the presence of the proximal droplet in the ejaculated spermatozoa is a sign of a defective sperm maturation process that could be associated with biochemical alterations interfering with the normal progress of capacitation [26], whereas in the epididymis the presence of the droplet represents a physiological condition of the gametes. For this reason, an in-depth study of fertilizing ability of epididymal spermatozoa retrieved from the entire organ would contribute to extend their use in assisted reproductive techniques.

Conclusions

Canine spermatozoa undergo several modifications in the epididymal environment. The acquisition of progressive motility, migration of cytoplasmic droplet and acrosomal reshaping lead to mature spermatozoa which are then stored in the cauda epididymis. From this site, spermatozoa can be retrieved and used in assisted reproductive techniques as a valuable tool for propagating genetic traits of high value individuals that dies accidentally or undergoes orchiectomy for medical purposes. Further investigations should focus on the potential use of spermatozoa recovered from other epididymal regions.

Competing interest

None of the authors have any conflict of interest to declare.

Author’s contributions

GCL and SV contributed to design the study, analysed the data and drafted the paper. Laboratory work was carried out by SV and VV. MF performed statistical analysis. All authors read and approved the final version of the manuscript.

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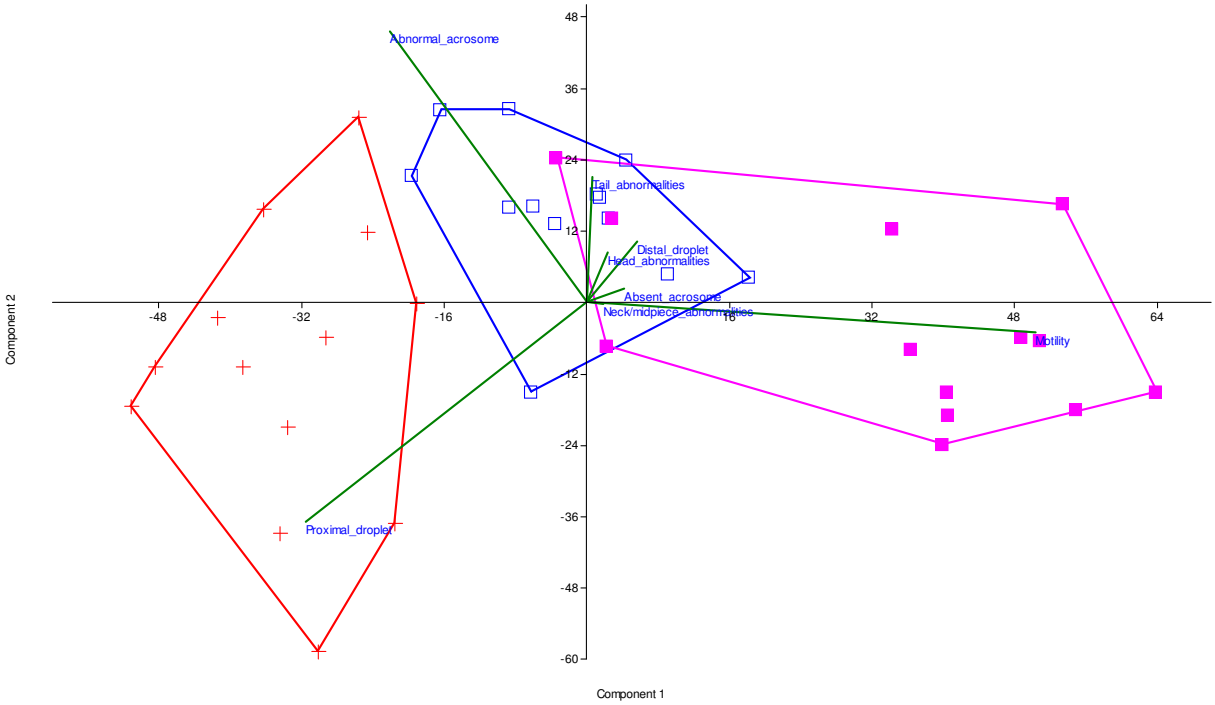
Figure 1.


Abnormal_acrosome
Tail_abnormalities
Distal_droplet
Head_abnormalities
Absent_acrosome
Neck/midpiece_abnormalities
Motility
Proximal_droplet
Component 2
Component 1
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CHAPTER 10

Paper II

Quality of canine spermatozoa retrieved by percutaneous epididymal sperm aspiration.

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PAPER

Quality of canine spermatozoa retrieved by percutaneous epididymal sperm aspiration

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Objectives: **To investigate the feasibility of percutaneous epididymal sperm aspiration in dogs and whether it might provide a population of epididymal spermatozoa similar to the population that can be obtained by processing isolated epididymis caudae.**

Methods: **Concentration and total sperm number, motility, morphology and acrosomal integrity of spermatozoa retrieved by percutaneous epididymal sperm aspiration, in vitro aspiration and mincing of the cauda of the epididymis were compared.**

Results: **Percutaneous epididymal sperm aspiration is a feasible procedure to retrieve a population of spermatozoa in dogs. Quality is similar to that of spermatozoa collected in vitro, although a wide variation amongst animals was observed.**

Clinical Significance: **In case of ejaculation failure due to pathological conditions in dogs, the collection of spermatozoa from the cauda of the epididymis could be an option for providing gametes for assisted reproductive technologies. Percutaneous epididymal sperm aspiration can be used in dogs with compromised reproductive performance, in which orchiectomy cannot be performed for medical or owner reasons. Further studies aimed to investigate whether the percutaneous epididymal sperm aspiration technique might be feasible for repeated semen collection and to accurately evaluate side effects are required.**

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INTRODUCTION

The fertilizing ability of epididymal spermatozoa has been demonstrated in several mammalian species including dogs (Dacheux & Paquignon 1980, Hewitt *et al.* 2001). Intravaginal and intrauterine artificial insemination with fresh and frozen spermatozoa retrieved from isolated epididymis caudae resulted in pregnancy and in the delivery of viable puppies (Marks *et al.* 1994, Hori *et al.* 2004, 2005, Klinc *et al.* 2005, Hori *et al.* 2011).

These studies indicate that the use of epididymal spermatozoa in assisted reproductive technologies (ART) might significantly contribute to the preservation of genetic material and to the generation of offspring from valuable males that die unexpectedly or undergo orchiectomy for medical reasons.

The retrieval of epididymal spermatozoa may also have relevance in individuals of high genetic or emotional value that cannot mate or ejaculate semen (Marks *et al.* 1994, Klinc *et al.* 2005). Neuropathic conditions or obstructive problems of the reproductive tract might cause erectile dysfunction or ejaculation problems (Johnston *et al.* 2001) and the collection of spermatozoa from in situ epididymides might be an option to obtain progeny.

When normal spermatogenesis occurs, the cauda of the epididymis is a reservoir of spermatozoa which can be cryopreserved for maintaining long-term availability of male germplasm for future use (Hewitt *et al.* 2001).

In humans, when the aetiology of infertility is obstructive azoospermia or when a previous surgical vasectomy was performed

(Collins *et al.* 1996, Glina *et al.* 2003), epididymal spermatozoa can be retrieved by percutaneous epididymal sperm aspiration (PESA) (Shah 2011) usually under local anaesthesia only or in association with intravenous sedation (Esteves & Agarwal 2011). It consists of needle aspiration of epididymal spermatozoa without scrotal incision. The epididymis is not directly visualized and the site of aspiration is guided by palpation (Shah 2011).

The epididymal spermatozoa retrieved by PESA are generally used in human ART as ICSI (intracytoplasmic sperm injection). In dogs, the low efficiency of in vitro embryo production has limited the application of in vivo collection of epididymal spermatozoa, but it cannot be excluded that high quality spermatozoa in adequate numbers for immediate use in artificial insemination and/or potential cryopreservation might be achievable by PESA.

The aim of this study was to investigate the feasibility of PESA in dogs and whether it might provide a population of epididymal spermatozoa similar to the population that can be obtained by processing isolated epididymis caudae. For this purpose, concentration, motility, morphology and acrosomal integrity of spermatozoa retrieved by PESA, in vitro aspiration and mincing of the cauda of the epididymis were compared.

MATERIALS AND METHODS

All chemicals were purchased from the Sigma Chemical Company unless otherwise stated.

Animals

Twenty healthy and sexually mature dogs, aged between one and seven years (9·5 to 52 kg bodyweight) presented to the Department of Health, Animal Science and Food Safety of the University of Milan for routine orchiectomy were included in this study. Informed owner consent was obtained regarding PESA.

Experimental design

In Experiment 1, PESA of the right epididymis cauda and in vitro aspiration of the left isolated epididymis cauda were performed in 12 dogs. In Experiment 2, PESA of the right epididymis cauda and mincing of the left isolated cauda of the epididymis were performed in eight dogs. Sperm concentration, motility, morphology and acrosomal integrity were compared in the samples retrieved by the different methods.

Percutaneous epididymal sperm aspiration (PESA)

In all cases, PESA was performed under routine general anaesthesia and analgesia directly before orchiectomy. The procedure was performed according to Shah (2011) with some modifications.

Briefly, the scrotum was cleaned with antiseptic solution and the cauda of the epididymis was palpated and stabilized between thumb and forefinger. A 26-G needle connected to a 2·5 mL syringe containing 0·2 mL of Ham's F-10 medium (HF10) supplemented with 2 mmol glutamine, 100 IU/mL Na-benzyl penicillin, 0·1 mg/mL streptomycin sulphate and 5% Fetal Bovine Serum, was inserted through the scrotal skin into the epididymis cauda. The aspiration was performed and a negative pressure was maintained while the tip of the needle was partially withdrawn and gently moved in and out within the epididymis in different directions for 360°. The collected samples were transferred into a tube and then processed for spermatozoa evaluation.

In vitro aspiration of the cauda of the epididymis

Fifteen minutes after surgical excision of the testis, in vitro aspiration was performed on the left epididymis.

The same procedure of PESA was adopted, with the only difference that epididymal spermatozoa were retrieved by aspiration of the cauda of the epididymis under direct visualization of the isolated organ.

In vitro mincing of the cauda of the epididymis

The left epididymis was dissected from testis. The blood vessels on the surface of epididymis were removed and the cauda was isolated and placed in a Petri dish containing 4 mL of HF10 medium. The organ was minced with a scalpel blade, and after 30 min of incubation at 37°C, 1 mL of suspension was collected and processed for spermatozoa evaluation.

Spermatozoa evaluation

Sperm concentration was determined with a Bürker chamber after dilution (1:200) of 5 L of semen samples. Total sperm number (concentration×total volume obtained by aspiration or mincing) was calculated. Motility was subjectively assessed with a light microscope with a heated stage at 38°C and 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 and 4, vigorous) (Mortimer 1994).

Morphology of spermatozoa was assessed following staining of the smear with Bengal Rose and Victoria Blue B. At least 100 spermatozoa were evaluated under light microscopy with oil immersion objective at ×100 magnification. Normal spermatozoa and site of defects in abnormal spermatozoa (head, neck/midpiece and tail) 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).

Evaluation of the acrosome patterns by FITC-PNA/PI was performed according to the procedure described by Cheng *et al.* (1996) for stallion spermatozoa. Staining solution was prepared with 90 µL of FITC-PNA (40 µg/mL in PBS) added with 10 µL of PI (340 µM in PBS.)

An amount of 10 µL of sperm suspension was smeared on a microscope slide and fixed in ethanol 96% for 30 seconds. The slide was dried in the dark, and then a droplet of 20 µL of FITC-PNA/PI was added to the slide. The slide was incubated in a moist chamber at +4°C and after 30 minutes it was rinsed with +4°C distilled water and air dried at +4°C in the dark over night. At least 100 spermatozoa were evaluated under fluorescent microscope (Axiovert 100, Zeiss).

The observed fluorescence images of ethanol-permeabilized spermatozoa, stained with FITC-PNA/PI, were classified into three patterns such as (1) spermatozoa displaying intensively bright fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane (intact acrosome); (2) spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane (vesiculated acrosome) and (3) 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 (acrosome residues or loss) (Cheng *et al.* 1996).

Statistical analysis

The data were not normally distributed and are summarized as median and range. Differences between the characteristics of epididymal spermatozoa retrieved using different techniques were evaluated with a Wilcoxon Signed Rank test for paired samples. P-values less than 0·05 were considered to be significant. All statistical procedures were performed by the software SAS release 9·13 for Windows platform.

RESULTS

In this study, PESA was performed in 20 epididymis caudae and semen samples were always collected. The results are summarized in Table 1. Sperm concentration and total number were similar to that of samples obtained with in vitro aspiration of the contralateral epididymis cauda (Experiment 1). In vitro mincing provided a higher concentration and total number of spermatozoa compared to PESA method (Experiment 2). Highly variable sperm concentrations among animals were observed within the same collection method, as evidenced by the wide ranges reported.

Sperm motility and morphology did not differ in samples retrieved by the different methods, although individual variability was observed.

Site and type of abnormalities were similar in all epididymal semen samples. In the samples retrieved with PESA, proportions of spermatozoa with intact acrosomes were slightly lower to those retrieved by in vitro aspiration, but higher compared to those obtained by mincing of the epididymis caudae.

DISCUSSION

The results of this study indicate that PESA is a feasible procedure to retrieve a population of spermatozoa in dogs. The quality is similar to that of spermatozoa collected in vitro, although a wide variation amongst animals was observed.

Hori *et al.* (2004) demonstrated that epididymal semen quality, although variable amongst animals, is almost the same in the bilateral epididymides of the same animal. According to this finding, a comparison between different collection techniques performed either on the right (PESA) or on the left (in vitro aspiration or mincing) cauda of the epididymis of the same subject supports PESA efficiency.

A similar concentration and total number of spermatozoa retrieved by PESA and in vitro aspiration demonstrate that the blind aspiration of the cauda of the epididymis in PESA ensures a retrieval of gametes comparable to that of the in vitro aspiration that was performed under direct visualization.

Sperm concentration obtained by mincing was higher than that obtained by PESA. Mincing results in the release of cells in

Table 1. Median values of different characteristics of canine spermatozoa retrieved by percutaneous epididymal sperm aspiration (PESA), in vitro aspiration and mincing of epididymis cauda

Spermatozoa characteristics		Experiment 1		Experiment 2	
		PESA right epididymis	In vitro aspiration left epididymis	PESA right epididymis	In vitro mincing left epididymis
Volume (L)		277 (221 to 326)	261 (198 to 298)	233·5[a] (200 to 290)	1000[b]
Concentration (sp. ×10⁶/mL)		111·2 (43·6 to 584)	93 (28·4 to 218)	27·8[a] (9·2 to 130·4)	104·2[b] (31·2 to 620·4)
Total number (sp. ×10⁶)		34·6 (11·4 to 177·5)	25·8 (7·2 to 57·3)	6·0[a] (2·3 to 30·1)	
Motility (%)		52·5 (10 to 80)	50 (5 to 80)	40 (15 to 80)	65 (10 to 90)
Normal morphology (%)		66·3 (47·2 to 85·3)	72·2 (45·8 to 83·9)	54·3 (26·1 to 66·9)	48·9 (31·5 to 69·3)
Head abnormalities	Pear-shaped/narrow at the base	0	0	0	0
	Detached	2·2	0·9	2·5	1·9
	Total abnormalities	2·2	0·9	4·3	5
Neck and midpiece abnormalities	Bent neck	2·8	1·4	3·4	3·6
	Cytoplasmic droplet proximal	0·8	0·8	1·7	0·4
	Cytoplasmic droplet distal	0	0	8·6	3·1
	Total abnormalities	3·9	3·1	15	8·8
Tail abnormalities	Single bent	23·4	25·5	19·2	19·1
	Coiled	1·6	1·2	2·1	2·7
	Broken	0·9	0	0·4	0·8
	Total abnormalities	24·3	25·9	22·9	26·2
Intact acrosomes (%)		68·5[a]	76[b]	72·3[a]	54·5[b]
Vesiculated acrosomes (%)		26·4	19·9	23·3[a]	38·2[b]
Acrosome residues/loss (%)		3·8	1·9	2·9[a]	7·3[b]

Within the same experiment different superscripts (a,b) within row indicate significant differences ($P < 0·05$).

a Petri dish after processing the whole epididymis cauda, therefore a high concentration was expected (104·2 sp.×10^6/mL) and consistent with that previously reported (100·8 ±115·8×10^6/mL; range, 4·6-400×10^6/mL; Hewitt *et al.* 2001). Furthermore, because of the different volumes of suspension obtained with PESA (about 0·2 mL) and in vitro mincing (1 mL) a higher total number of spermatozoa was expected in the latter method.

However, within the same collection method, a wide variability in the sperm concentrations and in the total number of spermatozoa was observed amongst animals.

Several factors of individual variability might affect the number of retrieved spermatozoa. A strong association between the degree of epididymal distension and the success of PESA has been documented, as spermatozoa are largely unobtainable form non-distended tubules (Collins *et al.* 1996). Furthermore, the number of spermatozoa in extragonadal site of storage is related to the period of sexual rest, unknown in these dogs, and to testicular size and bodyweight (Johnston *et al.* 2001). The latter relation could not be assessed in this study due to the limited number of dogs with different bodyweights.

The median values of sperm motility were similar in the samples collected by PESA and by in vitro techniques. However, the wide range of results further confirms the variability in animals previously reported, where the mean values of motility of fresh epididymal semen varied between 50 and 89·4% (Marks *et al.* 1994, Yu & Leibo 2002, Hishinuma & Sekine 2004, Hori *et al.* 2004, 2005, 2009, Klinc *et al.* 2005, Ponglowhapan *et al.* 2006).

In the samples collected with the different methods, no differences were observed in the proportions of morphologically normal spermatozoa (median values from 49 to 72%) and of abnormal spermatozoa with head, neck/midpiece or tail defects. Similar sperm morphology was expected because samples had the same origin (epididymis cauda), but it excludes that the negative pressure of aspiration (PESA or in vitro) might have exerted a damaging effect on the cells.

The aspiration techniques (PESA and in vitro) better preserve acrosomal integrity compared to mincing. There are no reliable data to explain the increased presence of acrosomal defects in the mincing procedure. It can be hypothesized that in these experimental conditions, the autolysis of tissue fragments and blood cells, during the incubation of minced tissue for the release of spermatozoa into the medium, might have affected the environmental/osmotic conditions to which spermatozoa were exposed with consequent membrane deterioration. In a previous study, no significant effect of blood and tissue fragments on the semen quality (motility, viability and morphology) was observed, but the status of the membranes was not evaluated (Hori *et al.* 2004).

As mentioned previously, artificial insemination with canine epididymal spermatozoa resulted in pregnancy and delivery of puppies. In most of these studies intrauterine insemination of frozen spermatozoa was adopted (Hori *et al.* 2004, 2005, 2011). Total number of spermatozoa used for insemination ranged between 2-3×10^8 and the motility after thawing was between 20 and 40%. Thus, pregnancies were obtained with the insemination of a total number of motile spermatozoa of approximately 50-60×10^6. In this study, PESA allows the retrieval of a similar total number of motile spermatozoa in some individuals, but only one epididymis was aspirated as the contralateral was used for in vitro retrieval. For potential application in ART, PESA could be accomplished in both epididymis caudae to obtain a sufficient number of gametes.

In humans, epididymal spermatozoa are used for ICSI; hence the aim of PESA is the retrieval of only few good quality spermatozoa and if the first retrieval is unsuccessful, the aspiration can be repeated either on the same or the other epididymis until an adequate specimen of spermatozoa is obtained (Glina *et al.* 2003, Esteves & Agarwal 2011).

From a technical point of view, PESA was a quick technique, easy to perform and no bleeding was noticed during or immediately after the procedure. The epididymis was well identified by palpation and the cauda was easily localized.

Epididymal aspiration has been used in dogs as a diagnostic tool to evaluate azoospermia and only rarely required sedation. However, the need of local anaesthesia, routinely used in humans, has not been investigated. In this study, discomfort or pain were not evaluated because PESA was performed in dogs under general anaesthesia and intravenous analgesia before orchiectomy. Moreover, due to the removal of testes, side effects could not be assessed. Previous reports on humans have established that PESA is a safe technique; the trauma to the epididymis is minimal (Rosenlund *et al.* 1998) as revealed by the absence of a developing haematoma or focal lesions at serial ultrasonographic examinations at variable time intervals after PESA (Ron-El *et al.* 1998). However, the histological examination of the rat epididymides evidenced inflammatory alterations after repeated PESA (Saade *et al.* 2008).

Among consequences of epididymal aspiration, there is also the potential loss of the integrity of blood-testis barrier with the resulting formation of anti-sperm antibodies. Attia *et al.* (2000) found that one of three dogs that underwent epididymal aspiration developed a transient formation of anti-sperm IgG on spermatozoa with no negative effect on total spermatozoa output or motility. Although this study concluded that epididymal aspiration is a safe procedure, side effects of PESA require further investigations.

In conclusion, the present study indicates that PESA is a feasible alternative to in vitro collection methods for spermatozoa retrieval. It can be applied to dogs with compromised reproductive performances, in which orchiectomy cannot be performed for medical or owner reasons. Further studies aimed to investigate whether the PESA technique might be feasible for repeated semen collection and to accurately evaluate side effects are required.

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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CHAPTER 11

Paper III

DNA integrity of fresh and frozen canine epididymal spermatozoa.

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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 the effect of cryopreservation on DNA integrity of canine epididymal spermatozoa and 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 by mincing the cauda epididymis. Samples were extended with and without 1mM melatonin and frozen.

In fresh and thawed samples sperm DNA integrity was assessed by a commercial kit specifically developed for canine semen (Sperm-Halomax®) and based on the sperm chromatin dispersion test.

The results showed a similar DNA fragmentation index in fresh (3.3 ± 3.6) and frozen samples with or without melatonin (4.2 ± 3.8 and 3.6 ± 3.7). Motility was significantly higher in fresh compared to frozen spermatozoa and the presence of melatonin in the freezing extender did not enhance the result. 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 affected by cryopreservation and melatonin did not exert any beneficial effect.

In conclusion, DNA integrity of canine epididymal spermatozoa is well preserved after cryopreservation and a protective effect of melatonin on post-thaw sperm quality has not been demonstrated. The DNA stability after thawing is particularly relevant for epididymal spermatozoa which potential use in assisted reproductive techniques is mainly after storage. However, other sperm characteristics as motility and acrosomal integrity are compromised by freezing and further investigations should be focused on their preservation.

Key words: dog, epididymal semen, freezing, DNA integrity

INTRODUCTION

Cryopreservation of epididymal spermatozoa is aimed at maintaining a long-term availability of male germplasm for a future use. This is particularly crucial for conservation of endangered species and for 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 the birth of offspring with a low conception rate (Marks et al. 1994; Hori et al. 2004, 2011), one of the reasons being the negative impact of freezing on sperm quality. The effects of cryopreservation on motility, membrane and acrosomal integrity of canine epididymal spermatozoa have been previously investigated (Hewitt et al. 2001; Ponglowhapan et al. 2006, Hori et al. 2009), but no information are available on its potential effect on DNA integrity.

Sperm DNA integrity has been evaluated in fresh ejaculated (Nunez-Martinez et al. 2005; Hidalgo et al. 2010; Lange Consiglio et al. 2010) and epididymal canine semen (Garcia-Masias et al. 2006). Only few reports have compared fresh and post-thaw chromatin integrity of canine ejaculated spermatozoa obtaining variable results (Rota et al. 2005; Koderle et al. 2009; Kim et al. 2010; Prinosilova et al. 2012).

The integrity of the paternal DNA is of crucial importance for the embryo development (Andrabi 2007) 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 (DFI) results in a significant decrease in pregnancy rates (Virro et al. 2004; Silva and Gardella 2006).

Nevertheless, there is no agreement neither on whether cryopreservation induces DNA fragmentation, nor on the mechanism which actually induces this damage (Di Santo et al. 2012). 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 (Koderle et al. 2009; Kim et al. 2010; Di Santo et al. 2012).

The role of antioxidant supplementations in protecting the sperm DNA from oxidative damage is still under investigation. Among antioxidants, it has been shown that melatonin (1mM), that directly neutralizes a high number of free radicals, has an effective action in protecting ram spermatozoa from the freezing injuries as evidenced by post-thaw DNA integrity, viability, motility, and fertilizing ability (Succu et al. 2011).

The aims of this study were to evaluate the effect of cryopreservation on DNA integrity of canine epididymal spermatozoa and the potential protective effect of melatonin on post-thaw sperm quality (motility, morphology, acrosomal and DNA integrity).

MATERIALS AND METHODS

All chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA) unless otherwise stated.

Animals

Ten healthy and sexually mature dogs, aged between 1 and 10 years (6 to 30 kg body weight) presented to the Department for routine orchiectomy were included in this study.

Epididymal spermatozoa retrieval

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 hematic 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 mmol 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 at 37°C, the suspension was collected from each dish and divided into three aliquots.

Semen freezing procedure

One aliquot was used as fresh control, and the others were frozen with or without 1mM melatonin (+M and -M) in the freezing extender.

After centrifugation (700 x g for 5 min) and removal of the surnatant, one aliquot (-M) was diluted (200 x 10^6spz⁄ml) with the following freezing extender: TRIS buffer with 5% glycerol, 1% Equex and 20% egg yolk and the other with the same extender supplemented

with 1mM melatonin (+M). Both aliquots were frozen according to the Uppsala system described by Linde-Forsberg (2002).

Briefly, this method consists of two extension steps before freezing in 0.5 ml straws. Straws were submerged in liquid nitrogen vapors in a Styrofoam box (10 min at −120°C) and subsequently immersed into liquid nitrogen. The straws were thawed in a water bath at 37°C for 30 sec.

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 (+M and -M).

Motility was subjectively assessed with a light microscope with a heated stage at 38°C and 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) (Mortimer 1994).

Morphology of spermatozoa was assessed following staining of the smear with Bengal Rose and Victoria Blue B. At least 100 spermatozoa were evaluated under light microscopy with oil immersion objective at 100x magnification. Normal spermatozoa and site of defects in abnormal spermatozoa (head, neck/midpiece, tail) 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).

Evaluation of the acrosome patterns by FITC-PNA/PI was performed according to the procedure described by Cheng and co-workers (1996) for stallion spermatozoa. At least 100 spermatozoa were evaluated under fluorescent microscope (Axiovert 100, Zeiss, Germany).

The observed fluorescence images of spermatozoa stained with FITC-PNA/PI, were classified into three patterns: 1) spermatozoa displaying intensively bright fluorescence of the acrosomal cap indicated an intact outer acrosomal membrane (intact acrosome); 2) spermatozoa displaying disrupted, patch-like, fluorescence of the acrosomal cap indicated the process of vesiculation and breakdown of the acrosomal membrane (vesiculated acrosome); 3) 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 (acrosome residues or loss) (Cheng et al. 1996).

Assessment of sperm DNA fragmentation

The sperm DNA fragmentation was assessed using the Sperm-Halomax® commercial kit specifically developed for canine semen (Halotech DNA SL, Madrid, Spain) and based on the sperm chromatin dispersion (SCD) test.

Sperm DNA fragmentation was evaluated in fresh and frozen samples (+M and -M) at the concentration of 50 x 10^6 spz/ml and processed following the manufacturer's instructions. Briefly, twenty-five microliters of diluted samples were added to a vial with fifty microliters of low melting agarose and mixed. Provided pre-treated slides were placed onto a metallic plate which was previously cooled at 4°C. A drop of the cell suspension (2 μl) was spread onto the treated face of the cooled slide, covered with a glass coverslip and

maintained at 4°C for 5 min. The coverslip was smoothly removed, and the layered sample was covered with the lysing solution provided in the kit. Finally, slides were washed for 5 min, dehydrated in sequential 70 and 100% ethanol baths and stained for 35 min in 1:1 Wright solution (Merck, Whitehouse Station, NJ, USA) and phosphate buffer (pH 6.88, Merck). When the slides were perfectly dried, they were mounted with Eukitt® and observed under bright-field microscopy (40x).

A minimum of five hundred spermatozoa was evaluated in each sample. Unfragmented sperm showed a small and compact halo, intensely coloured, around the spermatozoa head. Spermatozoa with fragmented DNA presented a widespread and soft halo of chromatin dispersion. Proportions of spermatozoa showing a halo of dispersion were considered positive for high DNA fragmentation index (DFI) as previously suggested (De Ambrogi et al. 2006).

Statistical analysis

Values are presented as mean ± standard deviation (SD). Significant differences ($P<0.05$) were determined by Student's t-test.

RESULTS

Spermatozoa concentration averaged 252.7±161.8 spz x 10^6/ml.

No effect of the cryopreservation procedure was observed on DNA status of canine epididymal spermatozoa as demonstrated by a similar DNA fragmentation index in fresh and frozen samples with or without melatonin (+M and -M; Table 1).

Motility was significantly higher in fresh compared to frozen spermatozoa and the presence of melatonin in the freezing extender did not enhance the result (Table 2).

Morphology of epididymal spermatozoa before and after freezing is summarized in Table 3. 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 affected by cryopreservation (Table 4). Proportions of spermatozoa showing vesiculated acrosome or acrosomal residues or loss were significantly higher in frozen samples (+M and -M) compared to fresh samples.

DISCUSSION

The results showed that the freezing procedure did not affect DNA integrity of canine epididymal spermatozoa. The presence of melatonin did not preserve motility and acrosome integrity that were severely affected by cryopreservation. Since sperm morphology and DNA integrity were not compromised by the freezing procedure, the potential protective effect of melatonin on their preservation could not have been proved.

The impact of cryopreservation on sperm DNA integrity is still a controversial matter in mammals including dogs. Some authors assessed the chromatin status of canine ejaculated spermatozoa and showed that the freezing/thawing procedure does not produce significant adverse effect on sperm DNA (Rota et al. 2005; Prinosilova et al. 2012). Conversely, Kim and co-workers (2010) found a higher level of DNA fragmentation in thawed compared to fresh spermatozoa.

Notably, in the present study, DNA fragmentation has been evaluated with the commercial kit Halomax® based on the sperm chromatin dispersion (SCD) test, and previously used for ejaculated canine semen (Hidalgo et al. 2010), whereas cited authors used different techniques as the Sperm Chromatin Structure Assay (SCSA) or the Acridine orange assay. However, it has been demonstrated in human spermatozoa that the results obtained with SCD, that is a simple, highly reproducible and inexpensive technique, are highly correlated to those obtained with SCSA (Fernandez et al. 2003).

Present results indicated that the DNA fragmentation index was similar in fresh and frozen canine epididymal spermatozoa and this finding confirms the resilience of canine sperm DNA to cold stress.

The sperm DNA stability after freeze/thawing can be a species-specific characteristic as recently suggested by Gosalvez and co-authors (2011). In a comparative study of sperm DNA fragmentation in eleven different mammalian species, a correlation between the structure of basic proteins, protamines 1 (P1) and 2 (P2), in the sperm head and the DNA status after thawing has been found. Particularly, the spermatozoa of those species lacking P2 resisted fragmentation more effectively during freeze/thawing than those that contained both P1 and P2. Unfortunately, the dog was not included in the aforementioned study, but the lack of P2 in the canine spermatozoa (Lee and Cho 1999) might explain the resilience of DNA to the cold damages.

Another factor that may preserve DNA stability during cryopreservation is the presence of seminal plasma in the sample. It has been demonstrated that post-thaw DNA integrity was improved when human spermatozoa were frozen with seminal plasma (Di Santo et al. 2012).

In dogs, the removal of plasma from the second fraction of the ejaculate before cryopreservation is not recommended, because the samples frozen with prostatic fluid showed a higher DNA stability (Koderle et al. 2009).

The beneficial effect of seminal plasma on DNA could be related to the presence of antioxidants (Koderle et al. 2009). As previously mentioned, one possible mechanism which induces DNA fragmentation in cryopreserved spermatozoa is the oxidative damage due to the imbalance between the concentrations of ROS and antioxidant compounds (Koderle et al. 2009; Kim et al. 2010; Di Santo et al. 2012).

Epididymal semen that does not benefit of the antioxidant effect of seminal plasma, might be more vulnerable to the oxidative stress occurring during cryopreservation.

In this study the supplementation with melatonin aimed at compensating for the lack of antioxidants was not able to demonstrate a protective effect on sperm DNA as cryopreservation did not damage it.

Sperm morphology was also not affected by cryopreservation. Kim and co-workers (2010) observed an increased DNA fragmentation index and a higher proportion of head abnormalities in thawed compared to fresh spermatozoa. It would have been interesting to evaluate the possible correlation between head anomalies and DNA fragmentation because spermatozoon head consists almost entirely of DNA and a correlation among the head shape and the chromatin status has been previously demonstrated in dogs (Nunez-Martinez et al. 2005; Lange Consiglio et al. 2010).

A potential protective effect of melatonin has also been evaluated on other sperm parameters. The results showed that sperm motility and acrosomal integrity were significantly affected by cryopreservation, but no effect of melatonin has been observed. The presence of 1 mM melatonin in the freezing extender of ram ejaculated semen, other than preserve sperm DNA integrity, had a protective effect on post-thaw motility (Succu et al. 2011). The incubation of thawed epididymal spermatozoa of red deer with the same concentration of melatonin showed instead only a limited protection in terms of different sperm parameters including motility and acrosomal integrity (Dominguez-Rebolledo et al. 2010).

It remains to be elucidated whether the lack of ameliorative effect of melatonin in canine cryopreserved semen was due to an inappropriate melatonin concentration or to an iatrogenic non-oxidative damage.

In conclusion, DNA integrity of canine epididymal spermatozoa is well preserved after cryopreservation and a protective effect of melatonin on post-thaw sperm quality has not been demonstrated.

The DNA stability after thawing is particularly relevant for epididymal spermatozoa which potential use in assisted reproductive techniques is mainly after storage. However, other sperm characteristics as motility and acrosomal integrity are compromised by freezing and further investigations should be focused on their preservation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

GCL and SV contributed to design the study, analyse the data and draft the paper.

Laboratory work was carried out by SV and VV.

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Table 1. Mean ± SD of motility (%) of canine epididymal spermatozoa fresh or frozen with (+M) and without 1mM melatonin (-M).

	Fresh	Frozen -M	Frozen +M
Motility (%)	74.5 ± 9.6a	37.5 ± 15.3b	36 ± 9.7b

Different superscripts (ab) within row indicate significant differences (P<0.0001).

Table 2. Mean ± SD of morphological abnormalities (%) of canine epididymal spermatozoa fresh or frozen with (+M) and without 1mM melatonin (-M).

Sperm morphology	Fresh	Frozen -M	Frozen +M
Normal	47.9 ± 24.9	49.9 ± 17.3	52.7 ± 12.9
Head abnormalities	13.5 ± 20.4	11.8 ± 25.0	11.3 ± 20.7
Neck/Midpiece abnormalities	19.7 ± 16	8.2 ± 8.8	10.3 ± 6.9
Tail abnormalities	18.8 ± 11.3a	30.2 ± 11.7b	25.7 ± 10.0ab

Different superscripts (ab) within row indicate significant differences (P<0.05).

Table 3. Mean ± SD of different acrosomal patterns (%) of canine epididymal spermatozoa fresh or frozen with (+M) and without 1mM melatonin (-M).

Acrosome patterns	Fresh	Frozen -M	Frozen +M
Intact (%)	66.6 ± 25.5a	37.2 ± 11.6b	36.3 ± 15b
Vesiculated (%)	29.3 ± 23.7a	52.1± 12.1b	46.7 ± 16.6ab
Residues/loss (%)	4.2 ± 5.5a	10.7± 3.5b	17.0± 7.5c

Different superscripts (abc) within row indicate significant differences ($P<0.05$).

Table 4. Mean ± SD of DNA fragmentation index (%) of canine epididymal spermatozoa fresh or frozen with (+M) and without 1mM melatonin (-M).

	Fresh	Frozen -M	Frozen +M
DNA fragmentation index (%)	3.3 ± 3.6	3.6 ± 3.7	4.2 ± 3.8

No significant differences were observed.