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Alternative approach

to feline epididymal spermatozoa characterization

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CHAPTER 1

Introduction

1. Introduction

1.1. Elements of anatomy and physiology of the epididymis

The epididymis is a single long and highly convoluted duct connected with the *rete testis* by multiple efferent ducts. Spermatozoa are transported from the *caput* to the *cauda* by peristaltic activity of smooth muscular cells surrounding the duct.

The epididymis is lined with a pseudostratified epithelium. The main cell type is the "principal cell", which is covered with *stereocilia*. Principal cells are characterized by structures associated with absorption, such as microvilli, vesicles and lysosomes and by structures characteristic of protein secretion, including rough endoplasmic *reticulum* and a large Golgi complex. Apart from the principal cells, there are basal cells and apical cells in the epithelium of the feline epididymal duct (Arrighi *et al.*, 1986). It has been also described the presence of periodic acid Schiff (PAS)-positive narrow cells (Sanchez *et al.*, 1998).

The epithelial lining of the duct has regional structural differences, which are likely to be related to functional differences (Nicander, 1957).

Anatomically, the epididymis can be grossly divided into the *caput*, *corpus* and *cauda* regions. Functionally and on the basis of its ultrastructure, it has also

been divided into initial, middle or intermediate and terminal segments (Glover *et al.*, 1971).

These ultrastructural segments can be further subdivided into six different regions, according to morphometric and histological characteristics (Fig. 1; Axnér *et al.*, 1999, 2006). Regions 1–4 are localized within the *caput*, region 5 in the *corpus*, and region 6 in the *cauda* of the feline epididymis.

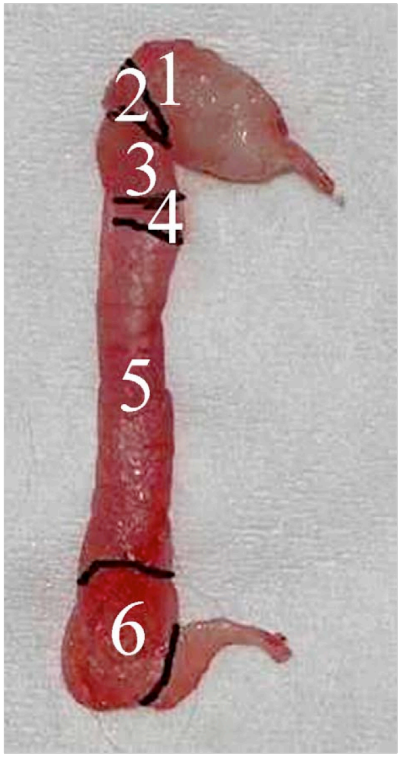

Fig. 1. Localization of the epididymal regions in the cat (Axnér *et al.*, 2006)

Region 1 shows the characteristics of the initial segment. In this segment, the concentration of spermatozoa is very low and the principal cells are long,

with tall *stereocilia* and have moderate pinocytotic activity, few vacuoles, and a conspicuous Golgi apparatus.

Regions 2–4 correspond to the middle segment. Region 2 seems to be the most active part of the duct. In this region, there is a conspicuous occurrence of droplets, granules and vesicles in the cytoplasm of the principal cells. In mature cats, region 2 can be identified macroscopically as a brown area in the apical curvature of the epididymis. Sperm concentration increases in the distal part of region 2, but it is not until region 3 that most of the sections are filled with spermatozoa. The pronounced occurrence of vesicles in the cytoplasm of epithelial cells in region 2 probably indicates absorption of fluid, which causes the increase in sperm concentration. Region 3 has a low epithelium and here the tubular diameter is the lowest in the feline epididymal duct. Supranuclear basophilic and PAS-positive granules are frequent. The tubular diameter of region 4 is slightly higher than that of region 3; it further increases in region 5 and is highest in region 6.

Regions 5 and 6 correspond to the terminal segment. The epithelium is short, with short *stereocilia*, and the lumen is wide. The cytoplasm of the principal cells has fewer vacuoles and granules than in the previous regions, indicating a less active epithelium. Short, relatively inactive epithelium with *stereocilia* resembling a brush border is characteristic of the terminal segment, as described in other species and indicative of a storage function (Glover *et al.*, 1971).

Intraepithelial cysts are often present in region 5 and, to some extent, in region 6. The cysts are filled with a PAS-positive material and do not contain spermatozoa. The role of the intraepithelial cysts in the feline epididymal epithelium, and the question whether this is physiological or pathological, remains unclear. Intraepithelial cysts have previously been described in other species, including bull, human and camel (Nicander, 1958; Hodges *et al.*, 1969; Singh *et al.*, 1980).

The composition of the epididymal fluid changes along the duct. Most of the fluid that leaves the testis is reabsorbed in the efferent ducts (Crabo, 1965) and most of the remaining fluid is reabsorbed in the *caput* of the epididymis with the consequent increase of sperm concentration as previously mentioned (Axnér *et al.*, 1999).

Concentrations of electrolytes in the epididymal fluids have not been reported in the cat. In other species, the osmotic pressure of the luminal fluid has been shown to increase from the *rete testis* to the *cauda* of the epididymis, leading to dehydration of the spermatozoa that contributes to the preservation of viability by keeping the spermatozoa in a quiescent state (Crabo *et al.*, 1975).

Few studies about epididymal fluid composition have been performed in cats, but taurine and hypotaurine are present in the epididymal fluid, as well as in ejaculated and epididymal cat spermatozoa (Buff *et al.*, 2001).

The concentration of alkaline phosphatase is higher in complete ejaculates, than in accessory gland fluids in cats, indicating testicular and/or epididymal origin (Johnston *et al.*, 1988). The epididymis is likely to be the main production site for alkaline phosphatase, as shown in the boar in which a high concentration of this enzyme in the seminal plasma indicates the presence of epididymal fluid (Einarsson *et al.*, 1976). Accordingly, in azoospermic ejaculates, alkaline phosphatase can be used as a diagnostic aid to distinguish between incomplete ejaculates, with only accessory gland fluid, and complete ejaculates containing fluids from the epididymis (Axnér *et al.*, 2002).

1.2. Maturational changes in spermatozoa

The epididymis is a dynamic organ in which substantial maturational changes of spermatozoa occur. During epididymal transit, spermatozoa acquire the capability to be motile, the cytoplasmic droplet migrates from a proximal to a distal position, the composition of the plasma membrane changes and sperm fertilizing ability develops. Maturational changes of spermatozoa have been studied in several species (Amann *et al.*, 1993), but few studies have been performed in felids (Axnér, 2006).

The mean duration of epididymal transit is 10–12 days in most species (Orgebin-Crist, 1965), but in the cat the transit time has not been estimated.

An obvious morphological change during sperm maturation is the migration of the cytoplasmic droplet (Nicander *et al.*, 1957). This always takes places in a specific region of the epididymis, which varies among species. In the cat, migration of the droplet takes place in region 4. Most distal cytoplasmic droplets are lost at ejaculation, while the percentage of proximal droplets is the same in the ejaculate as in the *cauda* of the epididymis (Axnér *et al.*, 1998). Failure of droplet migration therefore results in an increased percentage of proximal droplets in the ejaculate.

Although not studied specifically in the cat, spermatozoa with proximal droplets are likely to be infertile. It has been demonstrated in bulls that proximal droplets are associated with decreased fertility and that such spermatozoa do not penetrate the *zona pellucida* (Thundathil *et al.*, 2001). It is possible that persistence of the cytoplasmic droplet in a proximal position that indicates a general failure of sperm maturation, also affects the sperm membranes and the receptors that interact with the *zona pellucida.*

Morphological changes of spermatozoa during epididymal transit also involve acrosome modifications. The percentage of spermatozoa with abnormal (mostly swollen) acrosomes decreases during epididymal transit in the cat (Axnér *et al.*, 1999). The size of the acrosome decreases; they are swollen in the *caput* region and become more closely apposed on the surface of the sperm head along the epididymis.

The ability of spermatozoa to be motile develops during maturation (Amann *et al.*, 1982). Cat spermatozoa from regions 1 to 3 of the epididymis do not show forward motility and the most pronounced increase in motility is seen between regions 4 and 5. As previously underlined, this is also the site where migration of the cytoplasmic droplet takes place, indicating that region 4 is crucial for feline sperm maturation.

As spermatozoa pass through the epididymis sperm nuclei are stabilized by formation of disulfide bonds in the nuclear protamines (Rodriguez-Martinez *et al.*, 1990). During epididymal transit, there is a decrease in the proportion of spermatozoa with incompletely condensed chromatin, as determined by acidic aniline blue staining and the acridine orange test (Hingst *et al.*, 1995).

The fertilizing ability of the spermatozoon develops during maturation in the epididymis. Spermatozoa from the *caput* of the epididymis cannot fertilize oocytes, while spermatozoa from the *cauda* show a fertilizing ability similar to, and sometimes even better than, that of ejaculated spermatozoa (Crabo *et al.*, 1975; Gatti *et al.*, 2004). Although feline epididymal spermatozoa are used effectively to produce embryos *in vitro* (Pope *et al.*, 2006), the fertilizing ability of spermatozoa from different regions of the epididymis has not yet been studied.

The percentages of abnormal sperm heads, acrosomal defects and midpiece abnormalities decrease during transit from the efferent ducts to the *cauda* of the epididymis (Axnér *et al.*, 1999). This decrease in the rates of sperm

abnormalities might indicate that the spermatozoa are recognized as "abnormal" and removed. The hypothesis of removal of non-viable and immature cells during transit is further supported by the increase in the percentage of spermatozoa with intact plasma membranes in the *cauda* (Axnér *et al.*, 2002).

The mechanism of selective sperm removal can be explained with phagocytosis of spermatozoa by epithelial cells or macrophages. In the cat, sperm phagocytosis by epithelial cells has been observed in the efferent ducts and in the *vasa deferentia,* whereas phagocytosis by macrophages in the *vasa deferentia* only (Murakami *et al.*, 1984).

Another way for removal of abnormal spermatozoa could be the dissolution within the duct. Abnormal spermatozoa might be affected by a mechanism making them more sensitive to autolysis or phagocytosis. Different proteins bind to defective spermatozoa (NagDas *et al.*, 2000) and, among these, ubiquitin, secreted by the epididymal epithelium (Sutovsky *et al.*, 2001), is responsible of the mechanism of ubiquitination (see 1.6.1).

1.3. Retrieval and selection of epididymal spermatozoa

When normal spermatogenesis occurs, the *cauda* of the epididymis is a reservoir of spermatozoa which can be used in assisted reproductive technologies (ARTs). This might significantly contribute to the preservation of genetic material and to the generation of offspring from individuals of high

genetic or emotional value that die unexpectedly, undergo orchiectomy for medical reasons or that cannot mate or ejaculate semen (Marks *et al.*, 1994; Klinc *et al.*, 2005).

Thus, epididymal sperm preservation might benefit breeding programmes of domestic cats and of wild felids threatened with extinction.

1.3.1. Sperm retrieval

Epididymal spermatozoa can be collected from excised organs or can be surgically obtained from *in situ* testicles.

After the death of an animal it is important to transport the whole excised testes with attached epididymides to a well-equipped laboratory for sperm harvesting and processing. In this case, spermatozoa in the epididymis may quickly degenerate because of the tissue decomposition (Songsasen *et al.*, 1998). It has been demonstrated that the storage of the epididymis at +5°C prevents a decrease in sperm quality and fertilizing ability in different species (Yu *et al.*, 2002; Gañán *et al.*, 2009; Vieira *et al.*, 2013). Cat spermatozoa cold stored in the epididymis for 24h maintain good viability and velocity (Tittarelli *et al.*, 2006); whereas, when stored in an extender, epididymal cat spermatozoa can retain their quality throughout 3 weeks (Harris *et al.*, 2001).

The most common methods to collect epididymal spermatozoa in the cat are mincing the epididymal tissue or squeezing the *vasa deferentia* (Axnér *et al.*, 1998; Zambelli *et al.*, 2006; Hermansson *et al.*, 2007).

In the first method, the *cauda* of the epididymis is isolated and minced by scissors or scalpel blade in a Petri dish containing a specific medium (Axner *et al.*, 1999). A period of incubation is recommended to improve spermatozoa release from the tissue. Since mincing allows processing the whole *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.

In humans, different surgical methods such as Percutaneous Epididymal Sperm Aspiration (PESA) and Microsurgical Epididymal Sperm Aspiration (MESA) have been developed to retrieve spermatozoa from the *in situ* epididymis *cauda* (Esteves *et al.*, 2011). The PESA consists of needle aspiration of spermatozoa without scrotal incision. The epididymis is not directly visualized and the site of aspiration is guided by palpation. The MESA involves the exteriorization of the testis, the incision of the epididymal tunica and the

dissection and opening of epididymal tubules under an operating microscope to allow the aspiration of spermatic fluid (Shah, 2011).

In the dog only one study describes the retrieval of epididymal spermatozoa by PESA (Varesi *et al.*, 2013), while in the cat no information are available.

1.3.2. Sperm selection

In vitro selection of functionally competent spermatozoa is a pre-requisite for successful outcome of ARTs.

Among factors that can affect epididymal sperm sample there is the unavoidable (due to the method of collection) contamination with epithelial and red blood cells, leukocytes, bacteria, cell debris, etc. These contaminants, as well as abnormal and dead spermatozoa, produce reactive oxygen species (ROS), which impair fertilization (Nichi *et al.*, 2007). In addition to abnormal and dead cells, whole blood appeared to reduce post-thaw quality of dog sperm (Rijsselaere *et al.*, 2004), as well as *in vitro* fertilization capability in cattle (Verberckmoes *et al.*, 2004). Bacterial contamination in the semen can impair sperm motility (Kaur *et al.*, 1986) and can induce an acrosome reaction (El Mulla *et al.*, 1996).

To select a good quality sperm population, the swim-up technique is commonly used in assisted reproductive laboratories (Henkel *et al.*, 2003) and

appeared to enhance motility and viability in cryopreserved human sperm samples (Esteves *et al.*, 2000; Counsel *et al.*, 2004). In cats, although the swim-up process resulted in a higher percentage of motile and structurally-normal spermatozoa compared to simple sperm washing (Howard *et al.*, 1993), the low number of recovered spermatozoa is a disadvantage.

A novel sperm selection technique using single-layer centrifugation (SLC) through silica colloids, that allows a higher recovery rate compared to the swim-up process, was also reported (Chatdarong *et al.*, 2010).

In addition, magnetic separation techniques, based on the use of antibodies or proteins-coated magnetic beads (Olsvik *et al.*, 1994), are now widely used in research and clinical laboratories.

There are two main strategies for isolating a specific cell type with magnetic methods: "positive isolation" or "negative isolation". Positive cell isolation is defined as the method whereby a single cell type is directly drawn out using cell-specific antibodies or ligands linked to magnetic beads. In negative cell isolation the cell type of interest is separated by removing all other cells from the sample. The advantage of negative isolation is that the cells of interest have not been attached to the magnetic beads at any time, avoiding any possible damage.

1.4. Evaluation of epididymal spermatozoa

1.4.1. Conventional evaluation

Epididymal samples should be evaluated prior to the use in ARTs.

Macroscopic parameters, as appearance and volume, depend on the retrieval technique and the conventional evaluation generally also includes microscopic parameters as motility, morphology, concentration and viability. Motility is assessed as percent motility (from 0 to 100%) and progressive motility (scale from 0 to 5) using a subjective method with a phase-contrast microscope. To have an equal emphasis on both sperm percent motility and progressive motility, a sperm motility index (SMI) could be calculated (Howard, 1992). The sample is evaluated at x100–x400 magnification using a microscope equipped with a warming plate. In cat epididymal semen a mean percent motility of 65.5% and a mean progressive motility of 3.4 has been described (Thuwanut *et al.*, 2009).

Concerning sperm morphology, a percentage of 75.5% of normal spermatozoa has been found in the feline *cauda* epididymis, although the proportion of each abnormality changes in the different epididymal regions (Anxér *et al.*, 1999). Morphologic assessment is performed on at least 200 spermatozoa at x1000 magnification. The most common sperm abnormalities in cats are pyriform head, micro and macrocephaly, bicephalic and biflagellate abaxial flagellar attachment, coiled midpiece and abnormal midpiece formation,

immature sperm, proximal and distal retained cytoplasmic droplet, detached head, bent flagella or neck (Howard, 1992).

Sperm concentration is usually evaluated with a standard haemocytometer using both undiluted or diluted (dilutions of 1:10, 1:25, 1:50 or 1:100 with distilled water) sperm samples.

Sperm viability can be estimated using eosin–nigrosin; this dye differentiates live and dead spermatozoa and a mean viability of 67.5% has been reported in epididymal cat semen (Vizuete *et al.*, 2014).

1.4.2. Sperm functional integrity evaluation

Routinary semen analysis is based on the conventional evaluation, but, in some cases the assessment of other parameters as the plasma membrane integrity, the acrosomal status and the sperm capability of binding to the *zona pellucida* is required for a functional evaluation of spermatozoa.

Sperm plasma membrane protects spermatozoa against extracellular injuries and responds to physiological challenges. It plays a crucial role during sperm capacitation, in sperm–egg interaction and, finally, in fertilization. Membrane integrity can be properly assessed using transmission electronmicroscopy (Schmehl *et al.*, 1989) or fluorescence microscopy (Axnér, 2004) to detect minimal defects. The proportion of epididymal spermatozoa

with intact plasma membrane ranged from 67.5% to 93% (Thuwanut *et al.*, 2008).

Acrosomal integrity is a critical factor in determining the fertilizing potential of male gametes. Different methods can be used to assess acrosome integrity. Among these, fluorescence microscopy after staining the spermatozoa with Peanut agglutinin (PNA) conjugated with a fluorescein isothiocyanate (FITC) and propidium iodide (PI, Cheng *et al.*, 1996) is widely used. In cat epididymal samples the percentage of spermatozoa with intact acrosomes ranges between 22% and 67% (Luvoni *et al.*, 2006).

The functional integrity of spermatozoa may be also estimated using *in vitro* systems of sperm-oocyte interaction. These *in vitro* sperm function tests evaluate the capability of spermatozoa to bind to the oocyte *zona pellucida* (Zona Binding Assay) or to penetrate the oocytes (Oocyte Penetration Test) and contribute to the evaluation of sperm fertilizing ability that cannot be assessed by routine semen analysis.

Homologous or heterologous *zona* binding systems and oocyte penetration assays have been developed for feline spermatozoa (Goodrowe and Hay, 1993; Swanson *et al.*, 1998; Nelson *et al.*, 1999). In cats a mean number of 12 epididymal spermatozoa attached per *zona* and a percentage of *zonae* with attached spermatozoa of 89.7% have been reported (Goodrowe and Hay, 1993).

1.4.3. Computer-assisted sperm analysis (CASA)

The development of computerized system for semen evaluation has reduced subjectivity and variability mainly owing to the observer's experience, when the conventional semen evaluation is performed. In addition, computer-assisted sperm analysis (CASA) allows the evaluation of a high number of spermatozoa in a short time.

Computerized measuring devices generally consist of a phase-contrast microscope, a camera, an image digitizer and a computer to save and analyze the data. These devices operate as a cell motion analyzer, reconstructing sperm trajectories from the position of sperm heads in successive frames and calculating various motility and concentration parameters simultaneously. This system provides also the identification of subtle sperm characteristics, which cannot be detected by light microscopic evaluation. Sperm morphometric analysis gives very detailed information on sperm head dimensions (length, width, area, roundness, perimeter), tail length and tail abnormalities (bent, coiled, absent).

The CASA was originally described by Dott and Foster (1979) more than 30 years ago in a wide variety of species including cattle, horse, pig, rabbit, rat and sheep and has gained increasingly more interest in veterinary medicine during the last decades.

In dogs and cats, CASA was first described almost 20 years ago by Günzel-Apel *et al.* (1993) and Stachecki *et al.* (1993), respectively. Then, numerous commercial CASA systems including the CellSoft computer videomicrography, Strömberg-Mika Cell motion analyser, Hobson Sperm Tracker, Cell Track/s System, Hamilton-Thorne, Sperm Vision, Sperm Class Analyser were validated mainly in dogs and to a lesser extent in cats, both for research purposes and various clinical applications (Smith and England, 2001; Verstegen *et al.*, 2002; Rijsselaere *et al.*, 2003; Schäfer-Somi and Aurich, 2007; Dorado *et al.,* 2011).

In cats, CASA is rather poorly documented and studies which actually validate these systems are very scarce. Stachecki *et al.* (1993) compared epididymal sperm motion characteristics from normozoospermic and teratozoospermic cats. In another study, the same authors showed that motility-stimulating agents, such as caffeine, pentoxifylline and 2′deoxyadenosine, could enhance several motility parameters of cryopreserved epididymal and ejaculated cat spermatozoa without deleterious effects on longevity. Recently, CASA reference values of motility for cat epididymal sperm samples obtained after gradient density centrifugation were described (Filliers *et al.*, 2008).

Improvement of biotechnical methods that result in fast and precise semen quality assessment would however be interesting to further optimize ART in domestic cats and endangered wild felids.

1.5. Feline epididymal semen in ART

The fertilizing ability of spermatozoa obtained from *cauda* of the epididymis has been demonstrated in several mammalian species (Hewitt *et al*,. 2001) including cats (Pope *et al*., 1998; Tsutsui *et al*., 2003).

The potential use of epididymal semen in ARTs is mainly after storage and Tsutsui *et al.* (2003) reported the first birth of kittens after insemination with frozen-thawed epididymal cat spermatozoa. In addition, *in vitro* embryo production by *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with epididymal spermatozoa have also been successfully applied in domestic felids (Pope *et al*., 1998).

1.5.1. Conservation of epididymal spermatozoa

Short or long-term conservation of spermatozoa, either ejaculated or epididymal, can be performed by chilling (+4-5°C) or freezing (-196°C in liquid nitrogen) (Luvoni, 2006).

To preserve good sperm quality, it is important to know the physiological characteristics of the semen. Such traits are species-specific as demonstrated by previous poor results obtained by preserving cat spermatozoa using the same diluents and procedures developed for cattle (Luvoni, 2006).

Each step in semen preservation protocols may cause damage to the spermatozoa, thereby reducing their fertilizing potential and longevity. The

sensitivity of cat spermatozoa, in terms of loss of motility or cell membrane damage, to changes in osmolality and to different cooling rates has been examined for the purpose of defining "ideal" chilling or cryopreservation procedures.

Osmolality of cat epididymal fluids has not yet been determined; whereas, osmolality of seminal plasma is approximately 325 mOsm (Axnér, 2006) and the optimal diluent should be isotonic. It has been shown that swelling and membrane rupture of cat spermatozoa caused by hypotonic solutions are more detrimental than the shrinkage caused by hypertonic solutions. Moreover, sperm motility is more sensitive to changes in osmolality than is membrane integrity and removal of cryoprotectants in multiple steps with an isotonic solution minimizes the loss of sperm motility and reduces membrane disruption (Pukazhenthi *et al.*, 2002).

The steps in cryopreservation procedures for mammalian spermatozoa generally include gradual cooling of the sample to +4-5°C, equilibrating at +4-5°C for about 20 min, packaging the spermatozoa in straws and exposing the sample to liquid nitrogen vapours for 10-30 min before immersion and storage in liquid nitrogen. The cooling rates may also be controlled with a programmable freezer.

Epididymal cat spermatozoa are tolerant to cooling at the rates usually applied for preservation of spermatozoa. The presence of 20% egg yolk

appeared beneficial for maintenance of motility during cold storage at +4°C (Hermansson *et al.*, 2007). After thawing, loss of motility varied from 24% in spermatozoa frozen immediately after collection from freshly excised epididymis to 34% in samples collected and frozen after overnight storage of the epididymis at +4°C (Luvoni, 2006).

Acrosome damage is not induce by cooling (Hermansson *et al.*, 2007), while it has been observed in epididymal frozen–thawed semen. Hay and Goodrowe (1993) reported 30–50% loss of acrosomal integrity and Lengwinat and Blottner (1994) indicated a decreased acrosomal integrity from 69.5% to 22.5% after thawing.

A significant higher proportion of cat epididymal spermatozoa after thawing showed the pattern of acrosome reaction compared with fresh or diluted semen before freezing. The proportion of acrosome-reacted spermatozoa in frozen–thawed samples indicates that many cells are damaged during freezing and thawing and, therefore, are functionally compromised (Marinoni, 2001). Some authors have reported that frozen epididymal spermatozoa bind to homologous *zona pellucidae* in a larger number than fresh epididymal spermatozoa, and this seems to be caused by the loss of acrosomal integrity, which in turn reduces the fertilizing potential (Goodrowe and Hay, 1993). The increased interaction of spermatozoa with *zona pellucidae* was due to a modification of surface components on the sperm head (i.e.

capacitation/acrosome reaction-like alterations), and since a loss of fertilizing ability is associated with acrosomal damage.

Studies on the response of ejaculated feline semen to chilling and freezing found that rapid cooling (14°C/min) from +25°C to 0°C was more detrimental to the morphological integrity of cat spermatozoa than was slow cooling (0.5°C/min; Pukazhenthi *et al.*, 1999). Moreover, massive acrosomal membrane damage occurred without a concomitant decrease in motility, demonstrating that motility was relatively resilient, but that acrosome integrity was highly sensitive to cold-shock. A suitable freezing rate for cat spermatozoa was reported to be 10°C/min from +5°C to -80°C before immersion in liquid nitrogen (Pope *et al.*, 1991); however, it has been demonstrated that a slow freezing rate of 3.85°C/min from +5°C to -40°C before immersion in liquid nitrogen resulted in higher sperm motility and improved morphology as compared to that of faster freezing rates (from 9°C/min up to 43°C/min) (Zambelli *et al.*, 2002). These findings suggest that an optimal freezing or cooling rate for feline spermatozoa has yet to be defined.

1.5.2. Artificial insemination with epididymal spermatozoa

Although an optimal procedure to preserve motility and integrity of cat spermatozoa has not been developed, the site of deposition of semen in the genital tract of the queen greatly influences the conception rates.

Deep vaginal insemination may be done in domestic cats by inserting a French Tom cat catheter as far as possible into the vagina (Axnér, 1998) and laparoscopic insemination (intrauterine artificial insemination: IUAI) is often used in Felids (Howard *et al.*, 1992; Donoghue *et al.*, 1993, 1996; Barone *et al.*, 1994; Swanson *et al.*, 1996).

Pregnancy rates are higher after IUAI both in domestic and captive wild cats (Villaverde, 2009; Howard, 1999) and offspring from IUAI with frozen–thawed semen have been obtained in ocelot (*Felis Pardalis*), leopard cat (*Felis Bengalensis*), cheetah (*Acinonyx Jubatus*), snow leopard (*Pantera Uncia*), clouded leopard (*Neofelis nebulosa*) and tiger (*Pantera Tigris*) (Howard, 1999).

Although most of these studies were focused on ejaculated gametes, artificial insemination with frozen epididymal cat semen in which motility after thawing was approximately 24% resulted in conception rates of 27% (Tsutsui *et al.*, 2003).

1.5.3. In vitro embryo production with epididymal spermatozoa

Fertilization rates after IVF of domestic cat *in vitro* or *in vivo* matured oocytes vary between 40-50% and 60–80%, respectively, and after transfer of IVF-derived embryos live offspring has been obtained (Pope, 2006).

An important component of the progress that has been made in developing ARTs in cats is the skilful use of gamete micromanipulation for

producing embryos. The ICSI procedure allows fertilization with one single selected spermatozoon, and it should be taken into account when in the epididymal samples sperm concentration or quality is poor and IVF cannot be performed.

Blastocysts have been obtained from *in vivo* matured oocytes fertilized by subzonal insemination (SUZI) or ICSI (Pope *et al.*, 1998). Earlier attempts at comparing SUZI and ICSI were in favour of SUZI (Pope *et al.*, 1995), but improvements in sperm injection technique and visualization of ooplasm by centrifugation of oocytes improved results considerably in favour of ICSI.

Ejaculated spermatozoa and preovulatory oocytes from gonadotropin-treated donors (*in vivo* matured) were used in the original report on *in vitro* production of cat embryos by ICSI (Pope, 1998, 2006). Others authors have examined the ability of frozen/thawed spermatozoa obtained from excised epididymides to induce activation and subsequent cleavage after ICSI of *in vitro* matured oocytes (Bogliolo *et al.*, 2004).

In wild felids such as lion and fishing cat (*Prionailurus viverrinus*) preliminary trials were done using ICSI to produce embryos from *in vivo* matured oocytes recovered by laparoscopy from gonadotropin-treated donors (Howard, 1999).

1.6. Alternative approach to feline epididymal spermatozoa characterization

Other aspects, not included in the conventional analysis or in sperm functional integrity tests, can be evaluated to extend the characterization of spermatozoa. Among these, the assessment of sperm ubiquitination, DNA fragmentation and morphometry represent the alternative approach to feline epididymal spermatozoa characterization described in the present study.

1.6.1. Sperm ubiquitination

Ubiquitin is a 8.5 kDa peptide that tags other proteins for proteasomal degradation, and it is also involved in the regulation of protein function. This protein is a normal component of human blood, ovarian follicular fluid and seminal plasma (Nandi *et al.*, 2006) and might be responsible of the elimination of defective spermatozoa during transit through epididymis in humans and cattle (Baska *et al.*, 2008; Sutovsky *et al.*, 2004).

Results demonstrate that the increase of sperm ubiquitin is inversely associated with spermatic concentration, motility and normal morphology indicating that ubiquitination could be a biomarker of poor semen quality (Sutovsky *et al.*, 2004). Conversely, other authors (Muratori *et al.*, 2005) found a

positive correlation between sperm ubiquitin and good semen parameters suggesting a different role for sperm ubiquitination.

Therefore, the role played by ubiquitination of spermatozoa proteins and their function during the transit through epididymis is still an open question, even in species whose semen properties have been extensively studied, such as human and bovine. Regarding the domestic cat, few researches have been conducted and no positive or negative correlations between semen quality and ubiquitination have been observed (Mota *et al.*, 2005).

Magnetic cell separation techniques, based on the use of antibodies or proteins-coated magnetic beads, as magnetic ubiquitin beads, may allow the selective capture of ubiquitinated spermatozoa from semen, thus contributing to the identification of a potential correlation between semen quality, spermatozoa maturation and ubiquitination process. This would contribute to understand whether ubiquitin could be considered a biomarker of quality of epididymal feline semen.

1.6.2. Sperm DNA fragmentation and sperm morphometry

The evaluation of DNA status is not included in the standard semen analysis, but the frequency of spermatozoa containing fragmented DNA may be an important parameter of semen quality, and a useful index of fertility potential.

Spermatozoa with severe DNA damage remain functionally intact, with normal fertilizing ability, but a high incidence of DNA fragmentation results in a significant decrease in pregnancy rates (Virro *et al.*, 2004). The exact mechanism of sperm DNA damage has not yet been clarified, but environmental stresses, gene mutations, chromosomal abnormalities or oxidative damages might be involved.

Several methods have been developed to assess sperm DNA fragmentation such as *in situ* nick translation (ISNT), terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL), comet assay (CT), acridine orange test (AO) and the sperm chromatin structure assay (SCSA) (Schulte *et al*, 2010). However, these techniques are labor-intensive and require expensive instrumentations.

Recently, a kit (Sperm-Halomax®) based on the sperm chromatin dispersion (SCD) test and specifically developed for boar (Enciso *et al.*, 2006), bull (García-Macías *et al.*, 2007), stallion (Cortés-Gutiérrez *et al.*, 2009) and dog semen (Hidalgo *et al.*, 2010), but not for cat semen, became commercially available.

Spermatozoa are immersed in an agarose matrix on a slide and briefly incubated in a lysing solution to remove membranes and proteins. DNA fragmentation produces large halos, whereas those sperm with low levels of fragmentation show circumscribed halos. The evaluation can be performed by

fluorescence or light microscopy. This kit would allow the routine assessment of sperm samples for DNA fragmentation in laboratories dealing with andrological examination and ARTs.

As previously mentioned, significant advances in ART have been achieved thanks to the embryo production by ICSI (Pope *et al.,* 1998, 2006). Sperm selection for ICSI is based on motility and morphology patterns, and the evaluation of the DNA status is not generally performed (Celik-Ozenci *et al.*, 2004). Since the sperm head consists almost entirely of DNA, subtle differences in sperm head morphometry might be related to DNA content and organization, as demonstrated in dogs (Lange-Consiglio *et al.*, 2010) and humans (Mangiarini *et al.*, 2013).

The detailed study of sperm head morphometry cannot be carried out with the conventional light microscopic technique, but it would require computerized analysis. To the authors' knowledge similar studies have not been performed in cats.

CHAPTER 2

Objectives

2. Objectives

To understand whether ubiquitin could be considered a biomarker of quality of epididymal feline semen, and to give a better understanding of the ubiquitin role in feline sperm maturation. For this purpose the specific aims were 1) to verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads and identify structural (morphological and acrosomal patterns) differences between whole sample and separated gametes; 2) to extensively characterize all the proteins ubiquitinated in the spermatozoa retrieved in the three regions of the epididymis by a proteomic approach **(Paper I)**.

To enhance the evaluation of spermatozoa used in ART the specific aims were 1) to verify the suitability of Sperm-Halomax® assay, specifically developed for canine semen, for the evaluation of DNA status of epididymal cat spermatozoa comparing baseline values of DNA fragmentation obtained with Sperm-Halomax® and TUNEL; 2) to investigate whether a correlation between DNA status, sperm head morphology and morphometry assessed by Computer Assisted Semen Analysis (CASA) exists in cat epididymal spermatozoa **(Paper II)**.

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 50 healthy and pubertal domestic cats (*Felis catus*) presented to the Department of Health, Animal Science and Food Safety of this University for routine orchiectomy.

In the first study **(Paper I)** samples were collected from 22 cats. In Experiment 1 semen samples were obtained from ten pairs of isolated testes by squeezing *cauda* epididymis and *vasa deferentia* in a warm (37°C) Phosphate Buffered Saline (PBS) solution. Ubiquitination of the epididymal spermatozoa was evaluated by western blot analysis as described in detail in the following section. To verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads the sample was divided into two aliquots: the first was processed with magnetic ubiquitin beads and the second aliquot was not treated and used as control. Sperm parameters (concentration, motility, morphology, acrosomal integrity) were evaluated in the sample treated with beads (unbound

spermatozoa, see below) and in whole sample (control). In Experiment 2 twelve pairs of epididymides were processed. 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. Each pair of epididymides was macroscopically divided into three anatomical portions, *caput*, *corpus* and *cauda*, according to previous studies (Schimming *et al.*, 1997; Schimming and Vicentini, 2001) and each pair of portions was placed in a Petri dish containing 2 ml of PBS. 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. Each sample was processed with magnetic ubiquitin beads and proteomic analysis was assessed in spermatozoa bound to the beads to extensively characterize all the proteins ubiquitinated in the three regions of the epididymis.

In the second study **(Paper II)** epididymal spermatozoa were collected from 28 tomcats subjected to routine orchiectomy. Epididymides and *vasa deferentia* were dissected and squeezed to collect epididymal fluid in a warmed (37°C) PBS without calcium and magnesium. In Experiment 1 epididymal fluid collected from 10 cats was divided into two aliquots for the evaluation of DNA status by Sperm-Halomax® for canine spermatozoa (Halotech DNA SL, Madrid, Spain) and TUNEL Test (Calbiochem® FragEL™ DNA fragmentation

detection kit, Fluorescent–TdT Enzyme; EMD Millipore Billerica, MA, USA). In experiment 2 epididymal fluid collected from the remaining 18 cats was used for the evaluation of DNA status by Sperm-Halomax®, for the conventional sperm head morphology evaluation, and for the sperm head morphometry by CASA.

3.2. SDS-PAGE, 2D electrophoresis and western blot analysis (Paper I; Exp. 1)

Whole semen samples were suspended in 0.5 M Tris–HCl pH 6.8, 10% glycerol and 10% sodium dodecyl sulfate (SDS). The protein content was determined by Bradford method (Bradford, 1976) and separated on an homemade 11% polyacrylamide gel according to Laemmli (1970). 2D-electrophoresis was carried out as described in Tedeschi *et al.* (2005). For the first dimension, proteins were applied to rehydrated IPG strips (70 mm, 3–10 NL) (Amersham Biosciences, Cologno Monzese, Italy). Isoelectric focusing was performed at 15°C as follows: 600 V for 10 min, 900 V for 15 min, 1,500 V for 15 min, 2,500 V for 15 min and 3,500 V for 5 h and 15 min. Before the second dimension, each strip was rinsed with buffer (6 M urea in 0.375 M Tris–HCl pH 8.8, 2% SDS, 20% glycerol, bromophenol blue). The second dimension was performed on homemade 12% SDS minigels (8.5 x 6 x 0.15 cm) at 20 mA/gel.

Proteins in the gel were stained with ammonium silver stain or transferred from the gel onto a polyvinylidene difluoride membrane (PVDF, Immobilon P,

Millipore, Bedford, MA, USA) by electrophoretic elution at 180 mA for 1 hour. The transfer buffer was 10 mM CAPS and 10% methanol. Membranes were then probed for immunoreactivity as follows: for ubiquitin detection membrane was blocked with 5% dry milk in Tris Buffered Saline (TBS), washed five times with TBS-T 0.25%, incubated in rabbit anti-ubiquitin polyclonal antibody (AbCam, Cambridge, UK) to 1:1000 in 2% dry milk in TBS-T 0.25%. The membrane was washed five times with TBS-T 0.25%, incubated in anti-IgG rabbit HRP conjugated (Calbiochem Darmstadt, Germany) 1:1000 in 2% dry milk in TBS-T 0.25%.

For actin detection, the membrane was blocked with 10% dry milk in TBS-T 0.1%, washed three times with TBS-T 0.1%, incubated in mouse anti-actin monoclonal antibody (Sigma) to 1:4000 in 5% dry milk in TBS-T 0.1%. The membrane was washed three times with TBS-T 0.1%, incubated in anti-IgG mouse HRP conjugated (Calbiochem Darmstadt, Germany) 1:2000 in 5% dry milk in TBS-T 0.1%.

For tubulin detection membrane was blocked with 5% dry milk in TBS-T 0.05%, washed three times with TBS-T 0.05%, incubated in mouse anti-tubulin monoclonal antibody (AbCam, Cambridge, UK) to 1:1000 in 1% bovine serum albumin (BSA) in TBS-T 0.05%. The membrane was washed three times with TBS-T 0.05%, incubated in anti-IgG mouse HRP conjugated (Calbiochem Darmstadt, Germany) 1:20000 in 1% BSA in TBS-T 0.05%.

3.3. Sperm separation with magnetic ubiquitin beads (Paper I; Exp 1-2)

Magnetic ubiquitin beads (Li Starfish S.r.l., Cernusco S/N, Milan, Italy) suspended in 10 mM phosphate solution (pH 7.5) with 0.02% sodium azide were added to semen aliquots (80 µl beads/ml of semen) in a 1.5 ml tube. The tube was gently mixed for 20 min, to allow contact between the magnetic beads and the targeted spermatozoa before placing it in a magnetic field for 10 min. The separation procedure is a negative depletion in which the magnetic beads attach to the targeted surface marker and are collected against the wall of the tube by application of an external laboratory magnet. The separated sample was decanted and collected while the tube was still in the magnetic field, while the ubiquitinated spermatozoa bound to the beads remained attached to the wall of the tube as long as the magnet was in place. Thus, two fractions were obtained: spermatozoa bound to the beads and spermatozoa unbound in the tube.

3.4. Spermatozoa evaluation (Paper I; Exp 1)

Sperm concentration was determined with a Bürker chamber. 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) (Mortimer, 1994).

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 (Diaplan Leitz, Wetzlar, Germany) with oil immersion objective at 100x magnification. Normal spermatozoa and site of defects in abnormal spermatozoa (head, neck/midpiece, tail) were recorded (Christiansen, 1984). 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 (Cheng *et al.*, 1996). 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 96% ethanol for 30 sec. 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".

Mean±SD of sperm characteristics were analyzed by Student's t-test ($p<0.05$).

3.5. Mass spectrometry analysis (Paper I; Exp. 2)

In order to increase the homogeneity of the sample and overcome the inter- and intra-sample variability, epididymal spermatozoa of different cats collected from *caput* (A), *corpus* (B) or *cauda* (C) and bound to the magnetic beads were pooled.

The ubiquitin–conjugate proteins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the identities as well as the ubiquitination sites after tryptic digestion, and missed tryptic cleavage at the modified site. In particular, the spermatozoa bound to the magnetic beads were collected and lysed in a buffer containing 7 M urea, 2 M

thiourea, 50 mM ammonium bicarbonate and the complete protease inhibitor cocktail (Roche, Basel, Switzerland).

Upon sonication the lysates were clarified by centrifugation at 15000 rpm for 15 min and the total protein concentration was determined using the Bradford method (Bradford, 1976). Protein samples were reduced with 45 mM dithiothreitol, alkylated with 100 mM iodoacetamide and subsequently digested with Sequencing Grade Modified Trypsin overnight at 37°C. Digestion was stopped by adding 1 µl of 98% formic acid. The proteolytic digests were desalted on a ZipTipC18 (Millipore, Billerica, MA, USA) before MS analysis. Each sample was separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific, Waltham, MA, USA). Buffer A was 0.1% v/v formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 lm ID, 3 lm resin, Dionex). The gradient was as follows: 5% buffer B (10 min), 5-40% B (60 min), 40-50% B (10 min), 95% B (5 min) at a flow rate of 0.3 µL/min.

Mass spectrometry was performed using a LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nanospray source (Proxeon Biosystems, now Thermo Fisher Scientific, Waltham, MA, USA). Eluted peptides were directly electrosprayed into the mass spectrometer through a standard non-coated silica tip (New Objective, Woburn, MA, USA) using a

spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Three replicate analyses of each sample were performed.

Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific, Waltham, MA, USA).

3.6. Protein identification and computational analysis (Paper I; Exp. 2)

For identification of ubiquitinated proteins, raw data files were processed and analyzed using MaxQuant 1.3.0.5. Parent ion and MS2 spectra were searched against the Human and *Felis catus* Uniprot/SwissProt database using the Andromeda search engine. Search parameters allowed for 2 missed tryptic cleavages, a mass tolerance of 6 ppm in MS mode and 20 ppm in CID MS/MS mode, a static modification carbamidomethylation (Cys), and up to 3 total dynamic modifications [(N-acetylation (protein), oxidization (Met) and ubiquitination (Lys)]. To achieve highly reliable identifications, the following criteria were used: maximal protein, peptide and site FDR of 0.01 and minimal peptide length of 6. The default setting of the maximal peptide posterior error probability of 1 was used. The presence of ubiquitinated amino acids on each

peptide was confirmed upon visual inspection of the corresponding MS/MS spectrum by Perseus, discharging all the spectra presenting a modification at the C-terminal lysine.

3.7. Sperm-Halomax® assay (Paper II; Exp. 1-2)

To perform the Sperm-Halomax® assay, the semen was diluted in PBS to obtain a final concentration of 5-10x10^6 sp/ml. A vial of agarose was melted into a water bath at 90-100°C for 5 min, then equilibrated at 37°C for 5 min and an aliquot of the diluted sample was added and mixed thoroughly. A drop of suspension was placed onto the treated face of a slide (marked surface), covered with a glass coverslip in horizontal position and placed into the fridge for 5 min for the solidification of sample. After the removal of the coverslip, the slide was settled (in horizontal position) in the lysing solution and incubated for 5 min at room temperature. The slide was washed with distilled water for 5 min and a sequential ethanol baths (70, 90 and 100%, for 2 minutes each) and air dry were used to dehydrate the sample.

The slide was stained in Wright solution (Merck, Darmstadt, Germany) diluted 1:1 in phosphate buffer at pH 6.88 (Merck), as recommended by manufacturer's instruction. Keeping the horizontal position, a layer of the dying solution was placed to cover the slide for 15-20 min. The dying solution was removed and the slide was washed briefly and smoothly in tap water and air-

dried. The stained slide was analyzed under a light microscope with x100 magnification lens (Axiovert 100).

At least 500 spermatozoa were evaluated and classified as follows: spermatozoa with fragmented DNA those with a large and spotty halo of chromatin dispersion and a small head; spermatozoa with unfragmented DNA those with a small and compact halo of chromatin dispersion and a large head.

The sperm DNA fragmentation index (DFI) was then calculated as the percentage of spermatozoa with fragmented DNA over the total number of sperm counted per slide.

3.8. TUNEL test (Paper II; Exp. 1)

DNA sperm integrity was evaluated using DNA fragmentation detection kit (Calbiochem® FragEL™ DNA fragmentation detection kit, Fluorescent–TdT Enzyme; EMD Millipore Billerica, MA, USA). The principle of Fluorescein-FragEL is that terminal deoxynucleotidyl transferase (TdT) catalyzes the addition of fluorescein-labeled and unlabeled deoxynucleotides to the 30-OH ends generated by endonucleases during apoptotic degeneration.

Sperm samples were smeared on a slide and air-dried. Then smears were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and washed twice in Tris Buffered Saline (TBS) for 15 min. The slides were then covered with the permeabilization solution (Protein Kinase 2 diluted 1:100 in

TRIS solution 10mM), and incubated for 6 min in a moist chamber. The slides were washed twice in TBS solution and maintained in a moist chamber. In the dark, an aliquot of the equilibration solution (TdT Equilibration Buffer diluted 1:5 in sterile water) was added to each slide that was incubated for 30 min in a moist chamber at room temperature. After the removal of the equilibration solution, an aliquot of the labelling solution (Fluorescein-FraELTM TdT Labelling Reaction Mix diluted 1:20 in TdT Enzyme) was added to each slide that was incubated for 90 min in a moist chamber at 37°C. The slides were washed three times in TBS and a drop of an antifade reagent (Gel Mount; BiØmeda Corp., Foster City, CA, USA) was added. The slides covered with a coverslip were examined under fluorescent microscope (Eclipse E600, Nikon Corporation, Tokio, Japan) with x40 magnification lens and oil immersion. At least 500 spermatozoa of each sample were analyzed randomly to evaluate the percentage of TUNEL-positive sperm cells (bright green nuclear fluorescence in DNA fragmented sperm cells) (De Pauw *et al.*, 2003).

In order to assess the agreement between tests, considering the TUNEL procedure as reference method (gold standard), data of Sperm-Halomax® assay and TUNEL test were analyzed by the Bland-Altman plot technique (Bland and Altman, 1986).

3.9. Conventional sperm head morphology (Paper II; Exp. 2)

Undiluted samples were stained with a rapid Giemsa-Wright stain (Diff-Quick, Merck) and in each sample a total of 200 spermatozoa was evaluated under light microscope (Diaplan Leitz) with x100 magnification lens and oil immersion. Abnormal sperm heads included those that were pear-shaped, large, small, or amorphous were recorded.

3.10. CASA sperm head morphometry (Paper II; Exp. 2)

The stained slides were examined for the evaluation of the sperm head morphometry using a light microscope (Olympus BX51, Olympus America Inc., Melville, NY, USA) equipped with a video camera (Scion Corp. 1394, Frederick, MD, USA) interfaced to a computer. The software used for image acquisition and analysis was Image-Pro Plus 5.1; Media Cybernetics (Immagini & Computer, Bareggio, Italy).

Each sperm head was measured for different parameters: area (μm2), aspect (ratio between major and minor axes of the ellipse), perimeter (μm), maximum diameter (dmax, μm), minimum diameter (dmax, μm), maximum radius (radmax, μm), minimum radius (radmin, μm), radius ratio and roundness (Núñez-Martinez *et al.*, 2007).

To establish reference values for the morphological sperm variables (area; aspect; perimeter; dmax; dmin; radmax; radmin; radius ratio; roundness), a non-parametric approach (2.5-97.5 percentile of the distribution) was followed on 2425 spermatozoa.

Variables not determined on a single spermatozoon (i.e. DFI and head anomalies) were submitted to the calculation of the 95% confidence interval as indicative reference values.

Aiming to evaluate the multivariate relations between DFI and the morphological variables, a principal component analysis (PCA) was applied: data were submitted to PCA after normalization and the varimax rotation. The number of retained components was calculated when at least the 90% of the total variability was explained. Moreover, the Pearson univariate correlation between DFI and the morphological variables was calculated ($p<0.05$).

CHAPTER 4

Results and discussion

4. Results and discussion

4.1. Paper I

4.1.1. Exp. 1. SDS-PAGE, 2D electrophoresis and western blot analysis

The presence of ubiquitinated proteins in cat epididymal semen was evaluated by anti-ubiquitin antibodies upon separation on monoD electrophoresis of the whole-cell extract (Fig.1A-Paper I). The pattern of ubiquitination is very similar to the one described by Thompson *et al.* (2003) in bovine seminal plasma for the proteins at higher molecular weight (more than 50 kDa). The major differences can be observed at lower molecular weight (37-20 kDa) where few bands, clearly detectable in cat semen, are absent in the bovine sample.

The semen was analyzed by 2D-electrophoresis to better separate all the proteins and evaluate the ubiquitination pattern. Fig. 1B (Paper I) shows all the proteins detected by silver staining while Fig. 1C (Paper I) reports the corresponding western blot analysis using anti-ubiquitin antibodies which clearly shows that the proteins ubiquitinated are mainly present in the 70-40 kDa region except for few spots at lower molecular weight. The 70-40 kDa region was further investigated looking for the possible ubiquitination of the two most abundant proteins of the cytoskeleton: actin and tubulin, in order to better

understand the correlation between ubiquitination and morphological alterations of the spermatozoa.

The table in Fig 2A (Paper I) reports the expected molecular weight of the two proteins with and without the modification, while Fig. 2B (Paper I) reports the western blot analysis using anti-ubiquitin antibodies following anti-actin and anti-tubulin antibodies upon stripping of the membrane, as described in detail in the Methods section. The results clearly suggest that actin is indeed ubiquitinated in cat spermatozoa while the modification of tubulin was not detected. The same observation was further confirmed in the proteomic analysis described in the section below.

4.1.2. Exp. 1. Evaluation of spermatozoa treated with magnetic ubiquitin beads

A significant decrease of sperm concentration and sperm count was observed after treatment with magnetic ubiquitin beads in the fraction of spermatozoa unbound to the beads (Table 1-Paper I). No significant differences were observed in other sperm parameters (motility, morphology and acrosome integrity). These findings demonstrate the efficacy of beads in binding ubiquitinated spermatozoa as the sperm concentration and count were significantly decreased after treatment.

However, the unbound fraction was not characterized by higher sperm motility, morphology and acrosomal integrity. Therefore, the magnetic beads did not select a population of cat epididymal spermatozoa with better quality.

These results confirm the lack of correlation between ubiquitinated spermatozoa and morphological abnormal spermatozoa as previously reported by Mota *et al.* (2006).

4.1.3. Exp. 2. Mass spectrometry analysis, protein identification and computational analysis

The traditional proteomic approach contemplates a preliminary step of protein purification by 1D or 2D electrophoresis, followed by mass spectrometry identification of the proteins present. However, the availability of new, advanced and high throughput analytical platforms allow identification of thousands of proteins from a biological sample without a previous separation step, the so called shot gun proteomic approach, which relies on tandem mass spectroscopy for protein identification and characterization of post-translational modifications (PTM). This approach has been applied here to characterize ubiquitination of the proteins in the cat spermatozoa collected from different epididymal regions.

Each fraction enriched in ubiquitinated proteins was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the

identities and the ubiquitination sites of the proteins present in the samples. The proteomic analysis, carried out combining the results of three replicates, allowed to identify 766, 708 and 646 proteins in A, B and C, respectively, (see Table S1, S2, and S3 in Supplementary materials-Paper I) among which 212 proteins are common in the three epididymal tracts (A, B and C) and are reported in Table 2 (Paper I). No one of the 212 common proteins is ubiquitinated. Focusing only on the ubiquitinated proteins expressed in each epididymal tract, it was possible to identify 7 proteins in A, 17 in B and 6 in C, in total 30 proteins reported in Table 3 (Paper I). For each of them, one representative MS/MS sprectrum of a ubiquitinated peptide is reported as supplementary materials in Figures S1-S30 (Paper I). As indicated in Table 3 (Paper I), ubiquitination of many of these proteins have been previously observed in human or mouse, according to the PhosphoSite Plus Data Base.

The distribution of the 30 ubiquitinated proteins in terms of molecular function (GOMF), cellular components (GOCC) and biological processes (GOBP) are reported in the corresponding histograms of Fig. 3 (Paper I). Among the ubiquitinated proteins, there are cytoskeletal proteins or proteins interacting with the cytoskeleton and are key players in the cytoskeleton organization, in cellular differentiation and in the morphogenesis processes. Overall, more than 30% of the ubiquitinated proteins are involved in the anatomical structure development in keeping with the possible role of this

modification as a way to remove defective spermatozoa in the epididymal tract. Most are proteins important for the regulation of gene expression or are components of signal transduction pathways suggesting that ubiquitin, by playing a major role in the modulation of their homeostasis and expression level, may affect also the expression level and the activation of other proteins present in the epididymal environment.

However, to better understand the significance of the presence of specific ubiquitinated proteins in the different epididymal regions further investigations are required.

4.2. Paper II

4.2.1. Exp. 1: Sperm-Halomax® assay vs TUNEL test

Epididymal cat spermatozoa processed with Sperm-Halomax® produce images of similar characteristics to those obtained in dogs (Hidalgo *et al.*, 2010; Urbano *et al.*, 2013). Spermatozoa with unfragmented DNA do not show or show very small halos of dispersion of DNA loops, whereas those with DNA fragmentation release peripheral halos from the central core. Discrimination of the size of the halos was easy to establish in cat sperm samples because the size of the halos of DNA dispersion was large as those obtained in dogs.

No differences were observed in baseline values of DNA fragmentation index obtained with Sperm-Halomax® and TUNEL (4.34 ± 0.93% vs. 4.26 ± 0.83%; p = 0.84).

These data show that Sperm-Halomax® assay specifically developed for canine semen and based on SCD test, provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. Most of the differences between the DNA baseline values obtained with the Sperm-Halomax® assay and TUNEL test (considered the gold standard for the evaluation of DNA fragmentation) were within the 95% confidence interval limits, suggesting that the level of agreement between the two methods of analysis is satisfactory.

The conditions for sperm DNA fragmentation may not be the same in different animals, mainly because protamine residues, which form an important part of sperm chromatin, differ between species (Ausió *et al.*, 2007). However, the SCD test protocol designed for dogs has resulted equally efficient in analyzing DFI in cats. This finding is not surprising, given the relatively close phylogenetic relationship of the *Canidae* and *Felidae* within the *Carnivora* order and reinforces the idea that the sperm chromatin organization in these two taxa maintains some degree of structural atavism (Portas *et al.*, 2009).

In the present work DFI of cat epididymal spermatozoa ranged from 2.4% to 5.7%. These values are in agreement with those reported in the literature and obtained with different methods (Mota *et al.*, 2006; Thuwanut *et al.*, 2006).

In humans, semen with 30% of spermatozoa with fragmented DNA is considered of low or poor quality to be used in assisted reproduction (Evenson *et al.*, 2002). In feline sperm samples, additional data is necessary to establish a solid threshold value of this parameter.

4.2.2. Exp. 2: Correlation between sperm DNA status, conventional head morphology and CASA morphometry

To the authors' knowledge this is the first time that the relationship between conventional sperm head morphology, CASA morphometry and DNA status has been assessed in cat spermatozoa.

With CASA system, the post-acquisition processing of digitalized data offers an objective and detailed characterization of several sperm morphometric parameters which cannot be detected by conventional visual evaluation. The calculated reference values for the morphological variables of the sperm head were: area 7.34 – 15.59 mm2; aspect 1.69 – 2.86; perimeter 10.44 – 14.87 mm; dmax 4.09 – 6.19 mm; dmin 1.90 – 2.96 mm; radmax 2.12 – 3.20 mm; radmin 0.90 – 1.44 mm; radius ratio 1.83 – 3.24; roundness 1.11 – 1.44.

Thus, this analysis of more than 2400 spermatozoa representing 18 mature tomcats would contribute to the definition of normal values of morphometric measurements that can be used as a background for further

extended studies aimed at better investigating the phenomenon of teratozoospermia in this species.

The 95% confidence interval for DFI and head anomalies evaluated with conventional analysis were 0.037-0.044 % and 0.034-0.047 %, respectively.

The results for PCA analysis are reported in table 1 (Paper II); the first three components account for the 96.62% of the total variability. In particular, the morphological variables are mainly expressed in the first two PC with high correlations. The third component is represented by the DFI only, accounting for the 7.6% of the total variability. Being the PC orthogonal vectors, DFI seems to be independent from the other measured variables. The multivariate results are confirmed by the calculation of the Pearson correlation coefficients: none of the r coefficients resulted significant.

Present data indicate that DFI is independent from sperm head morphology and morphometry. This finding confirms what has been demonstrated in boar (Saravia *et al.*, 2007), but it is in contrast with the general assumption that head shape is mainly related to the status of sperm DNA due to the fact that most of the sperm head is compacted chromatin. Significant relationships among sperm morphometry and the percentage of denatured DNA has been described in dogs (Lange-Consiglio *et al.*, 2010; Núñez-Martinez *et al.*, 2005), bulls (Sailer *et al.*, 1996), brown bears (Alvarez *et al.*, 2008) and humans (Aziz *et al.*, 1998).

In feline epididymal spermatozoa it has been previously shown that head abnormalities are strongly correlated with, and could accurately predict, sperm DNA defects revealed by TUNEL test (Mota *et al.*, 2006). However, the conventional evaluation of sperm head morphology by Diff-Quick staining was only performed and no information on head morphometry were reported.

Morphometry provides a more objective evaluation of the sperm head shape compared to conventional examination of head morphology, and the results of the present study show that head shape is not a reliable predictor of DNA fragmentation in cat spermatozoa. Thus, different factors other than chromatin compaction might affect the sperm head shape (Saravia *et al.*, 2007).

In felids there are large individual variations in semen quality and many wild and domestic cats have a low percentage of normal spermatozoa (Axnér *et al.*, 2007; Swanson *et al.*, 2007). However, teratozoospermic cats may be fertile (Axnér *et al.*, 2007), and this further supports that sperm morphology alone should be interpreted with caution.

For this reason a sperm selection for ICSI typically based on motility and morphology attributes, might not ensure the use of a high quality spermatozoon. Sperm DNA integrity is of crucial importance for the embryo development and concerns have been raised regarding possible use of spermatozoa with DNA damage during ICSI (Seli *et al.*, 2005).

CHAPTER 5

Conclusions

5. Conclusions

The alternative approach to spermatozoa characterization based on the assessment of sperm ubiquitination, DNA fragmentation and morphometry demonstrates for the first time some peculiar aspects of cat epididymal spermatozoa.

The results show the presence of ubiquitinated proteins in cat epididymal semen. However, a correlation between semen quality and ubiquitination process has not been found. Thus, ubiquitin cannot be considered as a biomarker of quality, in terms of morphological and acrosomal patterns, of epididymal feline spermatozoa. Identification of all the ubiquitinated proteins of cat spermatozoa collected from different epididymal regions would contribute to a better understanding of the ubiquitin role in feline sperm maturation **(Paper I).**

Sperm-Halomax® assay specifically developed for canine semen and based on SCD test, provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. The availability of this simple technique could be useful to improve the feline semen evaluation in clinical practice and it could contribute to better select semen samples for biotechnological procedures. In fact, DFI is independent from sperm head morphology and morphometry and

the evaluation of the DNA status of spermatozoa would be of great interest in the completion of the standard analysis of fresh or frozen semen used for ICSI or other ARTs **(Paper II).**

CHAPTER 6

References

6. References

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CHAPTER 7

Summary

7. Summary

The *cauda* of the epididymis is a reservoir of spermatozoa which can be used in assisted reproductive technologies (ARTs). This might significantly contribute to the preservation of genetic material and to the generation of offspring from individuals of high genetic or emotional value that die unexpectedly, undergo orchiectomy for medical reasons or that cannot mate or ejaculate semen.

Epididymal samples should be evaluated prior to the use in ARTs. Routinary semen analysis is based on the conventional evaluation, and, in some cases, on the assessment of other functional integrity parameters.

Other aspects can be evaluated to extend the characterization of spermatozoa. Among these, the assessment of sperm ubiquitination, DNA fragmentation and morphometry represent the alternative approach to feline epididymal spermatozoa characterization described in the present study.

Ubiquitin is a 8.5 kDa peptide that tags other proteins for proteasomal degradation, and it is also involved in the regulation of protein function. It might be responsible of the elimination of defective spermatozoa during transit through epididymis but the exact biological function of this peptide in seminal

plasma has not yet been clarified. Magnetic cell separation techniques, based on the use of antibodies or proteins-coated magnetic beads, as magnetic ubiquitin beads, may allow the selective capture of ubiquitinated spermatozoa from semen, thus contributing to the identification of a potential correlation between semen quality, spermatozoa maturation and ubiquitination process.

The general aim of the first study was to understand whether ubiquitin could be considered a biomarker of quality of epididymal feline semen, and to characterize by a proteomic approach all the proteins ubiquitinated in the spermatozoa retrieved in the three regions of the epididymis.

Results demonstrate the presence of ubiquitinated proteins in cat epididymal semen. However, a correlation between semen quality and ubiquitination process has not been found. Thus, ubiquitin cannot be considered as a biomarker of quality, in terms of morphological and acrosomal patterns, of epididymal feline spermatozoa. Identification of all the ubiquitinated proteins of cat spermatozoa collected from different epididymal regions would contribute to give a better understanding of the ubiquitin role in feline sperm maturation.

Sperm DNA fragmentation is an important parameter to assess sperm quality and can be a putative fertility predictor. Since the sperm head consists almost entirely of DNA, subtle differences in sperm head morphometry might be related to DNA status. Several techniques are available to analyze sperm

DNA fragmentation, but are labor-intensive and require expensive instrumentations. Recently, a kit (Sperm-Halomax®) based on the sperm chromatin dispersion (SCD) test and specifically developed for spermatozoa of different species, but not for cat spermatozoa, became commercially available.

To enhance the evaluation of feline epididymal spermatozoa used in ART the first aim of the second study was to verify the suitability of Sperm-Halomax® specifically developed for canine semen, for the evaluation of DNA status of epididymal cat spermatozoa. For this purpose baseline values of DNA fragmentation obtained with Sperm-Halomax® and TUNEL were compared. The second aim of this study was to investigate whether a correlation between DNA status, sperm head morphology and morphometry assessed by Computer Assisted Semen Analysis (CASA) exists in cat epididymal spermatozoa.

No differences were observed in baseline values of DNA fragmentation index obtained with Sperm-Halomax® and TUNEL. This result indicates that Sperm-Halomax® assay provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa.

The DFI seems to be independent from all the measured variables of sperm head morphology and morphometry. Thus, the evaluation of the DNA status of spermatozoa could effectively contribute to the completion of the standard analysis of semen used in ARTs.

In conclusion, the alternative approach to spermatozoa characterization based on the assessment of sperm ubiquitination, DNA fragmentation and morphometry demonstrates for the first time some peculiar aspects of cat epididymal spermatozoa.

CHAPTER 8

Paper I

Sperm ubiquitination in epididymal feline semen

SPERM UBIQUITINATION IN EPIDIDYMAL FELINE SEMEN.

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AUTHOR CONTRIBUTIONS

GCL, VV and GT contributed to design the study, analyze the data and draft the paper. Laboratory work was carried out by VV, MGM, SN, EM and AN. All authors have approved the final article.

ABSTRACT

Ubiquitin is a 8.5 kDa peptide that tags other proteins for proteasomal degradation, and it is also involved in the regulation of protein function. It has been proposed that ubiquitination might be responsible of the elimination of defective spermatozoa during transit through epididymis in humans and cattle, but the exact biological function of this peptide in seminal plasma has not yet been clarified. In the domestic cat (Felis catus) no positive or negative correlations between semen quality and ubiquitination have been observed.

Magnetic cell separation techniques, based on the use of magnetic beads coated with anti-ubiquitin antibodies, may allow the selective capture of ubiquitinated spermatozoa from semen, thus contributing to the identification of a potential correlation between semen quality, spermatozoa maturation and ubiquitination process.

Moreover, the selective identification of all the proteins ubiquitinated in different epididymal regions could give a better understanding of the ubiquitin role in feline sperm maturation. Therefore, the aims of the present study were 1) to verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads and identify structural (morphological and acrosomal patterns) differences between whole sample and unbound gametes; 2) to extensively characterize all the proteins ubiquitinated in the spermatozoa retrieved in the three regions of the epididymis by a proteomic approach.

In Experiment 1 ubiquitination of the epididymal spermatozoa was evaluated by western blot analysis, then each sample was treated with magnetic ubiquitin beads to verify the possibility of separating ubiquitinated spermatozoa. Sperm parameters (concentration, motility, morphology, acrosomal integrity) were evaluated in the sample treated with beads and in whole sample. In Experiment 2 spermatozoa were retrieved from the three

anatomical portions of the epididymis (caput, corpus and cauda). Each sample was processed with magnetic ubiquitin beads and proteomic analysis was assessed in spermatozoa bound to the beads to extensively characterize all the proteins ubiquitinated in the three regions of the epididymis.

Results of the study demonstrate for the first time the presence of ubiquitinated proteins in cat epididymal semen. However, a correlation between semen quality and ubiquitination process has not been found. Thus, ubiquitin cannot be considered as a biomarker of quality, in terms of morphological and acrosomal patterns, of epididymal feline spermatozoa.

Identification of all the ubiquitinated proteins of cat spermatozoa collected from different epididymal regions would contribute to a better understanding of the ubiquitin role in feline sperm maturation.

Key words: cat, epididymal semen, ubiquitin

INTRODUCTION

High proportions of abnormal spermatozoa characterize the ejaculate of some domestic cats and many wild felids (Howard et al., 1984-1990). Neither the exact etiology, nor significance of the high incidence of abnormal spermatozoa in *Felidae* are known.

The proportion of morphologically abnormal spermatozoa in the domestic cat ejaculate is comparable to that found in the cauda epididymis (Axnér et al., 1998). In the epididymis sperm development and maturation occurs, and therefore the ability of spermatozoa to penetrate and fertilize the female gamete is enhanced. During epididymal transit the percentage of immature, unviable and abnormal spermatozoa decreases, indicating the existence of a mechanism that removes defective spermatozoa (Axnér, 2006).

Thus, the epididymis might acts as a quality-control organ to prevent misshapen, genetically abnormal or infertile spermatozoa from entering the ejaculate.

The hypothesis of epididymal removal of defective spermatozoa is based on the fact that a small proportion of bovine spermatozoa in the corpus epididymis are ubiquitinated and that cultured epididymal epithelial cells are capable of spermiophagy (Sutovsky et al., 2001).

Ubiquitin is a 8.5 kDa peptide that tags other proteins for proteasomal degradation, and it is also involved in the regulation of protein function. This protein is a normal component of human blood, ovarian follicular fluid and seminal plasma (Nandi et al., 2006) and its role in the elimination of defective spermatozoa during transit through epididymis has been described in humans and cattle (Baska et al., 2008; Sutovsky et al., 2004).

Results demonstrated that the increase of sperm ubiquitin is inversely associated with spermatic concentration, motility and normal morphology indicating that ubiquitination could be a biomarker of poor semen quality (Sutovsky et al., 2004). Conversely, other

authors (Muratori et al., 2005) found a positive correlation between sperm ubiquitin and good semen parameters suggesting a different role for sperm ubiquitination.

Therefore, the role played by ubiquitination of spermatozoa proteins and their function during the transit through epididymis is still an open question, even in species whose semen properties have been extensively studied, such as human and bovine. Regarding the domestic cat (*Felis catus*), few researches have been conducted and no positive or negative correlations between semen quality and ubiquitination have been observed (Mota et al., 2006).

Magnetic cell separation techniques, based on the use of magnetic beads coated with anti-ubiquitin antibodies, may allow the selective capture of ubiquitinated spermatozoa from semen, thus contributing to the identification of a potential correlation between semen quality, spermatozoa maturation and ubiquitination process. This would contribute to understand whether ubiquitin could be considered a biomarker of quality of epididymal feline semen. Moreover, the selective identification of all the proteins ubiquitinated in different epididymal regions could give a better understanding of the ubiquitin role in feline sperm maturation (Kuster et al., 2004; Suryawanshi et al., 2011; Souza et al., 2012; Yuan et al., 2013).

The evaluation of ubiquitination pattern of seminal protein has been applied to human semen (Oliva et al., 2009; Oliva and Castillo, 2011) and today, with the advances of proteomic techniques, thousands of proteins have been described as being part of normal human semen (Amaral et al., 2014). The challenges for analyzing protein ubiquitination are largely due to low stoichiometry of ubiquitinated species in cells (Peng, 2008). To overcome these limitations, it is essential to enrich for ubiquitinated proteins prior to mass spectrometry (MS) analysis. In the present study this strategy, combined to a shot gun

proteomic approach, has been applied for the first time to semen of domestic cat treated with magnetic ubiquitin beads before MS/MS analysis to identify all the proteins ubiquitinated in the epididymal tract.

Therefore, the aims of the present study were 1) to verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads and identify structural (morphological and acrosomal patterns) differences between whole sample and unbound gametes; 2) to extensively characterize all the proteins ubiquitinated in the spermatozoa retrieved in the three regions of the epididymis by a proteomic approach.

MATERIALS AND METHODS

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

Animals

Twenty-two healthy and pubertal cats presented to the Department for routine orchiectomy were included in this study.

Experimental design

In Experiment 1 semen samples were obtained from ten pairs of isolated testes by squeezing cauda epididymis and vasa deferentia in a warm (37°C) Phosphate Buffered Saline (PBS) solution. Ubiquitination of the epididymal spermatozoa was evaluated by western blot analysis as described in detail in the following section.

To verify the possibility of separating ubiquitinated spermatozoa with magnetic ubiquitin beads the sample was divided into two aliquots: the first was processed with magnetic

ubiquitin beads and the second aliquot was not treated and used as control. Sperm parameters (concentration, motility, morphology, acrosomal integrity) were evaluated in the sample treated with beads (unbound spermatozoa, see below) and in whole sample (control).

In Experiment 2 twelve pairs of epididymides were processed. 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. Each pair of epididymides was macroscopically divided into three anatomical portions, caput, corpus and cauda, according to previous studies (Schimming et al., 1997; Schimming and Vicentini, 2001) and each pair of portions was placed in a Petri dish containing 2 ml of PBS. 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. Each sample was processed with magnetic ubiquitin beads and proteomic analysis was assessed in spermatozoa bound to the beads to extensively characterize all the proteins ubiquitinated in the three regions of the epididymis.

SDS-PAGE, 2D electrophoresis and western blot analysis

Whole semen samples were suspended in 0.5 M Tris–HCl pH 6.8, 10% glycerol and 10% sodium dodecyl sulfate (SDS). The protein content was determined by Bradford method (Bradford, 1976) and separated on an homemade 11% polyacrylamide gel according to Laemmli (1970). 2D-electrophoresis was carried out as described in Tedeschi et al. (2005). For the first dimension, proteins were applied to rehydrated IPG strips (70 mm, 3–10 NL) (Amersham Biosciences, Cologno Monzese, Italy). Isoelectric focusing was performed at

15°C as follows: 600 V for 10 min, 900 V for 15 min, 1,500 V for 15 min, 2,500 V for 15 min and 3,500 V for 5 h and 15 min. Before the second dimension, each strip was rinsed with buffer (6 M urea in 0.375 M Tris–HCl pH 8.8, 2% SDS, 20% glycerol, bromophenol blue). The second dimension was performed on homemade 12% SDS minigels (8.5 x 6 x 0.15 cm) at 20 mA/gel.

Proteins in the gel were stained with ammonium silver stain or transferred from the gel onto a polyvinylidene difluoride membrane (PVDF, Immobilon P, Millipore, Bedford, MA, USA) by electrophoretic elution at 180 mA for 1 hour. The transfer buffer was 10 mM CAPS and 10% methanol. Membranes were then probed for immunoreactivity as follows: for ubiquitin detection membrane was blocked with 5% dry milk in Tris Buffered Saline (TBS), washed five times with TBS-T 0.25%, incubated in rabbit anti-ubiquitin polyclonal antibody (AbCam, Cambridge, UK) to 1:1000 in 2% dry milk in TBS-T 0.25%. The membrane was washed five times with TBS-T 0.25%, incubated in anti-IgG rabbit HRP conjugated (Calbiochem Darmstadt, Germany) 1:1000 in 2% dry milk in TBS-T 0.25%.

For actin detection, the membrane was blocked with 10% dry milk in TBS-T 0.1%, washed three times with TBS-T 0.1%, incubated in mouse anti-actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO, USA) to 1:4000 in 5% dry milk in TBS-T 0.1%. The membrane was washed three times with TBS-T 0.1%, incubated in anti-IgG mouse HRP conjugated (Calbiochem Darmstadt, Germany) 1:2000 in 5% dry milk in TBS-T 0.1%.

For tubulin detection membrane was blocked with 5% dry milk in TBS-T 0.05%, washed three times with TBS-T 0.05%, incubated in mouse anti-tubulin monoclonal antibody (AbCam, Cambridge, UK) to 1:1000 in 1% bovine serum albumin (BSA) in TBS-T 0.05%.

The membrane was washed three times with TBS-T 0.05%, incubated in anti-IgG mouse HRP conjugated (Calbiochem Darmstadt, Germany) 1:20000 in 1% BSA in TBS-T 0.05%.

Sperm separation with magnetic ubiquitin beads

Magnetic ubiquitin beads (Li Starfish S.r.l., Cernusco S/N, Milan, Italy) suspended in 10 mM phosphate solution (pH 7.5) with 0.02% sodium azide were added to semen aliquots (80 µl beads/ml of semen) in a 1.5 ml tube. The tube was gently mixed for 20 min, to allow contact between the magnetic beads and the targeted spermatozoa before placing it in a magnetic field for 10 min. The separation procedure is a negative depletion in which the magnetic beads attach to the targeted surface marker and are collected against the wall of the tube by application of an external laboratory magnet. The separated sample was decanted and collected while the tube was still in the magnetic field, while the ubiquitinated spermatozoa bound to the beads remained attached to the wall of the tube as long as the magnet was in place. Thus, two fractions were obtained: spermatozoa bound to the beads and spermatozoa unbound in the tube.

Spermatozoa evaluation

Sperm concentration was determined with a Bürker chamber. 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) (Mortimer, 1994).

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 (Christiansen, 1984). 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 (Cheng et al., 1996). 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 96% ethanol for 30 sec. 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".

Mean±SD of sperm characteristics were analyzed by Student's t-test ($p<0.05$).

Mass spectrometry analysis

In order to increase the homogeneity of the sample and overcome the inter- and intra-sample variability, epididymal spermatozoa of different cats collected from caput (A), corpus (B) or cauda (C) and bound to the magnetic beads were pooled.

The ubiquitin–conjugate proteins were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the identities as well as the ubiquitination sites after tryptic digestion, and missed tryptic cleavage at the modified site. In particular, the spermatozoa bound to the magnetic beads were collected and lysed in a buffer containing 7 M urea, 2 M thiourea, 50 mM ammonium bicarbonate and the complete protease inhibitor cocktail (Roche, Basel, Switzerland).

Upon sonication the lysates were clarified by centrifugation at 15000 rpm for 15 min and the total protein concentration was determined using the Bradford method (Bradford, 1976). Protein samples were reduced with 45 mM dithiothreitol, alkylated with 100 mM iodoacetamide and subsequently digested with Sequencing Grade Modified Trypsin overnight at 37°C. Digestion was stopped by adding 1 µl of 98% formic acid. The proteolytic digests were desalted on a ZipTipC_{18} (Millipore, Billerica, MA, USA) before MS analysis. Each sample was separated by liquid chromatography using an UltiMate 3000 HPLC (Dionex, now Thermo Fisher Scientific, Waltham, MA, USA). Buffer A was 0.1% v/v formic acid, 2% acetonitrile; buffer B was 0.1% formic acid in acetonitrile. Chromatography was performed using a PepMap C18 column (15 cm, 180 lm ID, 3 lm resin, Dionex). The gradient was as follows: 5% buffer B (10 min), 5-40% B (60 min), 40-50% B (10 min), 95% B (5 min) at a flow rate of 0.3 µL/min.

Mass spectrometry was performed using a LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a nanospray source (Proxeon Biosystems, now

Thermo Fisher Scientific, Waltham, MA, USA). Eluted peptides were directly electrosprayed into the mass spectrometer through a standard non-coated silica tip (New Objective, Woburn, MA, USA) using a spray voltage of 2.8 kV. The LTQ-Orbitrap was operated in positive mode in data-dependent acquisition mode to automatically alternate between a full scan (m/z 350–2000) in the Orbitrap and subsequent CID MS/MS in the linear ion trap of the 20 most intense peaks from full scan. Three replicate analyses of each sample were performed.

Data acquisition was controlled by Xcalibur 2.0 and Tune 2.4 software (Thermo Fisher Scientific, Waltham, MA, USA).

Protein identification and computational analysis

For identification of ubiquitinated proteins, raw data files were processed and analyzed using MaxQuant 1.3.0.5. Parent ion and MS2 spectra were searched against the Human and Felis catus Uniprot/SwissProt database using the Andromeda search engine. Search parameters allowed for 2 missed tryptic cleavages, a mass tolerance of 6 ppm in MS mode and 20 ppm in CID MS/MS mode, a static modification carbamidomethylation (Cys), and up to 3 total dynamic modifications [(N-acetylation (protein), oxidization (Met) and ubiquitination (Lys)]. To achieve highly reliable identifications, the following criteria were used: maximal protein, peptide and site FDR of 0.01 and minimal peptide length of 6. The default setting of the maximal peptide posterior error probability of 1 was used. The presence of ubiquitinated amino acids on each peptide was confirmed upon visual inspection of the corresponding MS/MS spectrum by Perseus, discharging all the spectra presenting a modification at the C-terminal lysine.

RESULTS AND DISCUSSION

Exp. 1. SDS-PAGE, 2D electrophoresis and western blot analysis

The presence of ubiquitinated proteins in cat epididymal semen was evaluated by anti-ubiquitin antibodies upon separation on monoD electrophoresis of the whole-cell extract (Fig.1A). The pattern of ubiquitination is very similar to the one described by Thompson et al. (2003) in bovine seminal plasma for the proteins at higher molecular weight (more than 50 kDa) (see Fig. 5C in Thompson et al., 2003 for comparison). The major differences can be observed at lower molecular weight (37-20 kDa) where few bands, clearly detectable in cat semen, are absent in the bovine sample.

The semen was analyzed by 2D-electrophoresis to better separate all the proteins and evaluate the ubiquitination pattern. Fig. 1B shows all the proteins detected by silver staining while Fig. 1C reports the corresponding western blot analysis using anti-ubiquitin antibodies which clearly shows that the proteins ubiquitinated are mainly present in the 70-40 kDa region except for few spots at lower molecular weight. The 70-40 kDa region was further investigated looking for the possible ubiquitination of the two most abundant proteins of the cytoskeleton: actin and tubulin, in order to better understand the correlation between ubiquitination and morphological alterations of the spermatozoa.

The table in Fig 2A reports the expected molecular weight of the two proteins with and without the modification, while Fig. 2B reports the western blot analysis using anti-ubiquitin antibodies following anti-actin and anti-tubulin antibodies upon stripping of the membrane, as described in detail in the Methods section. The results clearly suggest that actin is indeed ubiquitinated in cat spermatozoa while the modification of tubulin was not detected. The same observation was further confirmed in the proteomic analysis described in the section below.

Exp. 1. Evaluation of spermatozoa treated with magnetic ubiquitin beads

A significant decrease of sperm concentration and sperm count was observed after treatment with magnetic ubiquitin beads in the fraction of spermatozoa unbound to the beads (Table 1). No significant differences were observed in other sperm parameters (motility, morphology and acrosome integrity). These findings demonstrate the efficacy of beads in binding ubiquitinated spermatozoa as the sperm concentration and count were significantly decreased after treatment.

However, the unbound fraction was not characterized by higher sperm motility, morphology and acrosomal integrity. Therefore, the magnetic beads did not select a population of cat epididymal spermatozoa with better quality.

These results confirm the lack of correlation between ubiquitinated spermatozoa and morphological abnormal spermatozoa as previously reported by Mota et al. (2006).

Exp. 2. Mass spectrometry analysis, protein identification and computational analysis

The traditional proteomic approach contemplates a preliminary step of protein purification by 1D or 2D electrophoresis, followed by mass spectrometry identification of the proteins present. However, the availability of new, advanced and high throughput analytical platforms allow identification of thousands of proteins from a biological sample without a previous separation step, the so called shot gun proteomic approach, which relies on tandem mass spectroscopy for protein identification and characterization of post-translational modifications (PTM). This approach has been applied here to characterize ubiquitination of the proteins in the cat spermatozoa collected from different epididymal regions.

Each fraction enriched in ubiquitinated proteins was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the identities and the ubiquitination sites of the proteins present in the samples. The proteomic analysis, carried out combining the results of three replicates, allowed to identify 766, 708 and 646 proteins in A, B and C, respectively, (see Table S1, S2, and S3 in supplementary materials) among which 212 proteins are common in the three epididymal tracts (A, B and C) and are reported in Table 2. No one of the 212 common proteins is ubiquitinated. Focusing only on the ubiquitinated proteins expressed in each epididymal tract, it was possible to identify 7 proteins in A, 17 in B and 6 in C, in total 30 proteins reported in Table 3. For each of them, one representative MS/MS sprectrum of a ubiquitinated peptide is reported as supplementary materials in Figures S1-S30. As indicated in Table 3, ubiquitination of many of these proteins have been previously observed in human or mouse, according to the PhosphoSite Plus Data Base.

The distribution of the 30 ubiquitinated proteins in terms of molecular function (GOMF), cellular components (GOCC) and biological processes (GOBP) are reported in the corresponding histograms of Fig. 3. Among the ubiquitinated proteins, there are cytoskeletal proteins or proteins interacting with the cytoskeleton and are key players in the cytoskeleton organization, in cellular differentiation and in the morphogenesis processes. Overall, more than 30% of the ubiquitinated proteins are involved in the anatomical structure development in keeping with the possible role of this modification as a way to remove defective spermatozoa in the epididymal tract. Most are proteins important for the regulation of gene expression or are components of signal transduction pathways suggesting that ubiquitin, by playing a major role in the modulation of their

homeostasis and expression level, may affect also the expression level and the activation of other proteins present in the epididymal environment.

However, to better understand the significance of the presence of specific ubiquitinated proteins in the different epididymal regions further investigations are required.

CONCLUSIONS

The present data demonstrate for the first time the presence of ubiquitinated proteins in cat epididymal semen. However, a correlation between semen quality and ubiquitination process has not been found. Thus, ubiquitin cannot be considered as a biomarker of quality, in terms of morphological and acrosomal patterns, of epididymal feline spermatozoa.

Identification of all the ubiquitinated proteins of cat spermatozoa collected from different epididymal regions would contribute to a better understanding of the ubiquitin role in feline sperm maturation.

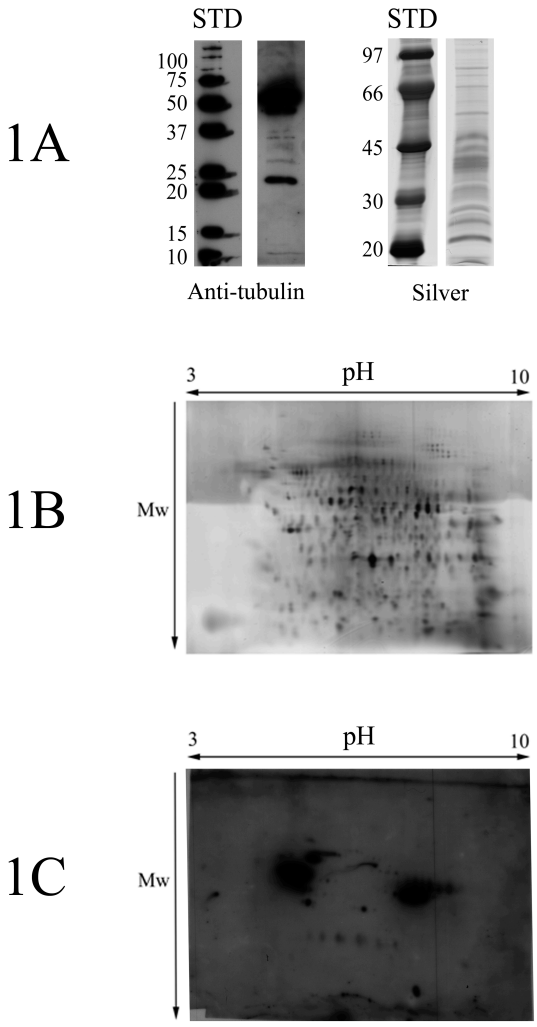
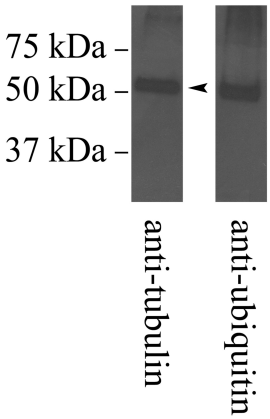


Fig. 1. 1D and 2D electrophoresis of whole cat semen and western blot analysis by anti-ubiquitin antibodies. Fig.1A 1D electrophoresis: proteins were separated on two homemade 11% polyacrylamide gels. One was blotted on a PVDF membrane to detect ubiquitinated proteins by anti-ubiquitin antibodies, while the other was stained by silver to detect all the proteins present in the sample. Fig.1B-C: semen proteins were separated by 2D electrophoresis and either stained by silver (B) or blotted and immunodecorated with anti-ubiquitin antibodies (C). STD=molecular weight standard.

2A

Proteins	no ubiquitin (kDa)	+1 ubiquitin (kDa)	+2 ubiquitin (kDa)	+3 ubiquitin (kDa)
Tubulin	49.9	58.4	66.1	75.4
Actin	42.0	50.5	59.0	67.5

2B



2C

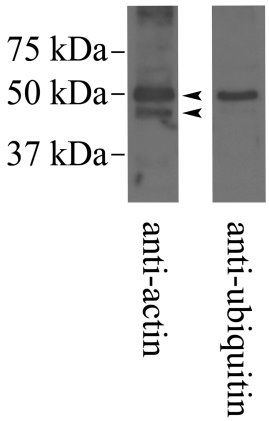


Fig.2. Possible ubiquitination of actin and tubulin. 2A: the table reports the expected molecular weights of actin and tubulin in the absence or in the presence of up to three ubiquitin chains. 2B: western blot analysis by anti-ubiquitin antibodies and anti-actin antibodies upon stripping of the membrane. 2C: western blot analysis by anti-ubiquitin antibodies and anti-tubulin antibodies upon stripping of the membrane.

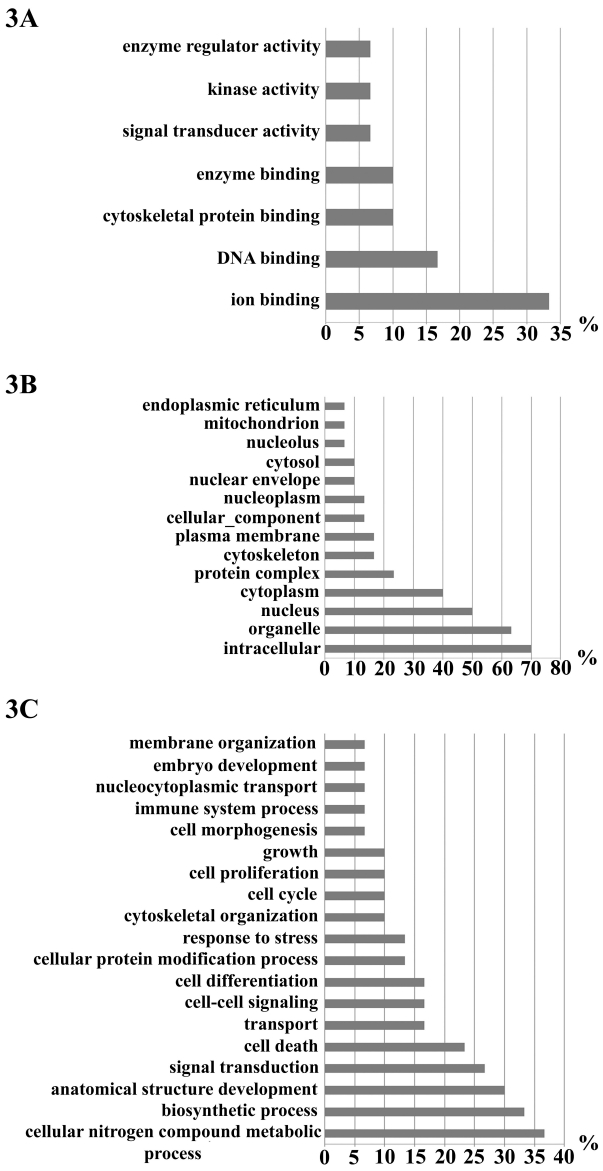


Fig. 3. Histograms of the distribution of the ubiquitinated proteins in the cat epididymal spermatozoa. The epididymal fluid was enriched in ubiquitinated proteins by using magnetic beads and bound spermatozoa were analyzed by a shot gun proteomics approach. The distribution of the ubiquitinated proteins in terms of molecular function (GOMF), cellular components (GOCC) and biological processes (GOBP) are reported in Fig. 3A, 3B and 3C, respectively.

Table 1. Sperm parameters in cat epididymal semen untreated or treated with magnetic ubiquitin beads.

	Sperm concentration (sp/ml)*106	Sperm count (sp/sample)*10^6	Normal morphology (%)	Motility (%)	Acrosome integrity (%)
Untreated control	148.9±102.8a	12.0±7.5a	44.5±16.4	51.0±16.0	76.5±15.1
Treated with beads	47.1±31.6b	6.7±5.2b	52.9±11.9	49.0±21.7	81.3±13.0

Different superscripts (ab) within columns indicate significant differences ($p<0.01$)

Table 2. Proteomic analysis of the proteins in cat seminal plasmasemen collected from three epididymal tracts: caputs (A), corpus (B) and cauda (C). The Table reports only the proteins common among the three epididymal regions, while the complete proteome is reported as Ssupplementary material. The table reports the number of razor plus unique peptides which allowed the identification of the corresponding proteins in caput (A), corpus (B) and cauda (C).

Table 2

Protein names	Gene names	Razor + unique peptidesA	Razor + unique peptidesB	Razor + unique peptidesC
F-actin-capping protein subunit alpha-2	CAPZA2	3	2	3
Aconitate hydratase, mitochondrial	ACO2	5	7	7
40S ribosomal protein SA	RPSAP58;RPSA	2	2	2
Annexin A6;Annexin	ANXA6	3	4	3
Glutathione S-transferase P	GSTP1	1	1	1
Vimentin	VIM	10	12	14
Plastin-3	PLS3	10	10	5
Calnexin	CANX	2	4	3
Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	ACADM	1	1	1
Voltage-dependent anion-selective channel protein 2	VDAC2	6	5	4
Prostaglandin E synthase 3	PTGES3	2	2	1
6-phosphogluconate dehydrogenase, decarboxylating	PGD	3	2	4
Histone H2B	HIST2H2BF	1	1	1
Polyubiquitin-C;ribosomal protein L40	UBC;UBB;RPS27A;UBA52	1	1	1
GTP-binding nuclear protein Ran	RAN	3	3	1
N-acetylglucosamine-6-sulfatase	GNS	2	2	2
3-ketoacyl-CoA thiolase	HADHB	4	4	1
Cellular retinoic acid-binding protein 1	CRABP1	1	3	1
Protein disulfide-isomerase A6	PDIA6	2	6	4
Alpha-actinin-1	ACTN1	8	7	8
Reticulocalbin-1	RCN1	2	1	1
V-type proton ATPase catalytic subunit A	ATP6V1A	4	2	4
Phosphoglycerate kinase 1;Phosphoglycerate kinase	PGK1	4	7	8
Protein-L-isoaspartate O-methyltransferase	PCMT1	2	2	2
Histone H2A.Z;Histone H2A.V;Histone H2A	H2AFZ;H2AFV	4	4	1
Fibrinogen gamma chain	FGG	1	2	1
Ran-specific GTPase-activating protein	RANBP1	2	2	2
Myotrophin	MTPN	2	2	1
Rho-related GTP-binding protein RhoC;Transforming protein RhoA	RHOC;RHOA	2	1	2
Fibrinogen beta chain;Fibrinopeptide B;Fibrinogen beta chain	FGB	3	4	1
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA	3	4	3

Acrosin-binding protein	ACRBP	2	2	2
Ezrin	EZR	1	5	6
Nucleoside diphosphate kinase	NME1-NME2	3	2	2
Rab GDP dissociation inhibitor beta	GDI2	4	3	6
Fibronectin;Anastellin;Ugl-Y1;Ugl-Y2;Ugl-Y3	FN1	3	3	2
Nucleobindin-2	NUCB2	1	2	1
Hypoxia up-regulated protein 1	HYOU1	4	9	4
26S protease regulatory subunit 6A	PSMC3	2	6	6
NSFL1 cofactor p47	NSFL1C	1	1	1
T-complex protein 1 subunit beta	CCT2	4	11	13
Mannose-6-phosphate isomerase	MPI	1	1	1
C-1-tetrahydrofolate synthase	MTHFD1	3	3	4
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha	PPP2R1A	4	2	5
Voltage-dependent anion-selective channel protein 3	VDAC3	1	3	3
Protein disulfide-isomerase	P4HB	7	8	6
Destrin	DSTN	1	3	3
Hsc70-interacting protein;Putative protein FAM10A5;Putative protein FAM10A4	ST13;ST13P5;ST13P4	2	2	2
Aldehyde dehydrogenase, mitochondrial	ALDH2	6	3	3
Serine/threonine-protein phosphatase PP1-gamma catalytic subunitsubunit	PPP1CC;PPP1CA;PPP1CB	2	1	4
Myosin light polypeptide 6	MYL6	6	6	6
Protein canopy homolog 2	CNPY2	2	3	3
Heat shock protein beta-1	HSPB1	1	2	1
Inositol-3-phosphate synthase 1	ISYNA1	2	1	4
ERO1-like protein alpha	ERO1L	1	2	1
Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	ALDH6A1	2	2	1
Izumo sperm-egg fusion protein 4	IZUMO4	1	1	2
Protein disulfide-isomerase A3	PDIA3	7	5	6
Calmodulin	CALM2;CALM1	3	8	6
Coiled-coil domain-containing protein 38	CCDC38	1	1	1
Creatine kinase B-type	CKB	4	6	2
Annexin A2;Annexin;Putative annexin A2-like protein	ANXA2;ANXA2P2	9	10	9
Fructose-bisphosphate aldolase A;Fructose-bisphosphate aldolase	ALDOA	6	8	7
Thioredoxin	PDIA3	1	5	1
Isovaleryl-CoA dehydrogenase,	IVD	2	3	3

mitochondrial				
Thioredoxin domain-containing protein 17	TXNDC17	1	3	2
Actin, cytoplasmic 2;Actin, cytoplasmic 2, N-terminally processed	ACTG1	12	1	1
Pigment epithelium-derived factor	SERPINF1	1	1	2
Rho GDP-dissociation inhibitor 1	ARHGDIA	3	4	2
Myosin regulatory light chain 12B;Myosin regulatory light chain 12A	MYL12B;MYL12A	2	5	4
Granulins	GRN	1	1	1
Calpain small subunit 1	CAPNS1	3	3	4
Membrane-associated progesterone receptor component 1	PGRMC1	2	2	2
Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	GAPDHS	1	4	6
Proteasome subunit alpha type	PSMA7;PSMA8	2	4	4
Alpha-actinin-4	ACTN4	8	7	3
WD repeat-containing protein 1	WDR1	3	3	3
Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	4	4	4
Cytochrome b5	CYB5A	2	4	3
Retinal dehydrogenase 1	ALDH1A1	1	9	8
Glutamate dehydrogenase 1	GLUD1;GLUD2	5	6	3
Carbonic anhydrase 2	CA2	1	3	3
Collagen alpha-1(I) chain	COL1A1	2	2	4
Prelamin-A/C;Lamin-A/C	LMNA	5	6	7
Fibrinogen alpha chain;Fibrinopeptide A;Fibrinogen alpha chain	FGA	1	1	1
Band 3 anion transport protein	SLC4A1	2	2	2
Transthyretin	TTR	1	1	1
ATP synthase subunit beta, mitochondrial;ATP synthase subunit beta	ATP5B	15	16	18
Alpha-enolase	ENO1	8	10	9
L-lactate dehydrogenase B chain;L-lactate dehydrogenase	LDHB	5	8	10
Tubulin beta chain	TUBB	10.5	14.5	3
Profilin-1	PFN1	2	2	2
Heat shock protein HSP 90-alpha	HSP90AA1	8.5	9.5	23
Heat shock 70 kDa protein 1A/1B	HSPA1A	8	12	1
Collagen alpha-2(I) chain	COL1A2	1	1	1
Heat shock protein HSP 90-beta	HSP90AB1	11	4	4
Annexin A5;Annexin	ANXA5	3	6	5
Galectin-1	LGALS1	2	2	2
Fructose-bisphosphate aldolase	ALDOC	3	3	3

Histone H2A type 1	HIST1H2AG;	4	4	1
Histone H1.4	HIST1H1E	2	3	2
60 kDa heat shock protein, mitochondrial	HSPD1	11	14	14
78 kDa glucose-regulated protein	HSPA5	18	27	23
Heat shock cognate 71 kDa protein	HSPA8	11	17	14
Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	PDHB	1	4	3
Spectrin beta chain, erythrocyte	SPTB	17	15	12
Elongation factor 2	EEF2	5	5	6
Protein disulfide-isomerase A4	PDIA4	1	6	4
Plastin-2	LCP1	10	10	12
Electron transfer flavoprotein subunit alpha, mitochondrial	ETFA	2	5	4
cAMP-dependent protein kinase type II-alpha regulatory subunit	PRKAR2A	1	5	10
Beta-enolase;Gamma-enolase;Enolase	ENO3;ENO2	1.5	1	1
Alcohol dehydrogenase [NADP(+)]	AKR1A1	1	3	4
Pyruvate kinase isozymes M1/M2;Pyruvate kinase	PKM2	6	7	2
Endoplasmin	HSP90B1	14	27	15
Glutamine synthetase	GLUL	6	5	6
Ubiquitin carboxyl-terminal hydrolase isozyme L3	UCHL3	1	4	4
Ankyrin-1	ANK1	15	10	8
Histone H1.2	HIST1H1C	2	3	3
Sarcoplasmic/endoplasmic reticulum calcium ATPase 2	ATP2A2	1	3	3
Desmin	DES	2	7	8
T-complex protein 1 subunit alpha	TCP1	1	4	7
Vinculin	VCL	5	4	3
Phosphoglycerate mutase 1;Probable phosphoglycerate mutase 4	PGAM1;PGAM4	1	4	5
Proteasome subunit beta type-1	PSMB1	1	2	2
Mimecan	OGN	3	3	3
Filamin-A	FLNA	11	12	17
Ubiquitin-like modifier-activating enzyme 1	UBA1	5	5	8
Heterogeneous nuclear ribonucleoproteins A2/B1	HNRNPA2B1	2	3	1
Peptidyl-prolyl cis-trans isomerase B	PPIB	6	9	5
NAD-dependent malic enzyme, mitochondrial	ME2	1	4	1
Adenosylhomocysteinase	AHCY	1	3	3
Cofilin-1	CFL1	4	7	6
Myosin regulatory light polypeptide 9	MYL9	4	4	3

ATP synthase subunit alpha, mitochondrial	ATP5A1	9	11	12
Proteasome subunit alpha type-1;Proteasome subunit alpha type	PSMA1	1	2	2
Moesin	MSN	4	1	3
Elongation factor 1-gamma	EEF1G	2	3	6
Calreticulin	CALR	3	6	4
Proteasome subunit alpha type-5	PSMA5	2	4	4
Cytosol aminopeptidase	LAP3	2	6	4
Endoplasmic reticulum resident protein 29	ERP29	2	6	6
Flavin reductase (NADPH)	BLVRB	3	2	2
Peroxiredoxin-5, mitochondrial	PRDX5	5	4	3
Enoyl-CoA hydratase, mitochondrial	ECHS1	1	2	2
Phosphatidylethanolamine-binding protein	PEBP1	1	3	2
S-adenosylmethionine synthase	MAT2A	1	2	3
Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	1	1	2
3-hydroxyisobutyrate dehydrogenase, mitochondrial	HIBADH	2	2	3
14-3-3 protein beta/alpha	YWHAB	2	3	4
Peroxiredoxin-2	PRDX2	4	5	4
Heat shock 70 kDa protein 1-like	HSPA1L	8	12	11
Myosin-9	MYH9	3.5	10	20
Myosin-11	MYH11	2	10	12
26S protease regulatory subunit 7	PSMC2	2	2	2
Transgelin-2	TAGLN2	5	5	6
Stress-70 protein, mitochondrial	HSPA9	6	5	7
T-complex protein 1 subunit zeta	CCT6A	3	6	9
Malate dehydrogenase	MDH2	6	7	7
Serpin B10	SERPINB10	1	1	1
T-complex protein 1 subunit gamma	CCT3	4	9	11
T-complex protein 1 subunit theta	CCT8	2	7	12
Heterogeneous nuclear ribonucleoprotein M	HNRNPM	4	9	3
Heat shock-related 70 kDa protein 2	HSPA2	1	5	6
26S proteasome non-ATPase regulatory subunit 4	PSMD4	2	1	5
Transitional endoplasmic reticulum ATPase	VCP	14	23	22
Triosephosphate isomerase	TPI1	5	7	8
Ras-related protein Rab-2A	RAB2A	2	6	5
ADP-ribosylation factor 3	ARF3;ARF1;ARF4;ARF5	1	2	2
Heterogeneous nuclear ribonucleoprotein K	HNRNPK	4	4	4
14-3-3 protein gamma	YWHAG	4	3	2

26S protease regulatory subunit 8	PSMC5	1	3	3
14-3-3 protein epsilon	YWHAE	5	3	3
Thymosin beta-4;Hematopoietic system regulatory peptide	TMSB4X;TMSL4	1	2	1
Actin, aortic smooth muscle;Actin, gamma-enteric smooth muscle	ACTA2;ACTG2	1	1	2
Histone H4	HIST1H4A	5	5	4
Peptidyl-prolyl cis-trans isomerase A;Peptidyl-prolyl cis-trans isomerase	PPIA	1	1	1
Peptidyl-prolyl cis-trans isomerase FKBP1A	FKBP1A;FKBP12-Exip2	2	2	1
14-3-3 protein zeta/delta	YWHAZ	7	6	9
Dynein light chain 1, cytoplasmic	DYNLL1	1	5	3
Thymosin beta-10	TMSB10	1	1	1
Actin, alpha cardiac muscle 1;Actin, alpha skeletal muscle	ACTC1;ACTA1	8	9	19
Elongation factor 1-alpha 1;Putative elongation factor 1-alpha-like 3	EEF1A1;EEF1A1P5	4	7	5
Tubulin beta-4B chain	TUBB4B	18	25	25
Clathrin heavy chain 1	CLTC	11	6	6
Lactoylglutathione lyase	GLO1	2	2	2
Proteasome activator complex subunit 1	PSME1	2	5	3
Peroxiredoxin-1	PRDX1	7	9	8
26S proteasome non-ATPase regulatory subunit 2	PSMD2	1	1	3
Tubulin beta-3 chain	TUBB3	18	25	2
Tubulin alpha-3C/D chain;Tubulin alpha-3E chain	TUBA3C;TUBA3E	1	3	21
Eukaryotic initiation factor 4A-II	EIF4A2;EIF4A1	1	4	4
Poly(rC)-binding protein 1	PCBP1	4	4	4
Regucalcin	RGN	2	1	1
NEDD8	NEDD8;NEDD8-MDP1	1	1	2
Outer dense fiber protein 2	ODF2	4	16	18
SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	1	2	1
Polymerase I and transcript release factor	PTRF	1	2	2
	PPIA	6	1	5
BTB/POZ domain-containing protein KCTD12	KCTD12	1	1	1
Dynein light chain 2, cytoplasmic	DYNLL2	1	2	5
Cytosolic non-specific dipeptidase	CNDP2	1	3	2
Protein DJ-1	PARK7	3	6	6
Synaptic vesicle membrane protein VAT-1 homolog	VAT1	2	2	2
3-hydroxyacyl-CoA dehydrogenase type-2	HSD17B10	3	3	4

T-complex protein 1 subunit eta	CCT7	4	7	8
Histidine triad nucleotide-binding protein 2, mitochondrial	HINT2	2	3	2
DnaJ homolog subfamily B member 11	DNAJB11	1	2	1
Proteasome activator complex subunit 2	PSME2	2	3	2
Talin-1	TLN1	11	12	14
Mannose-1-phosphate guanyltransferase beta	GMPPB	1	1	1

Table 3. Ubiquitinated proteins found in the three epididymal regions: caput (A), corpus (B) and cauda (C). The X in the PSPLUS column indicates proteins previously described ubiquitinated in human or mouse according to the PhosphoSite Plus Data Base.

Protein ID	Protein names	Gene names	Localization	PSPLUS
P23141-2	Liver carboxylesterase 1	CES1;CES1	A	X
H0Y8G3		IL7R	A	
H0YL13	uncharacterized protein	UNC13C	A	
O60290	Zinc finger protein 862	ZNF862	A	
Q8WWQ0	PH-interacting protein	PHIP	A	X
Q9UQ13	Leucine-rich repeat protein SHOC-2	SHOC2	A	X
Q96DR7	Rho guanine nucleotide exchange factor 26	ARHGEF26	A	
Q8NF91	Nesprin-1	SYNE1;SYNE1;SYNE1	B	X
B3KSY9	E3 SUMO-protein ligase PIAS1	PIAS1	B	
Q68DE3	Basic helix-loop-helix domain-containing protein KIAA2018	KIAA2018;KIAA2018	B	X
O95069	Potassium channel subfamily K member 2	KCNK2	B	
O95989	Diphosphoinositol polyphosphate phosphohydrolase 1	NUDT3	B	
A2A2Y4	FERM domain-containing protein 3	FRMD3	B	
E7EUH9	Condensin-2 complex subunit G2	NCAPG2	B	X
G3V5U4	Paired box protein Pax-2	PAX2	B	
Q8WXH0-2	Nesprin-2	SYNE2;SYNE2	B	X
H7C3W3	AF4/FMR2 family member 3	AFF3	B	
Q7Z7G2	Complexin-4	CPLX4	B	
O96028	Probable histone-lysine N-methyltransferase NSD2	WHSC1	B	X
P45983	Mitogen-activated protein kinase 8	MAPK8;MAPK8	B	X
P51813	Cytoplasmic tyrosine-protein kinase BMX	BMX	B	
Q8N8A8	Protein FAM169B	FAM169B	B	
Q8N9L7	Putative uncharacterized protein FLJ36925		B	
Q92908	Transcription factor GATA-6	GATA6	B	
O95257	inducible protein GADD45 gamma	GADD45G;GADD45G	C	
Q7Z4L5	Tetratricopeptide repeat protein 21B	TTC21B;TTC21B	C	X
Q16585	Beta-sarcoglycan	SGCB	C	X
Q5THR3-4	EF-hand calcium-binding domain-containing protein 6		C	
Q68DH5	LMBR1 domain-containing protein 2	LMBRD2	C	X
Q92620	Pre-mRNA-splicing factor ATP-dependent RNA helicase PRP16	DHX38	C	

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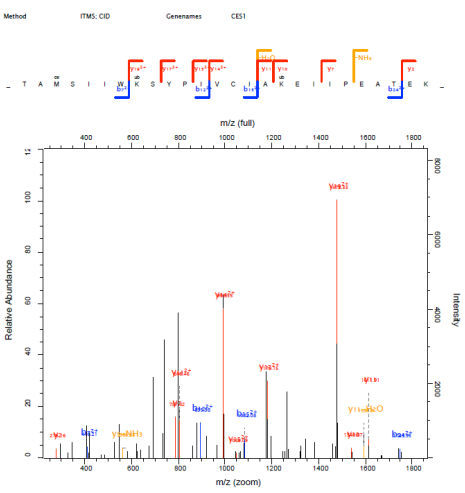
CHAPTER 9

Paper I

Supplementary files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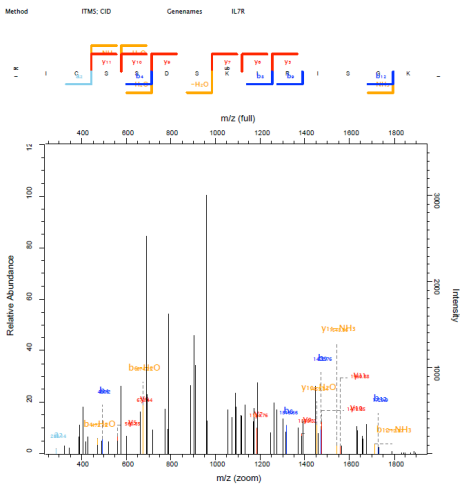
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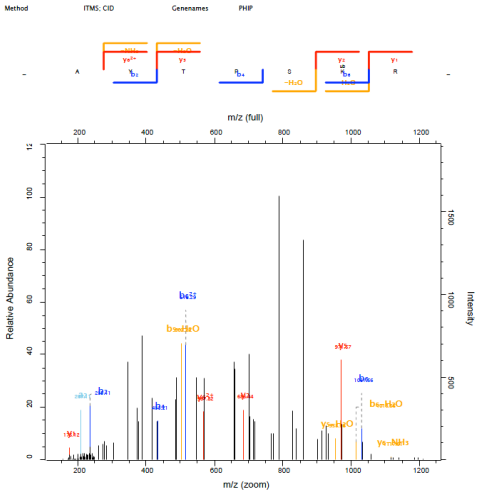
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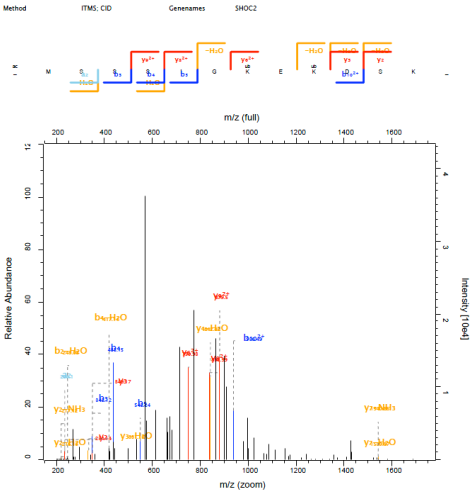
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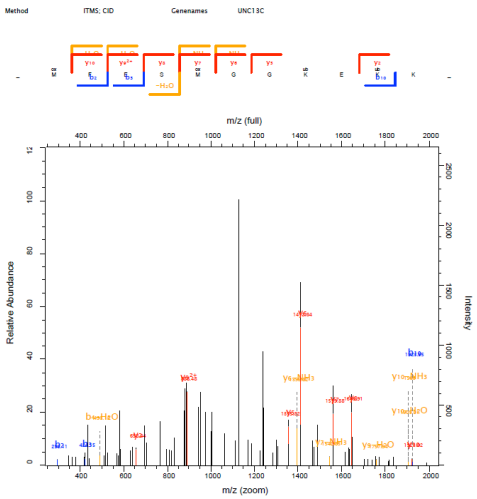
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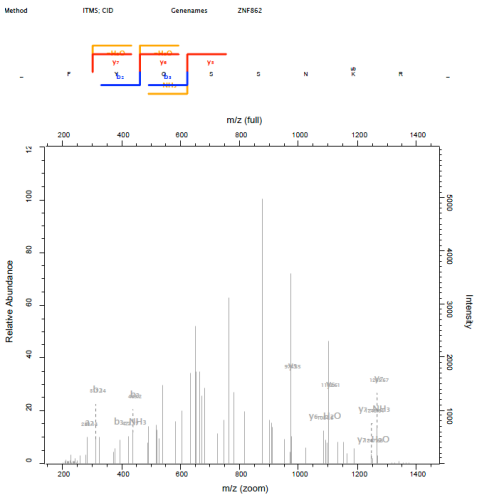
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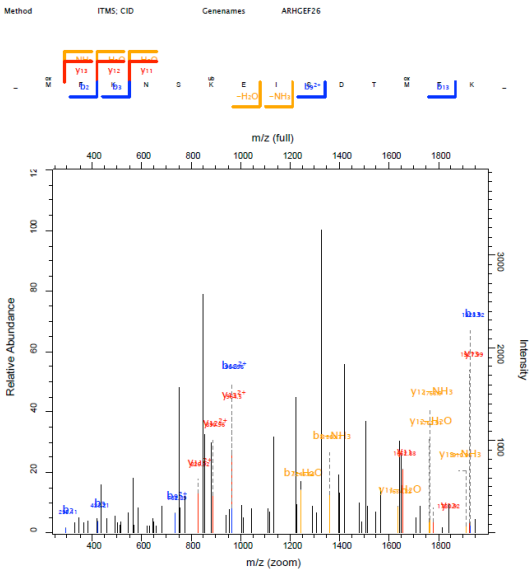
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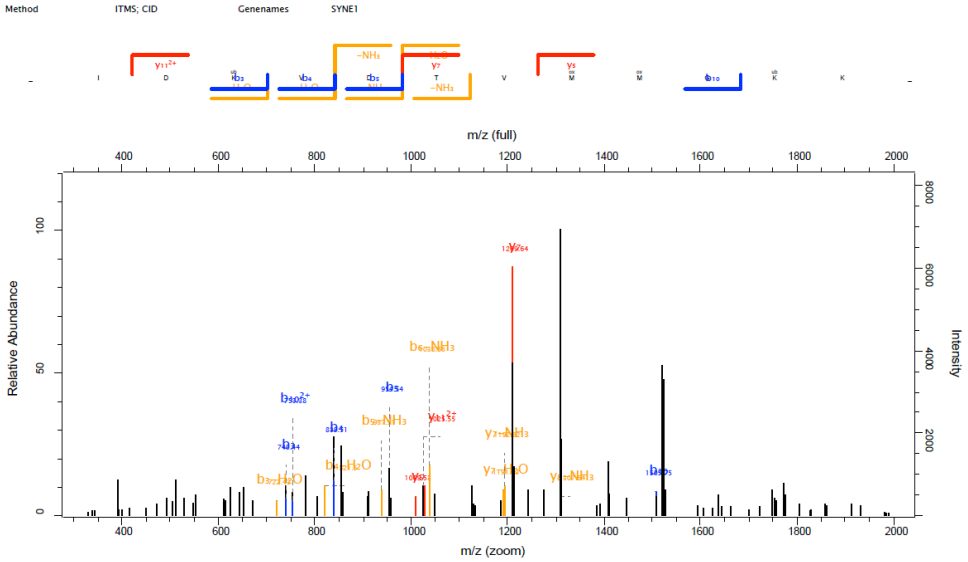
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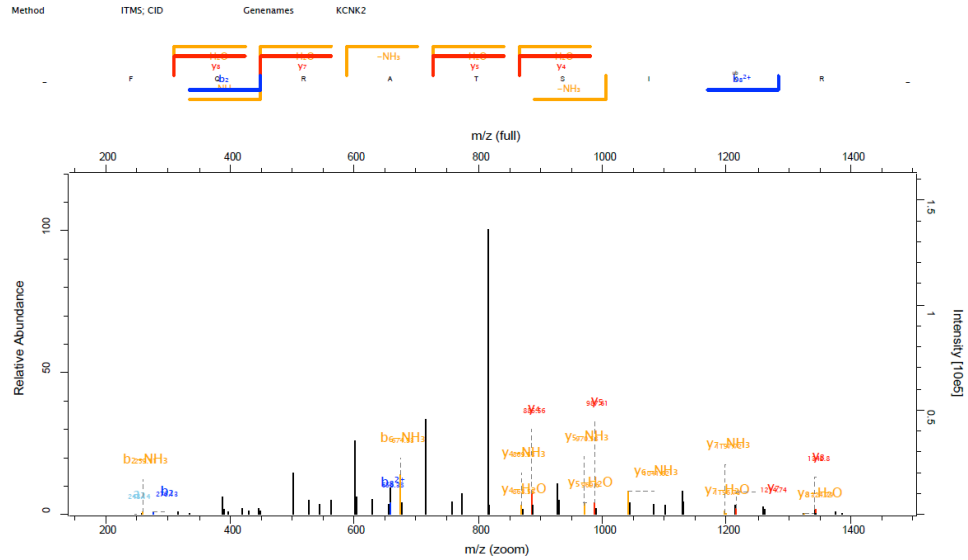
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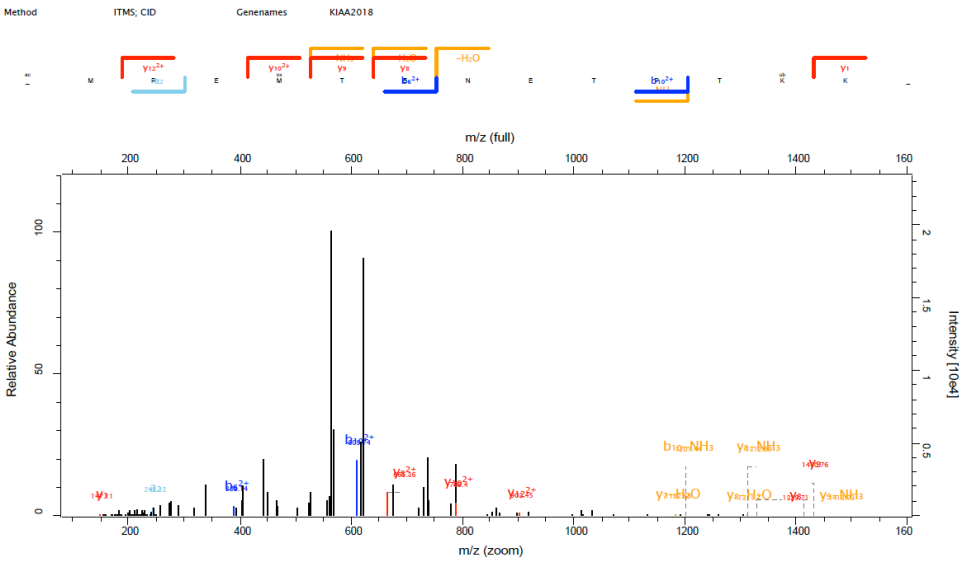
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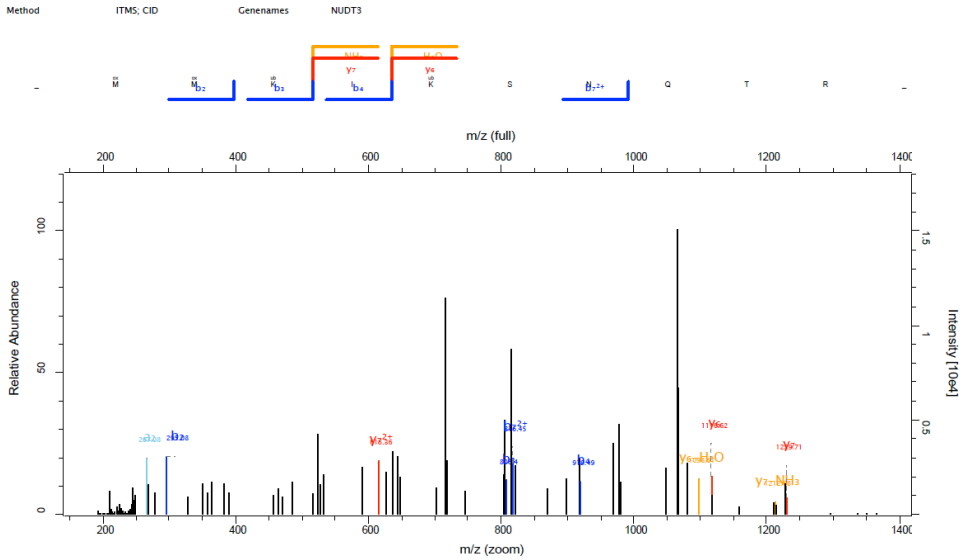
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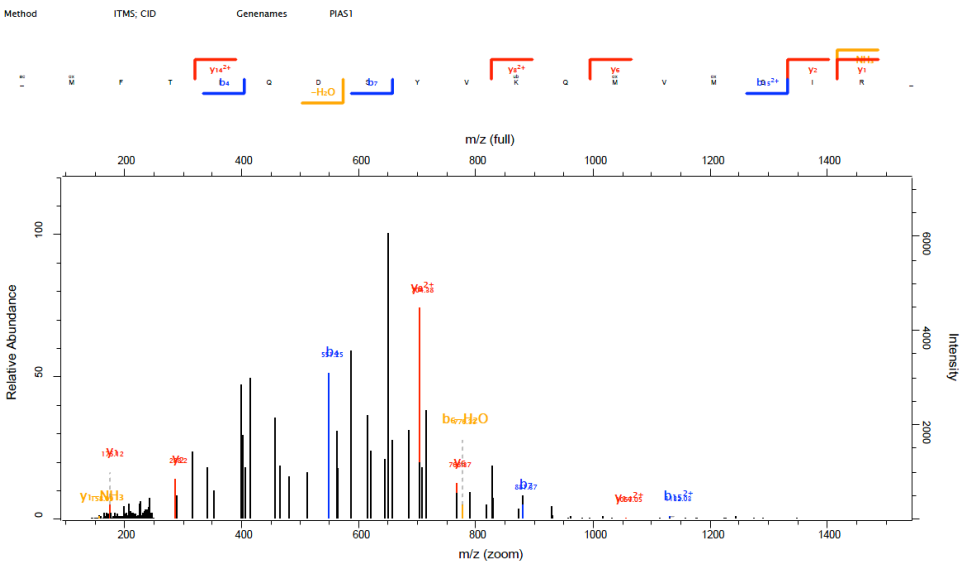
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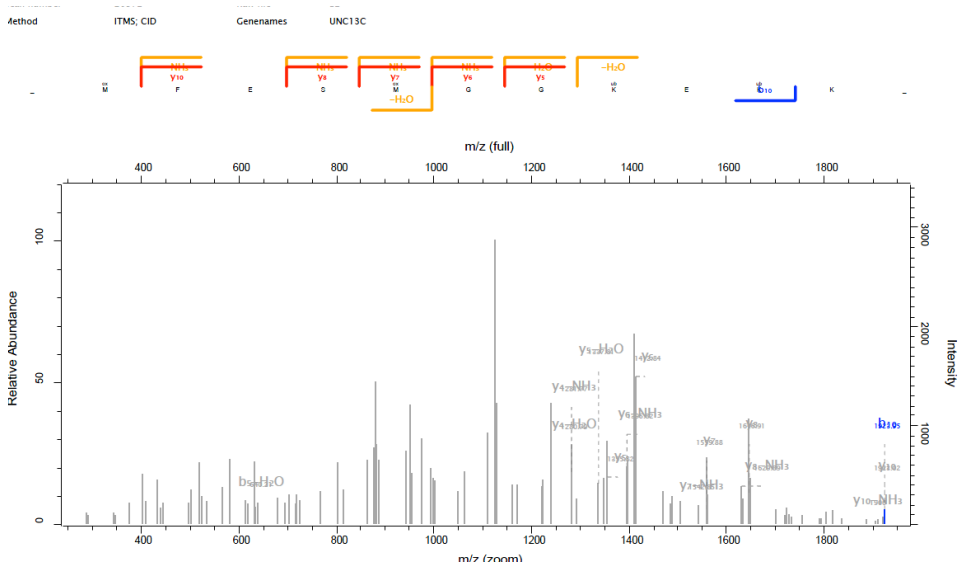
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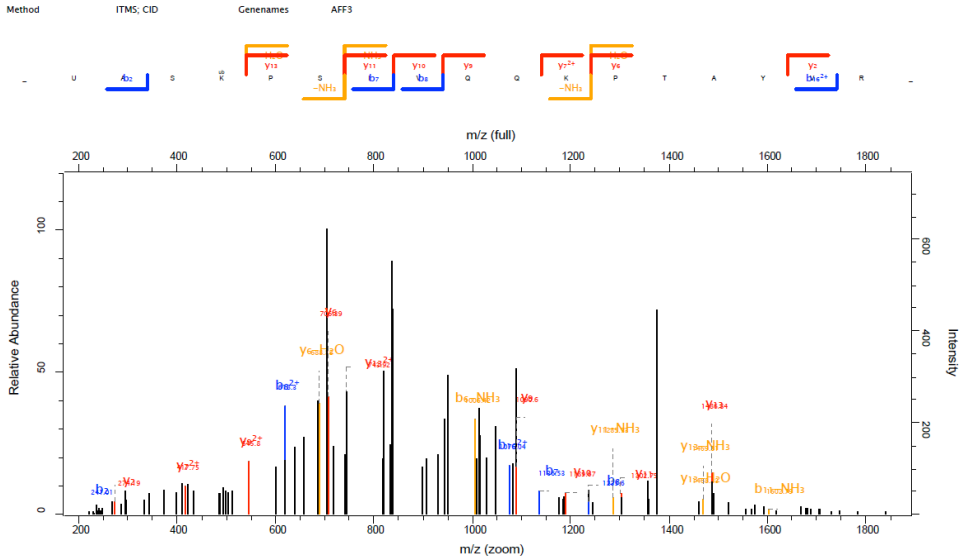
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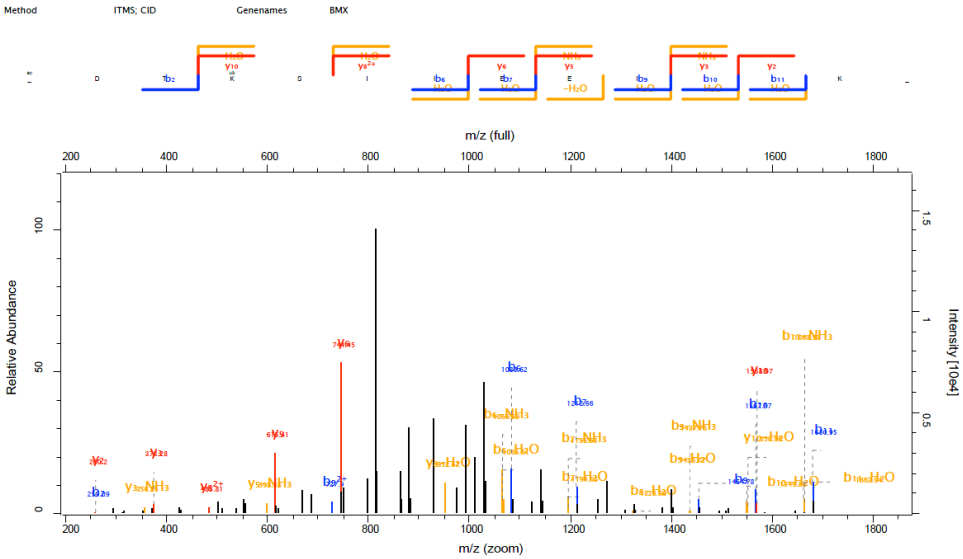
Intensity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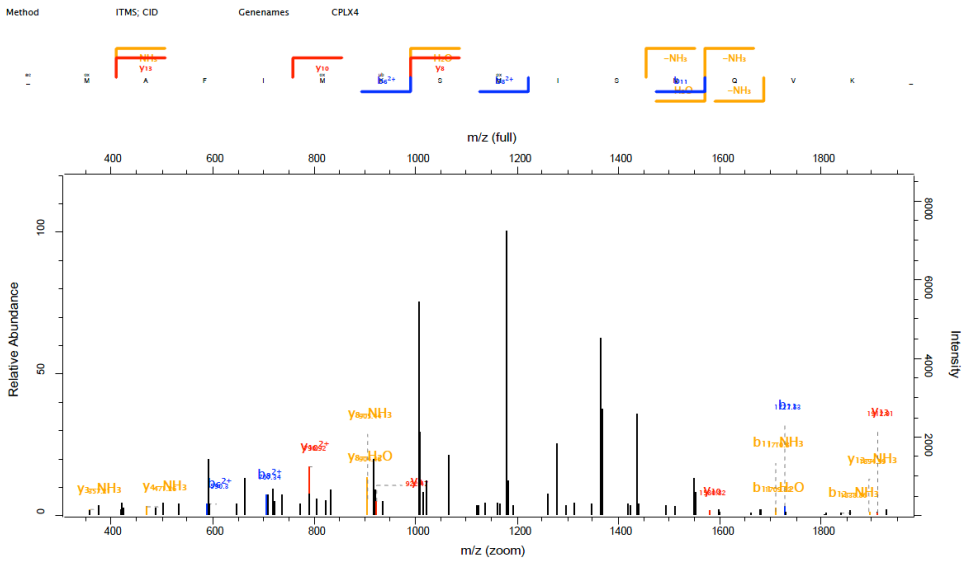
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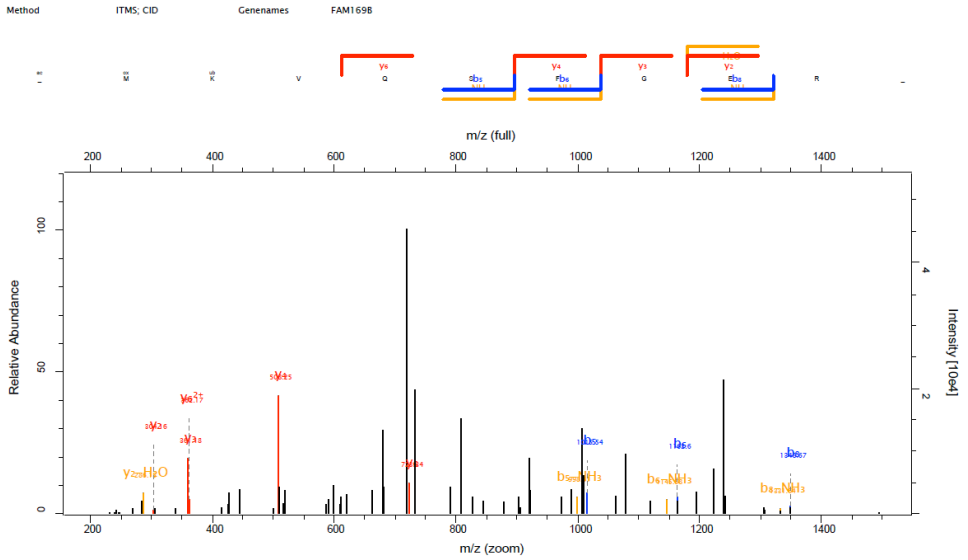
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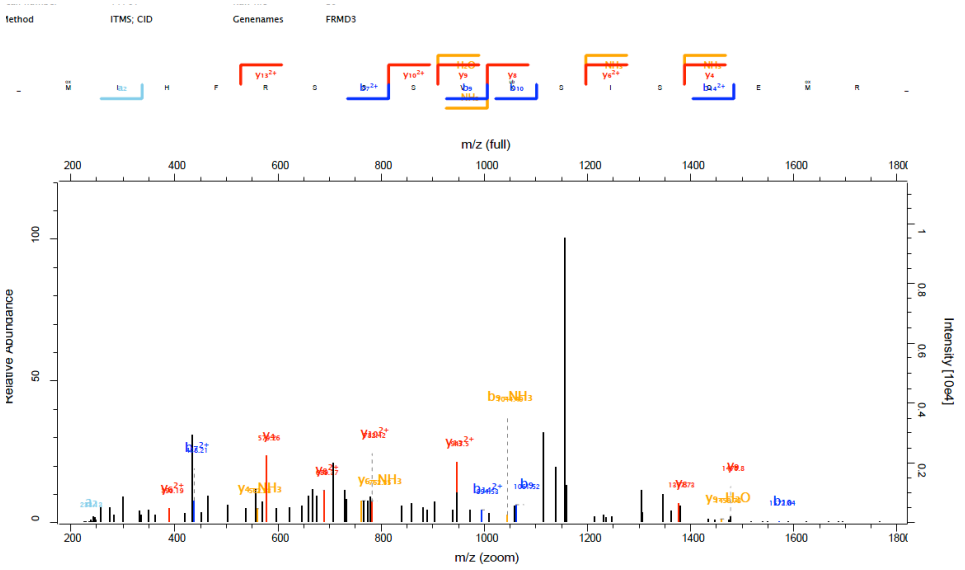
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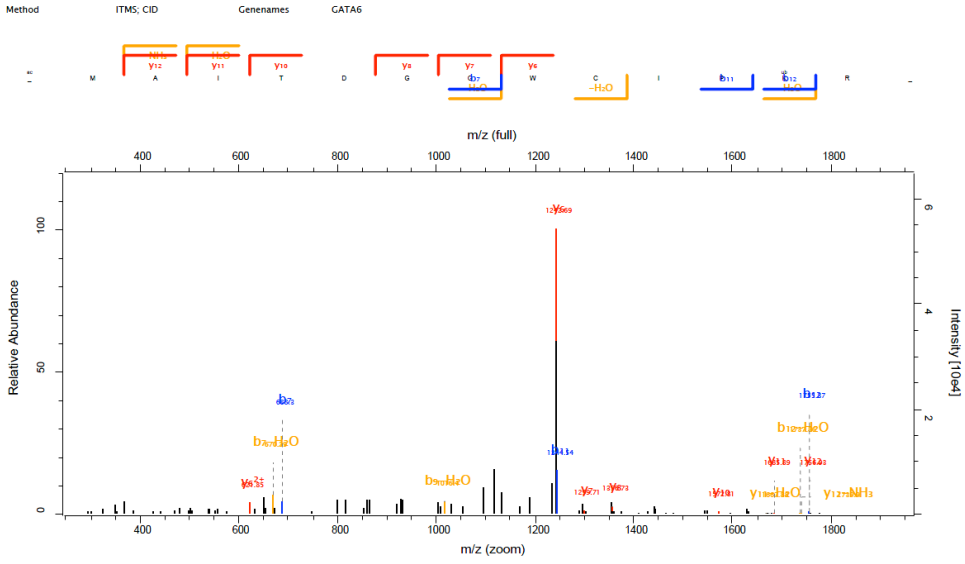
Intensity [10e4]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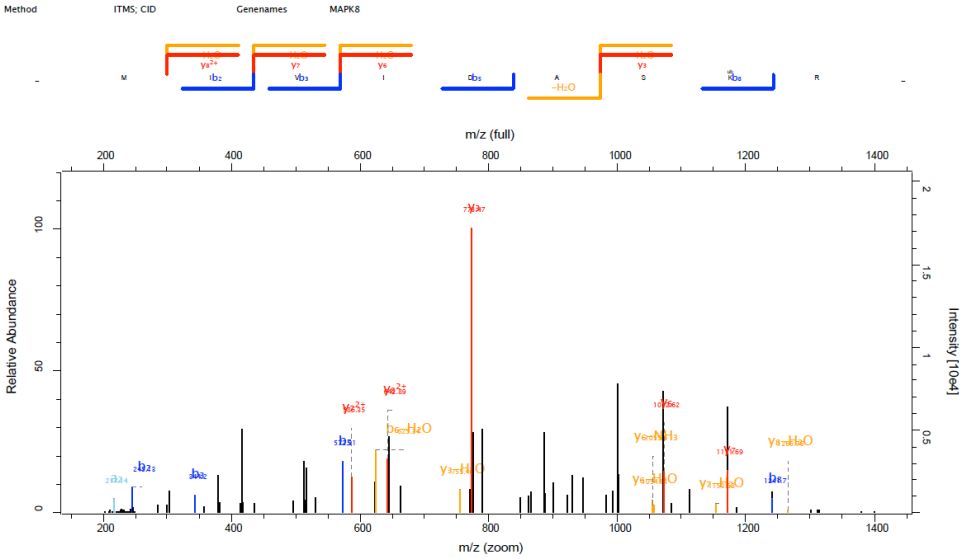
Intensity [10e4]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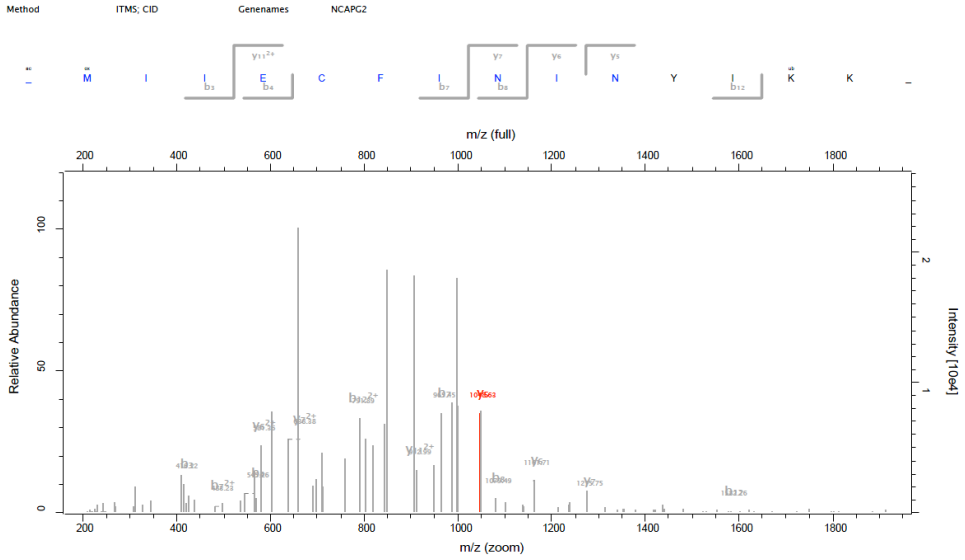
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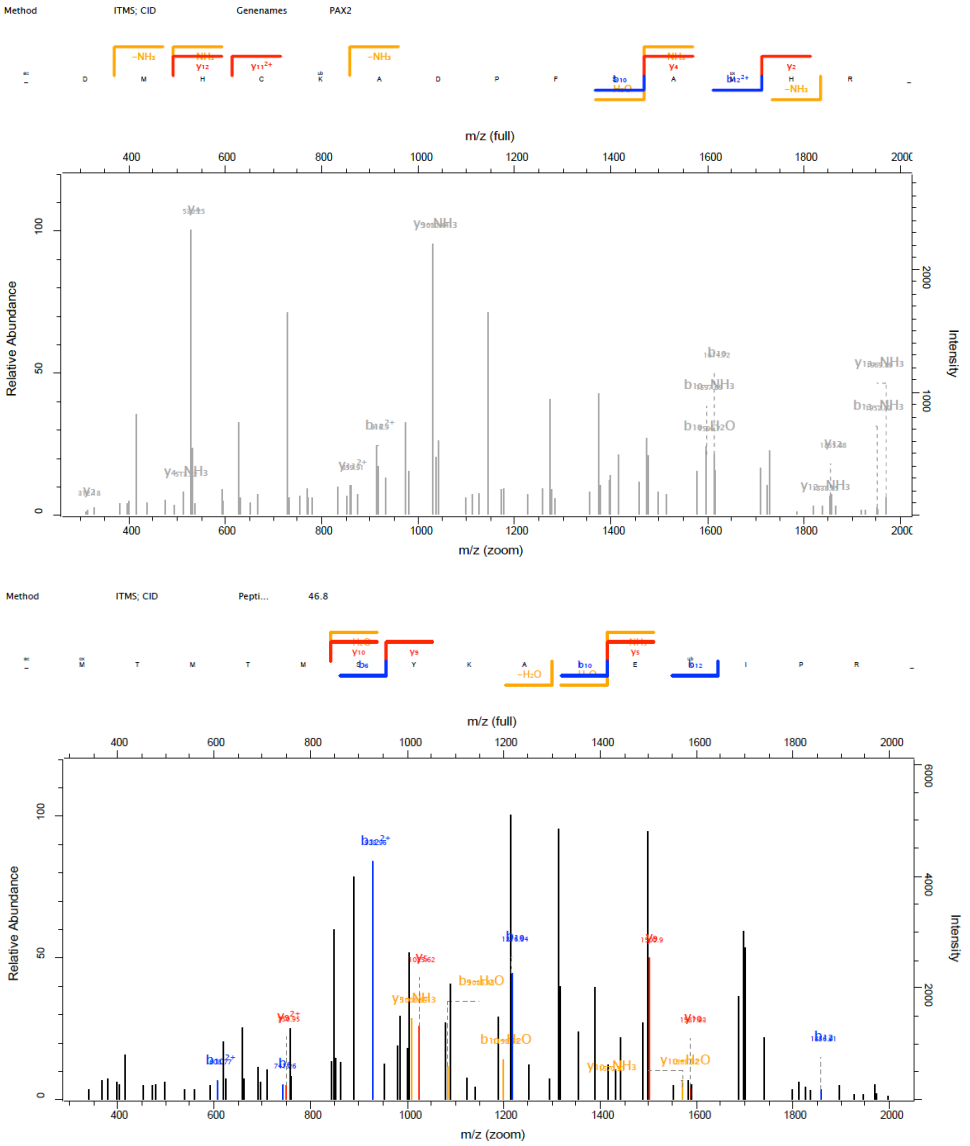
Intensity [10e4]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Genenames
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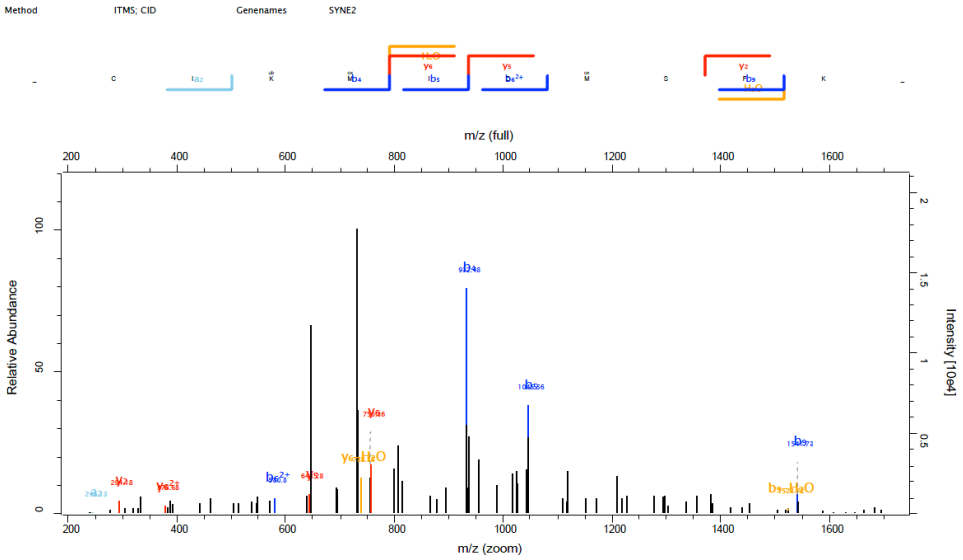
m/z (zoom)
Relative Abundance
Intensity
Method
ITMS; CID
Pepti...
46.8
m/z (full)
m/z (zoom)
Relative Abundance
Intensity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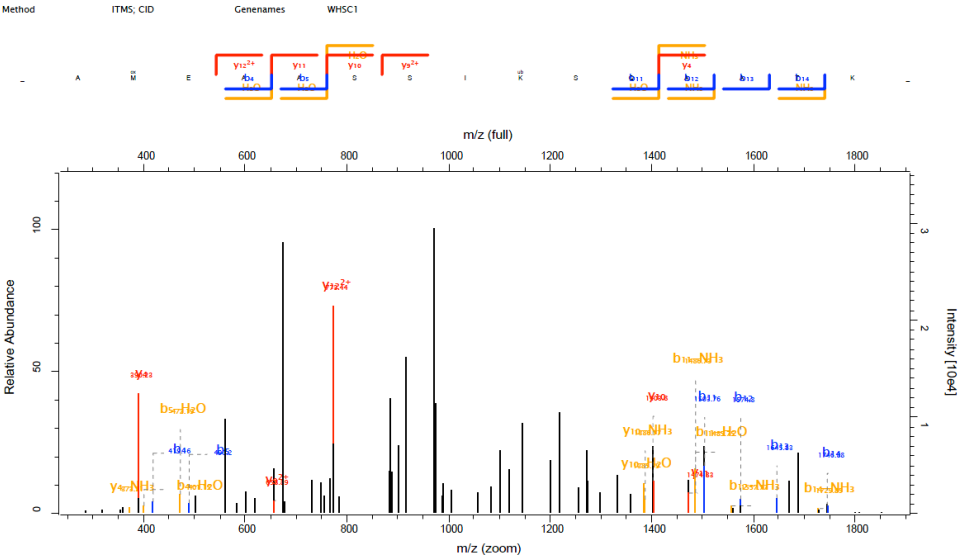
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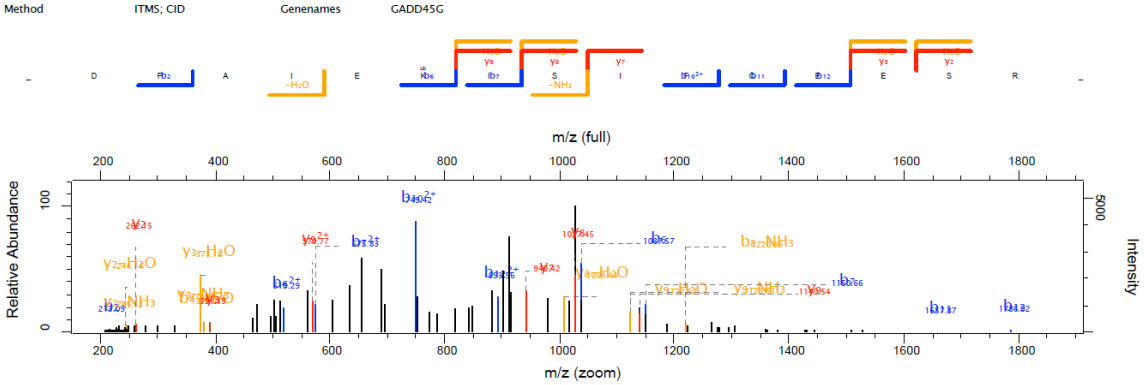
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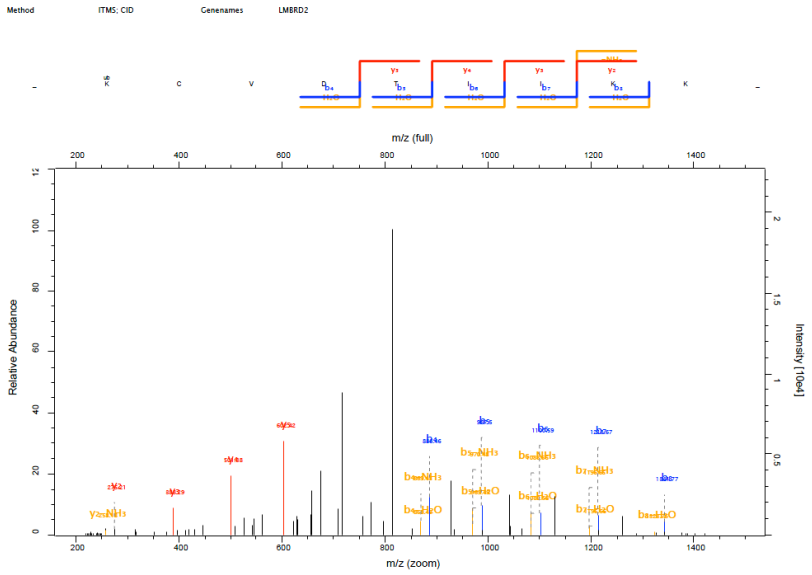
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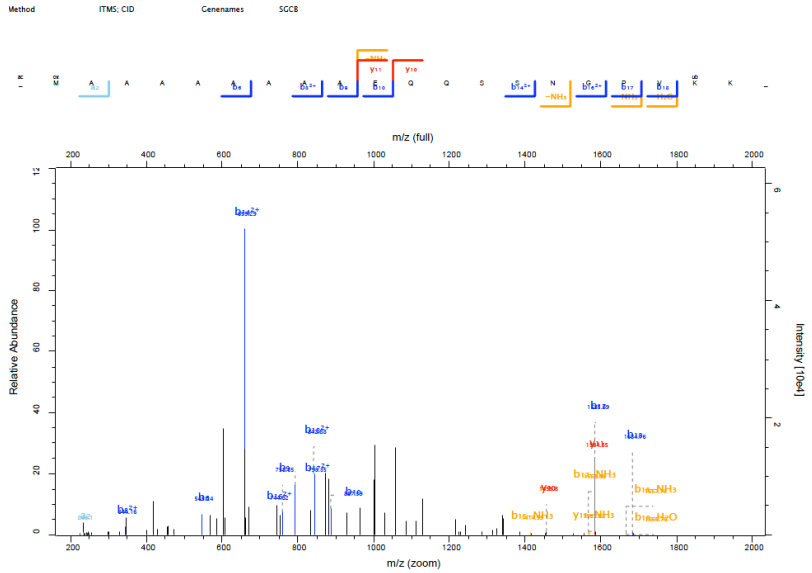
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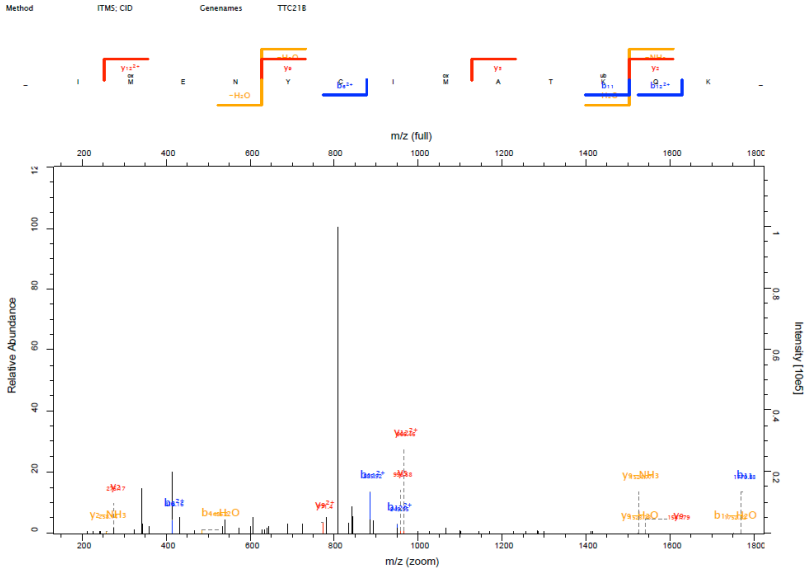
Intensity [10e4]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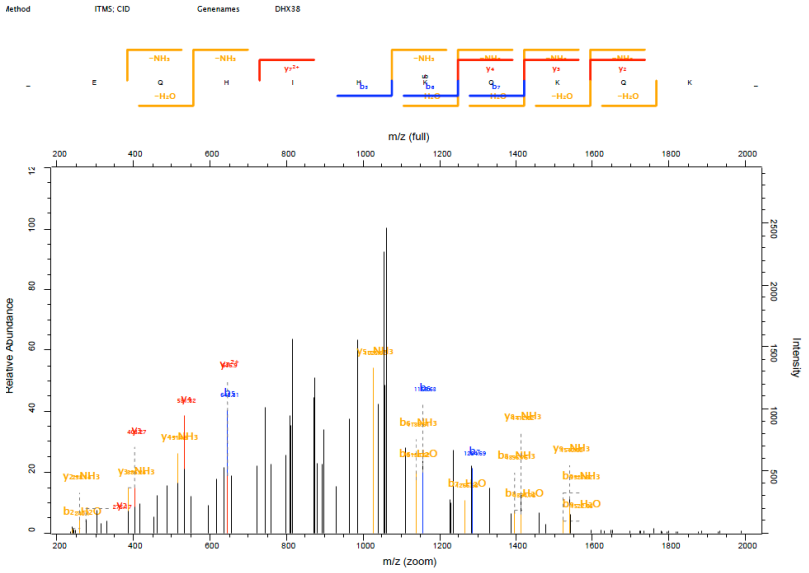
Method ITMS; CID Genenames DHX38
m/z (full)
m/z (zoom)
Relative Abundance
Intensity

CHAPTER 10

Paper II

DNA fragmentation and sperm head morphometry in cat epididymal spermatozoa

Submitted to:

Theriogenology

DNA FRAGMENTATION AND SPERM HEAD MORPHOMETRY IN CAT EPIDIDYMAL SPERMATOZOA

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Author contributions

GCL and VV contributed to design the study, analyze the data and draft the paper. MF performed statistical analysis. Laboratory work was carried out by VV, MGM and ALC. All authors have approved the final article.

Abstract

Sperm DNA fragmentation is an important parameter to assess sperm quality and can be a putative fertility predictor. Since the sperm head consists almost entirely of DNA, subtle differences in sperm head morphometry might be related to DNA status. Several techniques are available to analyze sperm DNA fragmentation, but are labor-intensive and require expensive instrumentations. Recently, a kit (Sperm-Halomax®) based on the sperm chromatin dispersion (SCD) test and specifically developed for spermatozoa of different species, but not for cat spermatozoa, became commercially available.

The first aim of the present study was to verify the suitability of Sperm-Halomax® assay, specifically developed for canine semen, for the evaluation of baseline values of DNA fragmentation of epididymal cat spermatozoa. For this purpose, DNA fragmentation indexes (DFIs) obtained with Sperm-Halomax® and TUNEL were compared. The second aim was to investigate whether a correlation between DNA status, sperm head morphology and morphometry assessed by Computer Assisted Semen Analysis (CASA) exists in cat epididymal spermatozoa.

No differences were observed in baseline values of DNA fragmentation index obtained with Sperm-Halomax® and TUNEL. This result indicates that Sperm-Halomax® assay provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa.

The DFI seems to be independent from all the measured variables of sperm head morphology and morphometry. Thus, the evaluation of the DNA status of spermatozoa could effectively contribute to the completion of the standard analysis of fresh or frozen semen used in assisted reproductive technologies.

Key words: cat, epididymal spermatozoa, DNA, morphometry

1. Introduction

The evaluation of DNA status is not included in the standard semen analysis, but the frequency of spermatozoa containing fragmented DNA may be an important parameter of semen quality, and a useful index of fertility potential.

Spermatozoa with severe DNA damage remain functionally intact, with normal fertilizing ability, but a high incidence of DNA fragmentation results in a significant decrease in pregnancy rates [1]. The exact mechanism of sperm DNA damage has not yet been clarified, but environmental stresses, gene mutations, chromosomal abnormalities or oxidative damages might be involved.

Several methods have been developed to assess sperm DNA fragmentation such as in situ nick translation (ISNT), Terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL), comet assay (CT), acridine orange test (AO) and the sperm chromatin structure assay (SCSA) [2]. However, these techniques are labor-intensive and require expensive instrumentations.

Recently, a kit (Sperm-Halomax®) based on the sperm chromatin dispersion (SCD) test and specifically developed for boar [3], bull [4], stallion [5] and dog semen [6,7], but not for cat semen, became commercially available. Spermatozoa are immersed in an agarose matrix on a slide and briefly incubated in a lysing solution to remove membranes and proteins. DNA fragmentation produces large halos, whereas those sperm with low levels of fragmentation show circumscribed halos. The evaluation can be performed by fluorescence or light microscopy. This kit would allow the routine assessment of sperm DNA fragmentation in laboratories dealing with andrological examinations and assisted reproductive technologies (ART).

In cats, significant advances in ART have been achieved thanks to the embryo production by intracytoplasmic sperm injection (ICSI) of mature oocytes [8]. Sperm selection for ICSI is based on motility and morphology patterns, and the evaluation of the DNA status is not generally performed [9]. Since the sperm head consists almost entirely of DNA, subtle differences in sperm head morphometry might be related to DNA content and organization, as demonstrated in dogs [10] and humans [11]. To the authors' knowledge, similar studies have not been performed in cats.

The first aim of the present study was to verify the suitability of Sperm-Halomax® assay, specifically developed for canine semen, for the evaluation of DNA status of epididymal cat spermatozoa. For this purpose, baseline values of DNA fragmentation obtained with Sperm-Halomax® and TUNEL were compared. The second aim was to investigate whether a correlation between DNA status, sperm head morphology and morphometry assessed by Computer Assisted Semen Analysis (CASA) exists in cat epididymal spermatozoa.

These data could contribute to achieve a better diagnosis in case of infertility due to male factors, to obtain a better evaluation of spermatozoa used in ART, and to refine the epididymal sperm selection criteria for ICSI.

The use of epididymal cat spermatozoa is currently a subject of interest with the purpose of establishing an efficient gene banking model for threatened and endangered wild felids and contributing to the preservation of genetic material from valuable males that die unexpectedly or undergo orchiectomy for medical reasons.

2. Materials and methods

2.1. Semen Collection

Epididymal spermatozoa were collected from 28 tomcats subjected to routine orchiectomy. Epididymides and vasa deferentia were dissected and squeezed to collect epididymal fluid in a warmed (37°C) Phosphate Buffered Saline (PBS) without calcium and magnesium.

2.2. Experimental design

2.2.1. Exp. 1: Sperm-Halomax® assay vs. TUNEL test.

Epididymal fluid collected from 10 cats was divided into two aliquots for the evaluation of DNA status by Sperm-Halomax® for canine spermatozoa (Halotech DNA SL, Madrid, Spain) and TUNEL Test (Calbiochem® FragEL™ DNA fragmentation detection kit, Fluorescent–TdT Enzyme; EMD Millipore Billerica, MA, USA).

2.2.2. Exp. 2: Correlation between sperm DNA status, head morphology and morphometry.

Epididymal fluid collected from the remaining 18 cats was used for the evaluation of DNA status by Sperm-Halomax®, for the conventional sperm head morphology evaluation, and for the sperm head morphometry by CASA.

2.3. Sperm-Halomax® assay

To perform the Sperm-Halomax® assay, the semen was diluted in PBS to obtain a final concentration of 5-10 x 10^6 sp/ml. A vial of agarose was melted into a water bath at 90-100°C for 5 min, then equilibrated at 37°C for 5 min and an aliquot of the diluted

sample was added and mixed thoroughly. A drop of suspension was placed onto the treated face of a slide (marked surface), covered with a glass coverslip in horizontal position and placed into the fridge for 5 min for the solidification of sample. After the removal of the coverslip, the slide was settled (in horizontal position) in the lysing solution and incubated for 5 min at room temperature. The slide was washed with distilled water for 5 min and a sequential ethanol baths (70, 90 and 100%, for 2 minutes each) and air dry were used to dehydrate the sample.

The slide was stained in Wright solution (Merck, Darmstadt, Germany) diluted 1:1 in phosphate buffer at pH 6.88 (Merck), as recommended by manufacturer's instruction. Keeping the horizontal position, a layer of the dying solution was placed to cover the slide for 15-20 min. The dying solution was removed and the slide was washed briefly and smoothly in tap water and air-dried. The stained slide was analyzed under a light microscope with x100 magnification lens (Axiovert 100, Zeiss, Germany).

At least 500 spermatozoa were evaluated and classified as follows: spermatozoa with fragmented DNA those with a large and spotty halo of chromatin dispersion and a small head; spermatozoa with unfragmented DNA those with a small and compact halo of chromatin dispersion and a large head.

The sperm DNA fragmentation index (DFI) was then calculated as the percentage of spermatozoa with fragmented DNA over the total number of sperm counted per slide.

2.4. TUNEL test

DNA sperm integrity was evaluated using DNA fragmentation detection kit (Calbiochem® FragEL™ DNA fragmentation detection kit, Fluorescent–TdT Enzyme; EMD Millipore Billerica, MA, USA). The principle of Fluorescein-FragEL is that

terminal deoxynucleotidyl transferase (TdT) catalyzes the addition of fluorescein-labeled and unlabeled deoxynucleotides to the 30-OH ends generated by endonucleases during apoptotic degeneration.

Sperm samples were smeared on a slide and air-dried. Then smears were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature, and washed twice in Tris Buffered Saline (TBS) for 15 min. The slides were then covered with the permeabilization solution (Protein Kinase 2 diluted 1:100 in TRIS solution 10mM), and incubated for 6 min in a moist chamber. The slides were washed twice in TBS solution and maintained in a moist chamber. In the dark, an aliquot of the equilibration solution (TdT Equilibration Buffer diluted 1:5 in sterile water (Sigma Chemical Co, St. Louis, MO, USA)) was added to each slide that was incubated for 30 min in a moist chamber at room temperature. After the removal of the equilibration solution, an aliquot of the labelling solution (Fluorescein-FraELTM TdT Labelling Reaction Mix diluted 1:20 in TdT Enzyme) was added to each slide that was incubated for 90 min in a moist chamber at 37°C. The slides were washed three times in TBS and a drop of an antifade reagent (Gel Mount; BiØmeda Corp., Foster City, CA, USA) was added. The slides covered with a coverslip were examined under fluorescent microscope (Eclipse E600, Nikon Corporation, Tokio, Japan) with x40 magnification lens and oil immersion. At least 500 spermatozoa of each sample were analyzed randomly to evaluate the percentage of TUNEL-positive sperm cells (bright green nuclear fluorescence in DNA fragmented sperm cells) [12].

2.5. Conventional sperm head morphology

Undiluted samples were stained with a rapid Giemsa-Wright stain (Diff-Quick, Merck) and in each sample a total of 200 spermatozoa was evaluated under light microscope (Diaplan Leitz, Wetzlar, Germany) with x100 magnification lens and oil immersion. Abnormal sperm heads included those that were pear-shaped, large, small, or amorphous were recorded.

2.6. CASA sperm head morphometry

The stained slides were examined for the evaluation of the sperm head morphometry using a light microscope (Olympus BX51, Olympus America Inc., Melville, NY, USA) equipped with a video camera (Scion Corp. 1394, Frederick, MD, USA) interfaced to a computer. The software used for image acquisition and analysis was Image-Pro Plus 5.1; Media Cybernetics (Immagini & Computer, Bareggio, Italy).

Each sperm head was measured for different parameters: area (μm2), aspect (ratio between major and minor axes of the ellipse), perimeter (μm), maximum diameter (dmax, μm), minimum diameter (dmax, μm), maximum radius (radmax, μm), minimum radius (radmin, μm), radius ratio and roundness [13].

2.7. Statistical analysis

Results of Exp. 1 (Sperm-Halomax® assay vs TUNEL test) have been evaluated by the Bland-Altman plot technique [14] in order to assess the agreement between tests, considering the TUNEL procedure as reference method (gold standard).

In Exp. 2 to establish reference values for the morphological sperm variables (area; aspect; perimeter; dmax; dmin; radmax; radmin; radius ratio; roundness), a non-

parametric approach (2.5-97.5 percentile of the distribution) was followed on 2425 spermatozoa.

Variables not determined on a single spermatozoon (i.e. DFI and head anomalies) were submitted to the calculation of the 95% confidence interval as indicative reference values.

Aiming to evaluate the multivariate relations between DFI and the morphological variables, a principal component analysis (PCA) was applied: data were submitted to PCA after normalization and the *varimax* rotation. The number of retained components was calculated when at least the 90% of the total variability was explained. Moreover, the Pearson univariate correlation between DFI and the morphological variables was calculated ($p<0.05$).

3. Results

3.1. Exp. 1: Sperm-Halomax® assay vs TUNEL test.

Epididymal cat spermatozoa processed with a SCD test as Sperm-Halomax® developed for dogs produce similar patterns than those described in dog spermatozoa [6]. Spermatozoa with unfragmented DNA do not show or show very small halos of dispersion of DNA loops, whereas those with DNA fragmentation release peripheral halos from the central core (Fig. 1).

No differences were observed in baseline values of DNA fragmentation index obtained with Sperm-Halomax® and TUNEL (4.34 ± 0.93% vs. 4.26 ± 0.83%; $p = 0.84$).

The Bland–Altman test was applied to evaluate the level of agreement between the TUNEL test and Sperm-Halomax®. The results showed that there was a good

agreement between the considered tests, since all points lay within the boundaries (Fig. 2).

3.2. Exp. 2: Correlation between sperm DNA status, conventional head morphology and CASA morphometry.

The calculated reference values for the morphological variables of the sperm head were: area 7.34 – 15.59 mm^2; aspect 1.69 – 2.86; perimeter 10.44 – 14.87 mm; dmax 4.09 – 6.19 mm; dmin 1.90 – 2.96 mm; radmax 2.12 – 3.20 mm; radmin 0.90 – 1.44 mm; radius ratio 1.83 – 3.24; roundness 1.11 – 1.44.

The 95% confidence interval for DFI and head anomalies evaluated with conventional analysis were 0.037-0.044 % and 0.034-0.047 %, respectively.

The results for PCA analysis are reported in table 1; the first three components account for the 96.62% of the total variability. In particular, the morphological variables are mainly expressed in the first two PC with high correlations. The third component is represented by the DFI only, accounting for the 7.6% of the total variability. Being the PC orthogonal vectors, DFI seems to be independent from the other measured variables. The multivariate results are confirmed by the calculation of the Pearson correlation coefficients: none of the r coefficients resulted significant.

4. Discussion

Present data show that Sperm-Halomax® assay specifically developed for canine semen and based on SCD test, provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. Most of the differences between the DNA baseline values obtained with the Sperm-Halomax® assay and TUNEL test (considered the gold

standard for the evaluation of DNA fragmentation) were within the 95% confidence interval limits, suggesting that the level of agreement between the two methods of analysis is satisfactory.

The conditions for sperm DNA fragmentation may not be the same in different animals, mainly because protamine residues, which form an important part of sperm chromatin, differ between species [15]. However, the SCD test protocol designed for dogs has resulted equally efficient in analyzing DFI in cats. This finding is not surprising, given the relatively close phylogenetic relationship of the Canidae and Felidae within the Carnivora order and reinforces the idea that the sperm chromatin organization in these two taxa maintains some degree of structural atavism [16].

Cat spermatozoa processed with Sperm-Halomax® produce images of similar characteristics to those obtained in dogs [6,7]. Discrimination of the size of the halos was easy to establish in cat sperm samples because the size of the halos of DNA dispersion was large as those obtained in dogs.

In the present work DFI of cat epididymal spermatozoa ranged from 2.4% to 5.7%. These values are in agreement with those reported in the literature and obtained with different methods [17,18].

In humans, semen with 30% of spermatozoa with fragmented DNA is considered of low or poor quality to be used in assisted reproduction [19]. In feline sperm samples, additional data is necessary to establish a solid threshold value of this parameter.

To the authors' knowledge this is the first time that the relationship between conventional sperm head morphology, CASA morphometry and DNA status has been assessed in cat spermatozoa.

With CASA system, the post-acquisition processing of digitalized data offers an objective and detailed characterization of several sperm morphometric parameters which cannot be detected by conventional visual evaluation. In the present study, the analysis of more than 2400 spermatozoa representing 18 mature tomcats would also contribute to the definition of normal values of morphometric measurements that can be used as a background for further extended studies aimed at better investigating the phenomenon of teratozoospermia in this species.

Present data indicate that DFI is independent from sperm head morphology and morphometry. This finding confirms what has been demonstrated in boar [20], but it is in contrast with the general assumption that head shape is mainly related to the status of sperm DNA due to the fact that most of the sperm head is compacted chromatin. Significant relationships among sperm morphometry and the percentage of denatured DNA has been described in dogs [10,21], bulls [22], brown bears [23] and humans [24].

In feline epididymal spermatozoa it has been previously shown that head abnormalities are strongly correlated with, and could accurately predict, sperm DNA defects revealed by TUNEL test [17]. However, the conventional evaluation of sperm head morphology by Diff-Quick staining was only performed and no information on head morphometry were reported.

Morphometry provides a more objective evaluation of the sperm head shape compared to conventional examination of head morphology, and the results of the present study show that head shape is not a reliable predictor of DNA fragmentation in cat spermatozoa. Thus, different factors other than chromatin compaction might affect the sperm head shape [20].

In felids there are large individual variations in semen quality and many wild and domestic cats have a low percentage of normal spermatozoa [25,26]. However, teratozoospermic cats may be fertile [25], and this further supports that sperm morphology alone should be interpreted with caution.

For this reason a sperm selection for ICSI typically based on motility and morphology attributes, might not ensure the use of a high quality spermatozoon. Sperm DNA integrity is of crucial importance for the embryo development and concerns have been raised regarding possible use of spermatozoa with DNA damage during ICSI [27].

4.1 Conclusions

In conclusion, Sperm-Halomax® assay specifically developed for canine semen and based on SCD test, provides a reliable evaluation of DNA fragmentation of epididymal feline spermatozoa. The availability of this simple technique could be useful to improve the feline semen evaluation in clinical practice and it could contribute to better select semen samples for biotechnological procedures. In fact, DFI is independent from sperm head morphology and morphometry and the evaluation of the DNA status of spermatozoa would be of great interest in the completion of the standard analysis of fresh or frozen semen used for ICSI or other ARTs.

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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Fig. 1. Spermatozoa processed with Sperm-Halomax® kit and stained with Wright solution. Those with a small halo have normal status of DNA and the spermatozoon with a large halo contains fragmented DNA. Bar represents 10 μm.

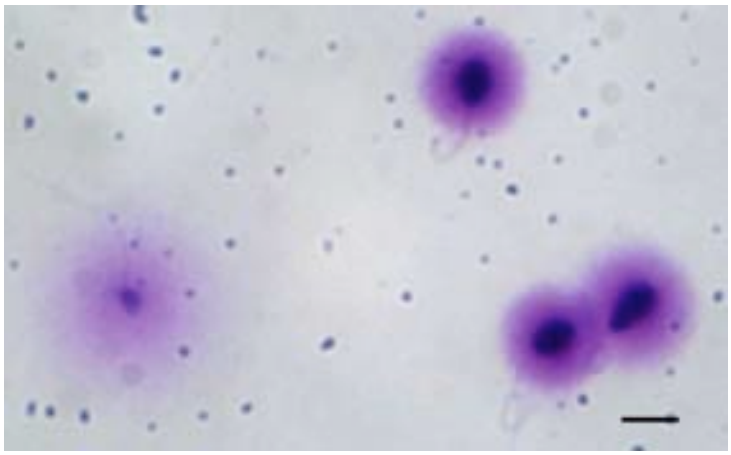

Fig. 2. Bland-Altman plot for Sperm-Halomax®/TUNEL DNA Fragmentation Index results agreement. The line boundaries indicate the 95% CI of the difference between variables.

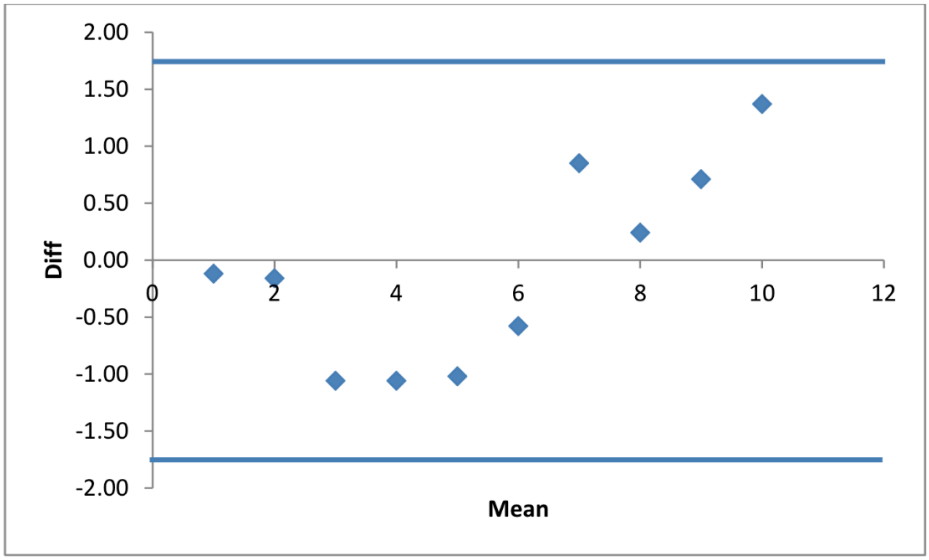

Table 1. Principal Component Analysis (PCA) loadings for morphological variables and DNA fragmentation index (DFI) on the first three components. The loadings with values >0.7 are bold typed. In brackets on the headers: variability explained by the PC.

Attribute	PC 1 (51.91%)	PC 2 (37.29%)	PC 3 (7.63%)
Roundness	**-0.93238**	-0.34192	-0.05891
Radius ratio	**-0.91686**	-0.37916	-0.06608
Aspect	**-0.90646**	-0.39293	-0.09087
Radius maximum (mm)	**-0.88915**	0.45212	0.01863
Diameter maximum (mm)	**-0.88565**	0.45974	0.00636
Head anomalies (%)	**-0.83809**	-0.14168	0.01752
Perimeter (mm)	**-0.74541**	0.66100	0.03964
Diameter minimum (mm)	0.23555	**0.96457**	0.10689
Radius minimum (mm)	0.26324	**0.95855**	0.08731
Area (mm^2)	-0.43677	**0.89489**	0.07500
DFI (%)	-0.17188	-0.41807	**0.89131**

CHAPTER 11

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CHAPTER 12

Appendix

Co-authorship

BSAVA BRITISH SMALL ANIMAL VETERINARY ASSOCIATION

JSAP JOURNAL OF SMALL ANIMAL PRACTICE

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 µL 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 (µL)		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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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

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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 × 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.

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).

The results for PCA analysis are reported in Figure 1.

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

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]
Abnormal	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
	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 ($P<0.05$).

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].

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 ($P<0.05$).

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,

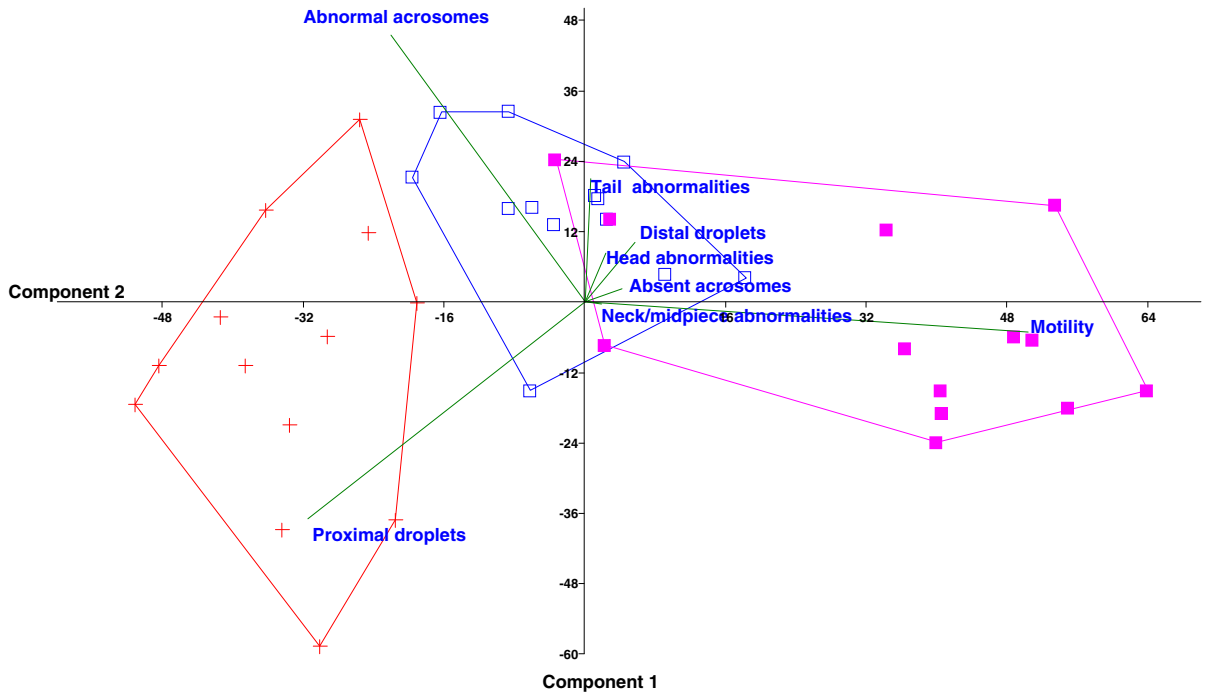

Figure 1 Principal component biplot of variables in the space of the first two principal components (PC). The length of each vector represents the weight of the variable in the plane, and the angle between variables quantifies the correlations among the variables. The objects (i.e. the samples) dispose 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).

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.

Authors' 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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DNA INTEGRITY OF FRESH AND FROZEN CANINE EPIDIDYMAL SPERMATOZOA

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AUTHOR CONTRIBUTIONS

GCL and SV contributed to design the study, analyse the data and draft the paper. Laboratory work was carried out by SV, MGM and VV. All authors have approved the final article.

ABSTRACT

The aims of this study were to evaluate the effect of cryopreservation on baseline values of DNA fragmentation 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 DNA fragmentation index was assessed by a commercial kit specifically developed for canine semen (Sperm-Halomax®) and based on the sperm chromatin dispersion test.

The results showed that baseline values of DNA fragmentation of canine epididymal spermatozoa were similar 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, no effect of cryopreservation was observed on baseline values of DNA fragmentation in canine epididymal spermatozoa and a protective effect of melatonin on post-thaw sperm quality has not been demonstrated. The evaluation of DNA status of thawed gametes is particularly relevant for epididymal spermatozoa which potential use in assisted reproductive techniques is mainly after storage.

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

1. 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 [1-3], 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 [4-6], but no information is available on its potential effect on DNA integrity.

Sperm DNA integrity has been evaluated in fresh ejaculated [7-10] and epididymal canine semen [11]. Only few reports have compared fresh and post-thaw chromatin integrity of canine ejaculated spermatozoa obtaining variable results [12-16], but the DNA stability after thawing of canine epididymal spermatozoa has not been investigated.

The integrity of the paternal DNA is of crucial importance for the embryo development [17] and a relationship between DNA damage and infertility has been demonstrated in humans. Spermatozoa with severe DNA damage remain functionally intact, with normal fertilizing ability, but a high index of DNA fragmentation results in a significant decrease in pregnancy rates [18,19].

Nevertheless, there is no agreement neither on whether cryopreservation induces DNA fragmentation, nor on the mechanism which actually induces this damage [20,21]. It has been hypothesized that the increase of reactive oxygen species (ROS) during cryopreservation and the decrease of antioxidant activity of the spermatozoa cause the peroxidative damage to the sperm plasma membrane and affect DNA integrity [13,14,20].

The role of antioxidant supplementation in protecting the sperm DNA from oxidative damage is still under investigation. Among antioxidants, it has been shown that melatonin (1-2 mM), that directly neutralizes a high number of free radicals, has an effective action in protecting ram and bull spermatozoa from the freezing injuries as evidenced by post-thaw DNA integrity, viability, motility, morphology and fertilizing ability [22,23].

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

2. MATERIALS AND METHODS

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

2.1 Animals

Ten healthy and pubertal privately owned stud 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.

2.2 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.

2.3 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 supernatant, one aliquot (-M) was diluted (200 x 10^6 sperm⁄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 [24].

Briefly, this method consists of two extension steps before freezing in 0.5 ml straws. Straws were submerged in liquid nitrogen vapour in a styrofoam box (10 min at 4.5 cm above liquid nitrogen) and subsequently immersed into liquid nitrogen. The straws were thawed in a water bath at 37°C for 30 sec.

2.4 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) [25].

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 (1000x 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 [26] 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) [26].

2.5 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.

Baseline values of sperm DNA fragmentation were evaluated in fresh and frozen samples (+M and -M) at the concentration of 50 x 10^6 sperm/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 (400x magnification).

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 as previously suggested [27].

2.6 Statistical analysis

Values are presented as mean ± standard deviation (SD). For each variable, the statistical differences between the treatment groups were calculated by a one-way analysis of variance (ANOVA), followed by the post-hoc Tukey test for multiple comparisons. Statistical significance was set to $P<0.05$.

3. RESULTS

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

No effect of the cryopreservation procedure was observed on DNA status of canine epididymal spermatozoa as demonstrated by similar baseline values of DNA fragmentation 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 1).

Morphology of epididymal spermatozoa before and after freezing is summarized in Table 2.

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 3). Proportions of spermatozoa showing vesiculated acrosome or acrosomal residues or loss were significantly higher in frozen samples (+M and -M) compared to fresh samples.

4. DISCUSSION

The results showed that baseline values of DNA fragmentation of canine epididymal spermatozoa were not affected by the freezing procedure. The presence of melatonin did not preserve motility and acrosome integrity that were severely affected by cryopreservation. Since in all samples (with or without melatonin) 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 [12,15,16]. Conversely, Kim and co-workers [14] 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 [9,16], 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 [28].

Present results indicated that the DNA fragmentation baseline values were similar in fresh and frozen canine epididymal spermatozoa, this finding confirms the resilience of canine ejaculated sperm DNA to cold stress found by some authors [12,15,16].

A progressive deterioration of DNA status has been observed after the exposure of fresh or thawed spermatozoa to different stressors (e.g. incubation time and temperature, toxicants, ROS, etc.) [21, 29].

This would indicate that the freezing-thawing procedure facilitates the destabilization of the chromatin structure of spermatozoa, resulting in an unstable DNA that is highly susceptible to fragmentation under certain stimuli [21]. Thus, DNA fragmentation is a dynamic process that depends on how quickly the iatrogenic DNA damage occurs.

Given that immediately after thawing DNA stability of epididymal spermatozoa is not compromised, further investigations on differential dynamic response on sperm DNA damage over incubation time would be advisable.

However, the factors that determine sperm DNA status during cryopreservation are not completely clarified and various hypotheses have been suggested.

For instance, it can be considered a species-specific characteristic as recently suggested by Gosalvez and co-authors [30]. 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 [31] might explain the resilience of DNA to the cold damages.

Another factor that may preserve DNA stability during freeze/thawing 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 [20].

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 [13].

The beneficial effect of seminal plasma on DNA could be related to the presence of antioxidants [13].

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 [13,14,20].

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

Hence, in this study the supplementation with melatonin was aimed at compensating for the lack of antioxidants; however it was not able to demonstrate a protective effect on sperm DNA as cryopreservation did not damage it. The effect of melatonin on sperm DNA damage over incubation time should be further investigated.

Sperm morphology was also not affected by cryopreservation. Kim and co-workers [14] 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 in the cited study 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 fresh ejaculated canine semen [7,8,10].

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 or 2 mM melatonin in the freezing extender of ram and bull ejaculated semen, other than preserve sperm DNA integrity, had a protective effect on post-thaw motility [22,23]. The incubation of thawed epididymal spermatozoa of red deer with melatonin showed instead only a limited protection in terms of different sperm parameters including motility and acrosomal integrity [32].

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, no effect of cryopreservation was observed on baseline values of DNA fragmentation in canine epididymal spermatozoa and a protective effect of melatonin on post-thaw sperm quality has not been demonstrated.

The evaluation of DNA status of thawed gametes 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 there is no conflict of interest that could be perceived as prejudicing the impartiality of the article reported.

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Table 1. Mean ± SD of DNA fragmentation index (%) and motility (%) 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
Motility (%)	74.5 ± 9.6a	37.5 ± 15.3b	36 ± 9.7b

Data derived from 10 replicates

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 ± 11.3	30.2 ± 11.7	25.7 ± 10.0

Data derived from 10 replicates

No significant differences were observed.

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 ± 16.6b
Residues/loss (%)	4.2 ± 5.5a	10.7± 3.5b	17.0± 7.5c

Data derived from 10 replicates

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