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Cytoplasmic droplet acting as a mitochondrial modulator during sperm maturation in dogs

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ABSTRACT

Motility acquisition during sperm maturation and passage through the epididymis is closely related to mitochondrial function and appears to occur in parallel with cytoplasmic droplet (CD) migration. However, such mechanism remains unclear in dogs. Thus, the aim of this study was to characterize the influence of sperm CD in the mitochondrial functionality during epididymal sperm maturation in dogs. Twenty-one adult dogs were submitted to elective bilateral orchiectomy. Testicles were stored for 18–24 h at 5 °C and epididymal sperm samples were then collected from different segments of the epididymis (caput, corpus and cauda). Samples were evaluated for computer-assisted motility analysis (CASA), presence of CD (eosin/nigrosin stain), ultrastructural CD analysis and sperm mitochondrial activity (3,3' diaminobenzidine technique) and membrane potential (JC-1 probe). Samples collected from the corpus epididymis showed higher motility and mitochondrial activity in comparison to the caput sperm. Moreover, corpus sperm had lower percentage of proximal droplets compared to caput samples, while mitochondrial membrane potential remained unchanged. Cauda samples showed higher motility, mitochondrial activity and potential, however, lower presence of sperm droplets (proximal and distal). In conclusion, the CD is essential for epididymal sperm maturation in dogs, showing important functions along the transit in the epididymis. In the corpus segment, the migration of the CD along the sperm midpiece provides a high mitochondrial activity and the onset of sperm motility. On the other hand, sperm from cauda epididymis lack CD but suffered lipid membrane changes which allow a maximum mitochondrial membrane potential and motility.

1. Introduction

During spermatogenesis, germinative cell cytoplasm is phagocytozed by the Sertoli cells, and only a remnant residue persists, forming the sperm cytoplasmic droplet (CD) (Cooper, 2005). In the caput epididymis, high percentage of spermatozoa possess a proximal droplet, however in the epididymal sperm maturation course, the CD migrates from a proximal to a distal position on the midpiece (Cooper, 2011). Simultaneously to CD migration, canine spermatozoa acquire motility in the corpus epididymis (Varesi et al., 2013; Angrimani et al., 2014a).

The exact mechanism related to the CD migration and the motility gain remains uncertain (Xu et al., 2013). Yuan et al. (2013) in

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mice, reported an energy source function of the CD during sperm maturation in the epididymis, suggesting that the droplet contains enzymes and substrates required for the proper functioning of sperm mitochondria. Moreover, several studies indicate that a failure during the translocation of the CD decrease sperm motility (Nothling et al., 1997; Cooper, 2005; Xu et al., 2013), which show that an important relationship between CD, mitochondrial function and sperm motility exists.

The relation between CD and sperm motility can be attributed to several typical features of the CD, such as the unique composition of lipids, RNAs, lipoproteins and hydrolytic enzymes in the cytoplasm residue (Cooper and Yeung, 2003; Rengan et al., 2012). Moreover, the CD is constituted of elements with Golgi characteristics, which are incorporated to the spermatic cell during droplet migration along the sperm maturation, been related to plasma membrane modifications (Oko et al., 1993; Cortadellas and Durfort, 1994) and acquisition of sperm motility (Xu et al., 2013).

In this context, studies have focused on the function of the CD and the pathways involved in motility acquisition. Therefore, the inhibition of sperm motility can contribute to the physiological knowledge of sperm maturation and ultimately allow the development of new male contraceptive or the elaboration of treatments for male infertility related to the loss of sperm motility (Ma et al., 2013). Despite such state of art, there are still few studies using the canine species, which is considered the experimental model of choice for the man (Kirchhoff, 2002). Thus, the aim of this study was to verify the influence of the sperm CD in the mitochondrial functionality during epididymal sperm maturation in dogs.

2. Materials and Methods

2.1. Animals

The present study was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science—University of São Paulo (protocol number: 2277/2011).

Twenty one healthy mature dogs, aged from 1 to 6 years, of different breeds (Poodle, Mixed Breed, Labrador Retriever, German Shepherd, Shetland Sheepdog and Schnauzer) and body weights (17.41 ± 2.6 kg) were used. All animals were privately owned and fed a commercial diet for dogs; water was provided ad libitum. Furthermore, animals were tested against brucellosis, using the *Antigen Rapid Canine Brucella Ab Ttest* (Bioeasy, Minas Gerais, Brazil), detecting the anti-Brucella canis IgG antibodies.

2.2. Sample collection

Animals were submitted to bilateral orchiectomy, and testicles-epididymis were immediately stored at 5 °C for not less than 18 h and not more than 24 h. Epididymal sperm samples were collected by performing incisions in the caput, corpus and cauda epididymides (Angrimani et al., 2017). Dissections were performed carefully to avoid cutting blood vessels and several incisions were performed in each epididymal region (caput, corpus and cauda) which were individualized using a hemostatic forceps. The flowing epididymal fluid was collected with an automatic pipette, and the total volume of the epididymal sample was resuspended in 300 µL of phosphate buffered saline medium (PBS). The dilution rate varied according to the epididymal size, similar to a previously described protocol (Kaabi et al., 2003). Tubes containing solely the PBS medium were weighted before (tube + PBS) and after the addition of the epididymal sample (tube + PBS + epididymal fluid) to calculate the epididymal fluid dilution factor. Due to the small volume recovered, the samples from the left and right epididymides of the same animal were pooled.

2.3. Assessment of sperm motility

Samples from the epididymal segments (i.e. caput, corpus and cauda) were immediately evaluated for computer-assisted sperm analysis (CASA; HTM-IVOS Ultimate 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA), according to a previously described protocol (Iguer-ouada and Verstegen, 2001). Briefly, 10 µL of each sample was deposited on microscope slides previously warmed at 37 °C and covered by coverslips. Eight fields of view were randomly selected and the following variables were assessed: VAP (average pathway velocity, µm/s), VSL (strait-line velocity, µm/s), VCL (curvilinear velocity, µm/s), ALH (amplitude of lateral head displacement, µm), BCF (beat cross frequency, Hz), STR (straightness—VSL/VAP, %), LIN (Linearity—VSL/VCL,%); MOT (motility, %), PROG (progressive motility, %). Sperm were also divided into four groups based on velocity: rapid (RAP, VAP > 50 µm/s, %), medium (MED, 30 µm/s < VAP < 50 µm/s, %), slow (SLOW, VAP < 30 µm/s or VSL < 15 µm/s, %) and non-moving spermatozoa (STATIC, %).

2.4. Evaluation of mitochondrial activity and presence of cytoplasmic droplets

To assess mitochondrial activity we used the cytochemical technique of 3,3' diaminobenzidine solution (1 mg/mL of DAB in PBS), which categorize the sperm into four classes: high (DAB—Class I), medium (DAB—Class II), low (DAB—Class III) and absence (DAB—Class IV) of mitochondrial activity (Hrudka, 1987). For this purpose, a sperm sample was incubated under light at 37 °C for 1 h with DAB in a ratio of 1:1 (20 µL of sample in 20 µL of DAB). After this period, samples were smeared on glass slides with subsequent fixation in 10% of formalin for 15 min. The evaluation was performed with a light transmitted microscope under 1000× magnification (Nikon, Eclipse E200, Japan) by counting 200 cells. The results were expressed in percentage (%).

To evaluate the presence of CDs, Eosin/Nigrosin stain was used. In brief, 5 µL of semen and 5 µL of the previously prepared stain were placed in a pre-warmed glass slide. The sperm smear was evaluated under light microscopy (Nikon, Eclipse E200, Japan) at