

## DELETERIOUS EFFECT OF HIGH CARNOSINE CONCENTRATIONS IN EXTENDERS DURING SPERM CRYOPRESERVATION IN DOGS EFECTO DELETÉREO DE ALTAS CONCENTRACIONES DE CARNOSINA EN DILUYENTES DURANTE LA CRIOPRESERVACIÓN ESPERMÁTICA EN PERROS

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### ABSTRACT

Cryopreservation is a key process among the canine reproductive biotechnologies. However, during sperm cryopreservation an excessive reactive oxygen species (ROS) generation occurs, leading to decrease in sperm quality. Therefore, several antioxidants were tested during sperm cryopreservation to prevent such effects, however the carnosine it has not used. Carnosine is a protein present in the seminal plasma, and unlike other antioxidants has the ability to remove products of lipid peroxidation (malondialdehyde), which are as harmful as ROS. Thus, the aim of this study was to evaluate the effects of different carnosine concentrations, during sperm cryopreservation in dogs. For this purpose, six dogs in reproductive age were used, and after sperm collection the samples were cryopreserved in Control (tris-citrate egg yolk extender), Carnosine 1mM, 50mM and 100mM groups. After thawing samples were analyzed by computer-assisted analysis of sperm motility, plasma membrane (eosin/nigrosin), acrosome integrity (fast green/rose Bengal), mitochondrial activity, DNA integrity and sperm resistance to oxidative stress (by TBARS). Decrease was observed in motility sperm kinetics (total and progressive motility) and reduced lipid peroxidation products in the group treated with 50mM and 100mM. On the other hand, 1mM was similar to control group. In conclusion, higher carnosine concentration (50 and 100mM) apparently promoted impairment in energy production and consequently was harmful to sperm kinetics. Thus, future studies must be performed using different carnosine concentrations and in association with substrates for glycolysis and oxidative phosphorylation.

**Key words:** Carnosine; Cryopreservation; Malondialdehyde; Oxidative Stress; Lipid Peroxidation; Canine.  
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### RESUMEN

La criopreservación es un proceso clave entre las biotecnologías reproductivas en caninos. Sin embargo, durante la criopreservación espermática se da una generación excesiva de especies reactivas de oxígeno (ROS), lo que lleva a una disminución en la calidad espermática. Por lo tanto, varios medios de congelación utilizando antioxidantes para evitar tales efectos han sido evaluados, aunque la carnosina todavía no se ha utilizada. La carnosina es una proteína presente en el plasma seminal que a diferencia de otros antioxidantes tiene la habilidad de remover productos de la peroxidación lipídica (malondialdehído), que son tan dañinos como los ROS. Por lo tanto, el objetivo de este estudio fue evaluar los efectos de diferentes concentraciones de carnosina durante la congelación espermática en perros. Para este propósito se utilizaron seis perros en edad reproductiva y después de la colectar los eyaculados, las muestras fueron criopreservadas en un diluyente Control (tris, citrato, yema de huevo), Carnosina 1mM, 50mM y 100 mM. Después del descongelado, las muestras fueron evaluadas mediante el análisis computerizado de la motilidad, integridad de membrana plasmática (eosina / nigrosina), integridad del acrosoma (Fast - green / rosa de Bengala), la actividad mitocondrial (3'3 Diaminobenzidina), la integridad del ADN (SCSA) y la evaluación de la resistencia al estrés oxidativo (TBARS). Se observó una disminución en la cinética de los espermatozoides (motilidad total y progresiva) y una reducción de los productos de la peroxidación lipídica en los grupos tratados con 50 mM y 100mM de carnosina. Por otro lado, el grupo con 1 mM de carnosina fue similar al control. En conclusión, una alta concentración de carnosina (50 y 100mM) parece afectar la producción de energía del espermatozoide y por lo tanto es perjudicial para la cinética del espermatozoide. Por lo tanto, futuros estudios deben realizarse utilizando diferentes concentraciones de carnosina y en asociación con sustratos para la glucólisis y la fosforilación oxidativa.

**Palabras clave:** Carnosina; Malonilaldehído; Estres oxidative; Peroxidación lipídica; Canino.  
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## INTRODUCTION

The cooling and sperm cryopreservation process are the main steps in canine assisted reproduction (Lucio et al. 2016ab; Jarosz et al. 2016). Seminal cryopreservation appears to be an alternative to store sperm samples during long periods, including post mortem, and then perpetuates the progenitor genetic characteristics (Lucio et al. 2016ab). But, it is estimated a 50% loss of post-thawing sperm quality (Farstad, 2009). This decrease in quality appears to be multifactorial, and may be caused by alterations in sperm plasma membrane permeability, osmotic changes, excessive cell dehydration and intracellular ice crystals formation (Watson, 2000). In this context, studies showed that the main cause of reduced post-thawing sperm quality is the excessive reactive oxygen species (ROS) generation, mainly due to mitochondrial dysfunctions and plasma membrane damage (Griveau & Le lannou, 1997ab).

ROS play a key role in sperm physiology, when in balance with the seminal antioxidant capacity, acts as triggers on biological processes, such as, hyperactivation, sperm capacitation, acrosomal reaction, binding of spermatozoon and oocyte and sperm penetration through the zona pellucida (de Lamirande et al. 1997). On the other hand, in situations of exacerbated ROS production or decreased antioxidant capacity characterizes the seminal oxidative stress (Kashou et al. 2013). In this case, ROS can be extremely deleterious, promoting damage to several sperm structures, proteins, carbohydrates and lipids (Griveau & Le Lannou, 1997ab). In addition to the ROS deleterious effect, lipid peroxidation produces malondialdehyde (MDA) which can be harmful as the reactive oxygen species (Sharma & Agarwal, 1996).

In fact, studies demonstrated that MDA can be extremely cytotoxic to spermatozoa, causing reduced motility, increasing DNA damage, and decreasing spermatozoa-oocyte binding, which affects the fertility rates (Ko et al. 2014; Moazamian et al. 2015). Since conventional antioxidants are not able to eradicate the malondialdehyde, some substances present in the seminal plasma ensure this function, such as, the carnosine protein (Ducci et al. 2006). The hydrophilic nature of carnosine allows the adhesion of the molecule to the fissures in the lipid bilayer of biological membranes, effectively attenuating the peroxidation products formed in damaged areas of these cellular structures (Kohen et al. 1988).

Also, carnosine binds to aldehydes derived from lipid peroxidation, acting as sacrificial nucleophiles, sequestering aldehydes, and cell macromolecules, thereby attenuating damage and progression of the oxidative process (Burcham et al. 2002). Despite carnosine beneficial effects, the removal of seminal plasma is necessary for sperm cryopreservation in dogs, which may compromise the oxidative balance of the spermatozoa (Lucio et al. 2016a). Thus, a possible alternative would be sperm treatment using carnosine during the cryopreservation process. Therefore, the purpose of this study was to evaluate the effects of different carnosine concentrations, during seminal cryopreservation in dogs, on oxidative status and post-thawing sperm quality.

## MATERIALS AND METHODS

### Animals

This study was according to the Bioethics Committee of the School of Veterinary Medicine and Animal Science - University of São Paulo. Furthermore, all chemicals and media were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned. Six dogs (aged 1 to 6 years) of several breeds (German Shepherd, Malinois, and Labrador Retriever) and body weights (average of 30 kg) were used. Ejaculate sperm-rich fraction was collected by digital stimulation and immediately after collection semen samples were macroscopically evaluated by appearance, color and volume. Samples were discarded if any abnormalities were detected (i.e. blood or urine contamination). Moreover, semen samples were evaluated for sperm motility subjectively, and a minimum criterion for inclusion was a total motility higher than 70%.

### Experimental Design

After semen collection, the sample of each animal were divided into 4 aliquots which were centrifuged (500 x g, for 10 minutes). The pellets formed were resuspended in diluents with tris-citrate egg yolk (Control group) cryoprotectant (0.26 M Tris-hydroxymethylaminomethane, 0.14 M Citric Acid monohydrate, 0.06 M D-fructose, 20% egg yolk, 0.02 M of gentamicin and distilled water, 1179 mOsm/L, 10% glycerol, pH=6.95) (Lucio et al 2016b). The composition of carnosine extenders was similar to control group except that 1mM, 50mM and 100mM carnosine was supplemented.

### Cryopreservation and thawing process

After dilution, semen samples reached a final concentration of  $100 \times 10^6$  sperm/mL; this sperm number was estimated using a hemocytometer counting chamber. The one step cryopreservation protocol was according to Brito et al. 2017. That is, diluted sperm were packaged in 0.5 mL straws, and samples at 37°C were submitted to slow cooling to 5°C for 1 hour. After this period, the semen was kept in nitrogen vapor (-70°C)

for 20 minutes and then submerged and stored in liquid nitrogen. For thawing procedure, semen samples were thawed at 37°C for 30 seconds (Brito et al. 2017).

### Semen analysis

Semen samples (Control, Carnosine 1mM, Carnosine 50mM and Carnosine 100mM groups) were analyzed after thawing, and one straw per group was thawed, one at a time.

### Computer-assisted analysis of sperm motility

Samples from each group (Control, Carnosine 1mM, Carnosine 50mM and Carnosine 100mM groups) after thawing were immediately evaluated for computer-assisted sperm analysis (CASA; HTM-IVOS Ultimate 12.3; Hamilton Thorne Biosciences, Beverly, MA, USA), according to previously protocol described for dogs (Iguer-ousada & Verstegen, 2001). In brief, 10 µL of each sample was placed on microscope slides warmed at 37°C and then covered by coverslips. Ten fields of view were selected and the following variables were evaluated: VAP (average pathway velocity, µm/s), VSL (straight-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, %). Also, the sperm were 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, %).

### Plasma membrane and acrosome integrity analysis

Eosin/nigrosin stain (Lagergren, 1953) were used to evaluate the plasma membrane permeability, the previously protocol were adapted for dogs (Angrimani et al. 2014). Briefly, 5 µL of semen and 5 µL of the prepared stain were placed in a pre-warmed (37°C) glass slide. Sperm smear was analyzed under light microscopy at 1000× magnification (Nikon, Eclipse E200, Japan). Were considered the intact sperm (membrane integrity) as cells with no stain; and sperm with lesions (membrane damage) as pink colored cells. Results were analyzed by counting 200 cells and expressed as percentage of plasma membrane lesion (%).

To assess sperm acrosome integrity were used the modify protocol of Fast Green/Rose Bengal stain, described by Pope et al. 1991, adapted for dogs (Angrimani et al. 2014). Briefly, 5µL of semen was mixed with 5µL of Fast Green/Rose Bengal stain in a pre-warmed glass slide (37°C). Smears were analyzed under light microscopy at 1000x magnification (Nikon®, Eclipse E200, Japan). If sperm acrosomal region stained was purple or darker than post-acrosomal area, sperm acrosome was considered as intact. Whence acrosomal region was unstained or brighter than post-acrosomal area, the acrosome was considered as damaged. Percentage of acrosomal integrity was analyzed by counting 200 cells (%).

### Evaluation of mitochondrial activity

The assessment to mitochondrial activity, were performed using the cytochemical technique of 3,3' diaminobenzidine solution (1 mg/mL of DAB in PBS), which classified the sperm into four classes of mitochondrial activity: high (DAB - Class I), medium (DAB - Class II), low (DAB - Class III) and absence (DAB - Class IV) (Lucio et al. 2016). For this, a sperm sample aliquot was incubated under light at 37°C for 1 hour with DAB in a ratio of 1:1 (25 µL of sample in 25 µL of DAB). Then, smears were performed on glass slides with subsequent fixation in 10% formalin for 15 minutes. Evaluation was under a microscope with transmitted light under oil immersion objective at 1000× magnification (Nikon®, Eclipse E200, Japan), with 200 sperm counted. Results were expressed as a percentage (%).

### Assay of the sperm chromatin structure

To chromatin analysis with fluorescent probe were used the Guava EasyCyte™ Mini System (Guava® Technologies, Hayward, CA, USA), a 488 nm laser argon and the following filters (photodetector): PM1 (583 nm) to yellow fluorescence, PM2 (680 nm) to red and PM3 (525 nm) to green. In each assay were evaluated a minimum of 20,000 spermatozoa. Data were analyzed using FlowJo v8.7 Software (Flow Cytometry Analysis Software — Tree Star Inc., Ashland, Oregon, USA). To assess the chromatin susceptibility to acid-induced denaturation were used a previously protocol for dogs (Lucio et al. 2016), based on the sperm chromatin structure assay (SCSA) (Evenson & Jost, 2000). Thus, the chromatin instability was quantified by flow cytometric measurement of the metachromatic shift from green (double-strand DNA) to red (denatured single-strand DNA) of acridine orange (AO) fluorescence. Samples were diluted in 100 µL of TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) and mixed with 400 µL of an acidified detergent solution (0.08 M HCl, 0.1% Triton X-100, 0.15 M NaCl, pH 1.2). After 30 seconds, spermatozoa were stained by mixing 600 µL of AO staining solution (0.037 M citric acid, 0.126 M Na<sub>2</sub>HPO<sub>4</sub>, 0.0011 M disodium EDTA, 0.15 M NaCl, pH 6.0). After 3 to 5 minutes of staining, samples were examined by flow cytometry. Then, DNA fragmentation was calculated based on the percentage of spermatozoa external to the main population in a histogram of αT (ratio between red fluorescence and total fluorescence) and calculated using FlowJo system (Version Mac) (Evenson & Jost 2000; Minervini et al. 2013)

### Sperm resistance to oxidative stress

After thawing, samples were two times centrifuged (800 x g, 10min, 4°C) to remove the remains of extender and then resuspended in physiological saline solution (NaCl 0.9%). Lipid peroxidation was induced by adding ferrous sulphate (100µL; 4mM) and sodium ascorbate (100µL; 20mM) to 0.4mL of sperm suspension, subsequently combination was incubated for 2 hours at 37°C (Gomez et al. 1998). Then, levels of thiobarbituric acid reactive substances (TBARS) were prepared in accordance with Nichi et al. 2007 and to Lucio et al. 2016a. The method is based on the reaction of two molecules of thiobarbituric acid with one molecule of malondialdehyde, at high temperatures and low pH, resulting in a pink chromogen that can be quantified with a spectrophotometer. After the 2 hours period, 500µL of the incubation mixture and 1000µL of a 10% solution (v:v) of trichloroacetic acid (TCA 10%) were mixed and centrifuged (18.000 x g, 15 min, 15°C), with the purpose to precipitate protein. Following this, 500µL of the supernatant was mixed in 500µL of 1% (v:v) thiobarbituric acid (TBA, 1% diluted in 0.05 N sodium hydroxide) in a glass tube and placed into a boiling water bath (100°C) for 20 min, and immediately cooled in an ice bath (0°C) to stop the chemical reaction. The thiobarbituric acid reactive substances (TBARS) were then quantified using a spectrophotometer (U-2001 spectrophotometer, Hitachi High Technologies America, Inc., San Jose, CA, US) at a wavelength of 532nm. Results were compared to a standard curve previously prepared with a standard solution of malondialdehyde. The TBARS concentration was determined using the value of  $1.56 \times 10^5$  x M/mL as the MDA extinction coefficient (Buege & Aust, 1978). The lipid peroxidation index is described as nanograms of TBARS/ $10^6$  sperm.

### Statistical Analysis

All data were evaluated using the SAS System for Windows (SAS Institute Inc., Cary, NC, USA). Differences The effect of sperm group (Control, Carnosine 1mM, Carnosine 50mM and Carnosine 100mM groups) was determined using parametric (LSD test) and nonparametric (Wilcoxon) tests, according to the residue normality (Gaussian distribution) and variance homogeneity of variables. A probability value of  $P < 0.05$  was considered statistically significant. Results are reported as untransformed means  $\pm$  SEM.

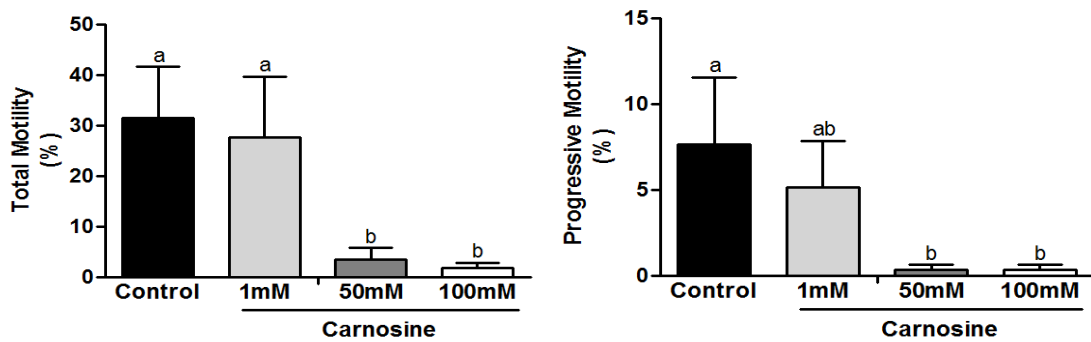
## RESULTS

Any protective effects of carnosine during the cryopreservation process were observed. On the other hand, the high carnosine concentrations (50mM and 100mM) used in the present experiment promoted a deleterious effect for some sperm variables. The Computer Assisted Sperm Analysis (CASA) showed significant and progressive decrease in sperm kinetics while the concentration of carnosine was rise. Total and progressive motility, fast and medium sperm velocity, showed that Control presented higher values than Carnosine 50mM and 100mM groups (Figure 1 and Table 1). Moreover, slow sperm velocity was higher in Carnosine 1mM than Carnosine 50mM and 100mM groups (Table 1). On the other hand, average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) was higher in the Control group than Carnosine 50mM (Table 1). Furthermore, Carnosine 100mM presented higher percentage of static sperm than Control and Carnosine 1mM (Table 1). In contrast, no differences were observed between the groups for the variables sperm beat cross-frequency (BCF), amplitude of lateral head displacement (ALH), sperm straightness (STR) and sperm linearity (LIN, Table 1).

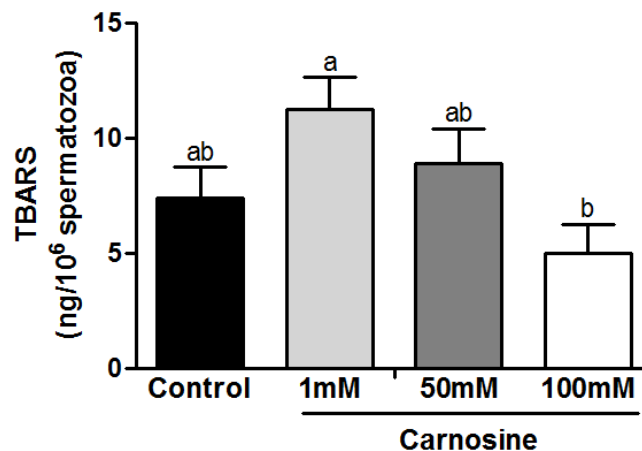
**Table 1. Effect of carnosine treatments (control group, carnosine 1mM, 50mM, and 100mM) on sperm kinetic variables in dogs.**

	Control	Carnosine 1mM	Carnosine 50mM	Carnosine 100mM
Percentage of fast sperm (%)	9.5 $\pm$ 4.9 <sup>a</sup>	6.3 $\pm$ 3.4 <sup>ab</sup>	0.5 $\pm$ 0.5 <sup>b</sup>	0.3 $\pm$ 0.3 <sup>b</sup>
Percentage of medium sperm (%)	21.8 $\pm$ 6.3 <sup>a</sup>	21.3 $\pm$ 8.6 <sup>a</sup>	3.1 $\pm$ 2 <sup>b</sup>	1.6 $\pm$ 0.9 <sup>b</sup>
Percentage of slow sperm (%)	17.8 $\pm$ 3.2 <sup>ab</sup>	20.8 $\pm$ 8.1 <sup>a</sup>	4.3 $\pm$ 3.7 <sup>b</sup>	4.6 $\pm$ 3.7 <sup>b</sup>
Percentage of static sperm (%)	50.6 $\pm$ 12.7 <sup>b</sup>	51.6 $\pm$ 15.6 <sup>b</sup>	75.3 $\pm$ 16.1 <sup>ab</sup>	93.1 $\pm$ 4.6 <sup>a</sup>
Sperm velocity average pathway (µm/s)	71.4 $\pm$ 7.7 <sup>a</sup>	51.8 $\pm$ 16.6 <sup>ab</sup>	27.8 $\pm$ 13 <sup>b</sup>	40.3 $\pm$ 13.9 <sup>ab</sup>
Sperm velocity straight line (µm/s)	61 $\pm$ 5.8 <sup>a</sup>	43.5 $\pm$ 14 <sup>ab</sup>	16.8 $\pm$ 9.5 <sup>b</sup>	36.3 $\pm$ 12.6 <sup>ab</sup>
Sperm curvilinear velocity (µm/s)	100.2 $\pm$ 11.4 <sup>a</sup>	69.9 $\pm$ 22.3 <sup>ab</sup>	27.2 $\pm$ 14.5 <sup>b</sup>	55.6 $\pm$ 18.8 <sup>ab</sup>
Sperm beat cross-frequency (Hz)	17.9 $\pm$ 1.2	9.4 $\pm$ 3.1	18.6 $\pm$ 10.8	11.5 $\pm$ 4.1
Amplitude of lateral head displacement (µm/s)	6.2 $\pm$ 1.3	4.6 $\pm$ 1.5	15.8 $\pm$ 14.6	2 $\pm$ 1.5
Sperm straightness (%)	86.1 $\pm$ 1.2	55.5 $\pm$ 17.5	39.3 $\pm$ 18.2	60.3 $\pm$ 19.1
Sperm linearity (%)	64.8 $\pm$ 1.9	43.3 $\pm$ 13.7	28.2 $\pm$ 17.3	45 $\pm$ 14.4

<sup>a-b</sup>indicate significant differences between groups ( $P < 0.05$ ).



**Figure 1.** Effect of carnosine treatments (control group, carnosine 1mM, 50mM, and 100mM) on total and progressive motilities in dogs. <sup>a-b</sup> indicate significant differences between groups ( $P < 0.05$ ).



**Figure 2.** Effect of carnosine treatments (control group, carnosine 1mM, 50mM, and 100mM) on sperm lipid peroxidation (TBARS) in dogs. <sup>a-b</sup> indicate significant differences between groups ( $P < 0.05$ ).

Regarding the sperm analysis, it was possible to observe higher amounts of TBARS in Carnosine 1mM than Carnosine 100mM group (Figure 2). Moreover, no differences were observed between control and carnosine groups in the integrity of plasma and acrosomal membranes, mitochondrial activity and DNA integrity (Table 2).

**Table 2. Effect of carnosine treatments (control group, carnosine 1mM, 50mM, and 100mM) on sperm attributes (mitochondrial activity, Integrity of plasma and acrosomal membranes and DNA fragmentation) in dogs.**

	Control	Carnosine 1mM	Carnosine 50mM	Carnosine 100mM
High mitochondrial activity (%)	90.6±2.6	90.5±2.5	89.7±2.6	85.4±3.7
Medium mitochondrial activity (%)	5±2.3	3.2±1.9	2.2±1.6	6.8±2
Low mitochondrial activity (%)	2±0.5	1.2±0.7	3±0.5	3.8±1.1
Absence of mitochondrial activity (%)	2.3±0.8	5±1.4	5±1.2	4±0.8
Sperm plasma membrane integrity (%)	19.8±3	21.5±5.4	20.3±6.2	40.3±13.5
Acrosome membrane integrity (%)	19.5±2	18.8±2.1	20±3.8	16±2.3
DNA Fragmentation (%)	0.65±0.3	0.51±0.2	0.28±0.1	0.28±0.1

## DISCUSSION

Cryopreservation process promotes a reduction in sperm viability (Farstad 2009). Among the main reasons for this decrease in sperm quality, the exacerbated production of reactive oxygen species (ROS), characterizing the seminal oxidative stress, is highlighted (Griveau & Le Lannou, 1997ab). In addition to the deleterious effect of ROS, the lipid peroxidation products, such as malondialdehyde (MDA), have a cytotoxic effect on spermatozoa, which may cause sperm DNA fragmentation and reduce oocyte binding (Sharma & Agarwal, 1996). Despite conventional antioxidants are not able to remove MDA, proteins present in seminal plasma such as carnosine have this function, promoting an antioxidant activity (Ducci et al. 2006).

However, removal the seminal plasma is necessary for cryopreservation process in dogs (Brito et al. 2017). Consequently, carnosine is removed during seminal processing, which may promote oxidative imbalance, increase of lipid peroxidation products and thus reduce post-thawing sperm quality (Ogata et al. 2015). Therefore, the scope of our study was to evaluate the effects of different concentrations of carnosine associated to tris-citrate egg yolk extender for cryopreservation on the oxidative status and post-thawing sperm functionality in dogs.

Regards to TBARS concentration, we observed that the group treated with 100mM of carnosine presented lower susceptibility to lipid peroxidation in relation to the group treated with 1mM. Thus, we suggest that carnosine at this concentration was efficient to remove the excess of lipid peroxidation products (i.e. MDA). In fact, several studies showed that carnosine, besides acting as an antioxidant, is extremely efficient in removing lipid peroxidation products in several biological systems (Pagano et al. 1997; Sanchez et al. 1997; Guiotto et al. 2007). Additionally, this dipeptide is effective in preventing aging due to the prevention of shortening of the telomeres (Hipkiss et al. 2016) besides acting effectively in healing processes (Dunnett et al. 2002). On the other hand, there is an absence of data demonstrating the effect of carnosine on sperm physiology. For that reason, this study is promising for the future supplementation using this protein in sperm biotechnologies.

Despite the beneficial effect of carnosine at 100mM, we observed a drastic reduction in total and progressive motility and the percentage of rapid cells in the groups treated with 50 and 100mM carnosine compared to untreated group (control group). In fact, carnosine has the ability to metabolize intermediates substrates of glycolysis, and may alter energy production by this pathway (Holliday & Mcfarland, 1996). Although, in this study the ATP levels were not evaluated, we can suggest that are changes in spermatid kinetics due to this deleterious effect of carnosine at high concentrations.

Previously studies showed that glycolysis appears to be efficient as oxidative phosphorylation in ATP synthesis for sperm motility (Mukai & Okuno, 2004). Furthermore, the ATP generated by the mitochondria in the intermediate piece does not appear to diffuse efficiently in the more distal portions of the flagellum, being extremely necessary the performance of the glycolytic pathway for this function (Turner, 2003). Thus, it seems evident that high carnosine concentrations may cause changes in energy production and consequently in spermatid kinetics.

Additionally, by interfering the glycolytic pathway, carnosine may reduce pyruvate levels (glycolysis product), which is extremely important for mitochondrial function (Krebs cycle and oxidative phosphorylation) (Holliday & Mcfarland, 1996). Thus, a possible alternative in the carnosine use during cryopreservation process is the association with pyruvate and glycolysis substrates (Holliday & Mcfarland, 1996). Moreover, different concentrations of carnosine should be considered in the presence of these substrates. This was a pioneer study, and we test large concentrations (1, 50 and 100mM), in order to evaluate how carnosine acts at different levels. Apparently, concentration ranges between 50 and 100mM may show promising results, as the higher concentration (100mM) reduced cell susceptibility to lipid peroxidation. However, these results should be carefully interpreted since the motility was low in these same groups, showing a deleterious effect in sperm viability.

Finally, it would be extremely important to evaluate in future studies the effect of these treatments on sperm capacitation and fertility. Because, despite carnosine prevent excessive amounts of reactive oxygen species and lipid peroxidation products, high concentrations of carnosine may inhibit physiological events such as acrosomal reaction, sperm hyperactivation, sperm and oocyte binding, and consequently reduce of fertility (Lamirande, Gagnon 1992).

## CONCLUSIONS

Regarding the results obtained in this study, carnosine can efficient reduce the lipid peroxidation products in the group treated with 100mM. On the other hand, the higher carnosine concentration (50 and 100mM) apparently promoted impairment in energy production and consequently

was harmful to sperm kinetics. Thus, we can suggest in future experiments the spermatogenic therapies with carnosine in association with substrates for glycolysis and oxidative phosphorylation, such as glucose and pyruvate, aiming the reduction of lipid peroxidation without harm the energy production of canine spermatozoa post-thawing.

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