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EVALUATION OF ARACHIDONIC ACID SUPPLEMENTATION IN THE EXTENDER ON THE INTEGRITY OF THE ACROSOME MEMBRANE IN POST-FREEZING GOAT SEMEN

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Abstract: Seminal cryopreservation causes damage to the integrity of sperm membranes, caused by oxidative stress, impairing the functional capacity of sperm cells. However, maintaining the integrity of the acrosomal membrane after cryopreservation is essential for fertilization to occur. Therefore, it is necessary to add substances that can improve the antioxidant defenses of biological systems, combating the high production of ROS. In this sense, the addition of arachidonic acid, a polyunsaturated fatty acid, was able to improve membrane fluidity, reduce the action of free radicals and maintain the integrity of sperm membranes in bovine and swine species. Thus, the objective was to evaluate the supplementation of arachidonic acid in the TRIS-yolk extender on the acrosome integrity during the cryopreservation of goat spermatozoa. Six ejaculates/animal were collected from four Anglo-Nubian goats, using artificial vagina. After immediate analysis, they were pooled, diluted in TRIS-Gema and divided into treatments: control and supplementation with 0.5μM, 5 μM and 50μM of arachidonic acid. Then the semen was cryopreserved. After thawing, the acrosomal membrane integrity analysis was performed using the FITC-PNA dye. 20μL aliquots of FITC-PNA were placed on smears of slides containing sperm and incubated for 20 minutes in a humid chamber at 4°C, in the absence of light. Then slides were rinsed twice in refrigerated PBS and dried in the dark. Immediately prior to evaluation, 5μL of UCD mounting medium was placed on the slide and covered with a coverslip. 200 spermatozoa/slide were evaluated in an epifluorescence microscope (400x), using an emission filter LP 515nm and BP 450-490nm for excitation. After the analyses, it was found that the supplementation of 50μM of arachidonic acid in the extender significantly differed from the other treatments in relation to the acrosomal

integrity. A possible explanation for the lower values after the addition of 50μM arachidonic acid would be its excessive concentration, considering that arachidonic acid, through the action of cyclooxygenase, is converted into prostaglandin E2, inducing the influx of Ca2+ through the membrane. sperm, leading to membrane fusion. It is concluded that the addition of 0.5μM and 5μM of arachidonic acid to the extender maintained the integrity of the acrosome membrane in post-cryopreservation goat semen, being beneficial for the process of sperm capacitation and fertilization, suggesting further research aiming at a greater accuracy of the concentrations of arachidonic acid as an antioxidant.
Keywords: Antioxidants, arachidonic acid, acrosomal membrane, cryopreservation.

INTRODUCTION

Cryopreservation was notable due to its benefits, being widely used, allowing the expansion of animal reproduction programs. This biotechnique allows the storage of genetic material indefinitely, in addition to a better use of animals of high zootechnical value (PURDY, 2006). However, during the cryopreservation process, numerous damages are caused to the sperm cell, which reduces the number of viable cells, as well as their functional capacity (WATSON, 2000).

In this sense, the maintenance of the integrity of the acrosomal membrane after cryopreservation is essential for the acrosomal reaction to occur, that is, the fusion of the sperm plasma membrane with the outer membrane of the acrosome, and consequent release of hydrolytic enzymes, such as acrosin and hyaluronidase, which result in the penetration of the ovum, in addition to indicating a good freezeability of the semen. (HAFEZ; HAFEZ, 2004).

During aerobic metabolism, spermatozoa produce reactive oxygen species (ROS) in certain amounts, and when ROS production increases, the antioxidant potential of sperm cells can be depleted, generating oxidative stress (AITKEN, 1995). Therefore, the greater the exposure of these cells to ROS, the greater the damage caused by lipid peroxidation (LPO), due to their high content of polyunsaturated fatty acids (PUFAs) in the membrane and low cytoplasmic antioxidant capacity. (DARBANDI *et al.*, 2019).

Therefore, strategies have been developed to improve the resistance of sperm cells to the cryopreservation method, such as the addition of substances that can improve the antioxidant defenses of biological systems, combating the high production of ROS (CASTELO BRANCO, 2018). Thus, some polyunsaturated fatty acids have been tested as additives in freezing medium, showing beneficial effects for the seminal cryopreservation process, such as arachidonic acid (EJAZ et al., 2014). These PUFAs were able to improve membrane fluidity, reduce the action of free radicals and maintain the integrity of sperm membranes from other species (NASIRI et al., 2012; TOWHIDI; PARKS, 2012). Regarding research involving arachidonic acid, it has been shown to improve the motility and viability of wild boar sperm (HOSSAIN et al., 2007) and increase motility, DNA integrity and sperm viability in bulls (EJAZ et al., 2007). al., 2014). So, this research was carried out with the objective of evaluating the effect of arachidonic acid supplementation in the TRIS-yolk extender on the acrosome integrity during the cryopreservation of goat spermatozoa.

MATERIAL AND METHODS

ANIMALS

Four Anglo-Nubian goats, aged 1.5 to 3 years, with body condition score 4, on a scale of 1 to 5, were used. This breed was chosen

due to its rusticity and excellent adaptation to the bioclimatological conditions of the region. North East. Previously, andrological exams were carried out, verification of offspring of the sires and assessment of general health.

SEMEN COLLECTION AND INITIAL EVALUATION

Semen collections were performed once a week, for six weeks (totaling 6 ejaculates/ animal), with the help of a female in estrus, using the artificial vagina method. Then, the ejaculates were evaluated individually according to the recommendations of the Brazilian College of Reproduction (CBRA, 2013). After this step, the samples were mixed to form a *"pool"* by collection, being evaluated again in relation to sperm quality.

SEMINAL DILUTION

Subsequently, the TRIS-yolk dilution medium was added to the *pool*, to finally supplement with different concentrations of ARACHIDONIC ACID (ARACHIDONIC ACID – ≥ 98.5%(GC), Sigma-Aldrich®, USA), and evaluate how much to sperm quality. The samples were divided into the following treatments: Control (Tris-yolk without the addition of arachidonic acid); Tris-yolk + 0.5 µM arachidonic acid; Tris-yolk + 5 µM arachidonic acid and Tris-yolk + 50 µM arachidonic acid.

SEMEN CRYOPRESERVATION

After dilution and fractionation, the semen samples were packaged in 0.25mL straws, with a final concentration of 20 × 106 viable spermatozoa / straw, and frozen in a freezing machine. Subsequently, it was thawed in a water bath at 37ºC for 30 seconds to perform the analysis of achromosomal integrity.

ACROSOMAL INTEGRITY ANALYSIS

According to the technique described by Roth et al. (1998), to perform this test, the dye fluorescein isothiocyanate conjugated to Peanut agglutinin (FITC-PNA; Sigma-Aldrich®, USA) was used. 20µL aliquots of FITC-PNA were placed on smears of slides containing sperm, which were incubated for 20 minutes in a humid chamber at 4°C, in the absence of light. Afterwards, the slides were rinsed twice in refrigerated PBS (4°C) and placed to dry in the absence of light. Immediately before evaluation, 5µL of UCD mounting medium (4.5mL of glycerol, 0.5mL of PBS, 5mg of sodium azide and 5mg of p-phenylenediamine) was placed on the slide and covered with a coverslip. 200 spermatozoa per slide were evaluated under an epifluorescence microscope (400x), using a 515nm long pass (LP) emission filter and 450-490nm band pass (BP) emission filter for excitation. Sperm cells were classified into spermatozoa with intact acrosomes, when they presented the acrosomal region stained with green fluorescence, or with reacted acrosomes, presenting a fluorescent green band in the equatorial region of the sperm head or not showing green fluorescence throughout the head region.

DESIGN AND STATISTICAL ANALYSIS

Initially, the variables were submitted to the normality test, verifying the normal distribution of the data. Subsequently, the means and standard deviations of the variables studied were calculated and submitted to analysis of variance (ANOVA) using the general linear models procedure (Proc GLM). Duncan's test was used to compare the means, with a probability of 5%. The analyzes were performed using the *Statistical Analysis System program* (SAS Institute Inc, 2013).

RESULTS AND DISCUSSION

After analyzing the acrosomal integrity (Table 1) of post-cryopreservation goat spermatozoa, it was observed that the treatment with 50 μM of arachidonic acid altered ($p < 0.05$) the acrosomal integrity in relation to the other treatments.

Treatments	AC (%)
Control	60.00 ± 7,84 [a]
0.5 μM of arachidonic acid	53.20 ± 9.85 [a]
5 μM of arachidonic acid	51.60 ± 7.50 [a]
50 μM of arachidonic acid	32.00 ± 17.17 [b]

Mean values with different superscript letters in the same column indicate significant differences ($p < 0.05$) by the DUNCAN test.

Table 1- Means and standard deviations of acrosomal integrity (AC) of post-thawed goat spermatozoa after supplementation of arachidonic acid to the extender.

The understanding of biochemical changes and the production of free radicals during cryopreservation has led to research involving the use of various fatty acids in the role of antioxidants. However, in goats, the use of arachidonic acid and its benefits as a possible antioxidant had not yet been evaluated.

Regarding the evaluation of the acrosomal integrity of goat spermatozoa after arachidonic acid addition, it was observed that the treatment with 50 μM of arachidonic acid altered ($p < 0.05$) the CA in relation to the other treatments (Table 1), showing that the other concentrations of arachidonic acid maintained the structural quality parameters of cryopreserved goat sperm.

A possible reason for the results of treatments with 0.5 μM and 5 μM of arachidonic acid not showing an improvement in relation to the control, may have been the insufficient incorporation of fatty acid into the sperm membrane. According to Ejaz et al. (2014), there is a probability that fatty acids are transported through the plasma membrane, and when they cross the outer membrane and enter the hydrophobic nucleus, they are protonated, allowing them to be included in the inner leaflet, improving membrane integrity rates. which was not found in this experiment.

On the other hand, the lower results after supplementation with a concentration of 50 μM of arachidonic acid may be related to a possible excessive dose of the antioxidant, taking into account that arachidonic acid is converted into prostaglandin E2, through the action of cyclooxygenase, inducing the influx of Ca2+ through the sperm membrane, generating membrane fusion (JOBIM et al., 2009; VIANA, 2018). That is, the influx of calcium and bicarbonate has been related to increased pH, regulation of cAMP levels and hyperpolarization of the plasma membrane (NISHIGAKI et al., 2014), causing damage to sperm cells.

Therefore, it is concluded that the supplementation of 0.5 μM and 5 μM of arachidonic acid to the TRIS-yolk extender was able to maintain the acrosomal integrity of goat spermatozoa after cryopreservation, opening precedents for the continuity of research involving this poly-fatty acid. as an antioxidant, adjusting the concentration for a possible improvement in this percentage.

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