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Cholesterol-loaded metil-β- cyclodextrin in alpaca semen cryopreservation (*Vicugna pacos*) The methodological alternative

Ciclodextrina cargada con colesterol en la criopreservación de semen de alpaca *(Vicugna pacos*) Una alternativa metodológica

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Article Data

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Abstract

The objective of the study was to evaluate the effect of three levels (0, 1.5 and 3 mg) of cholesterol loaded with methyl-β-cyclodextrin (MCD) in the cryopreservation of alpaca semen. Six male alpacas of the Huacaya breed from 6 to 8 years old were used, three for the collection of semen by the vaginal aspiration method (PC), and from three the EPZ were recovered by deviation of the vas deferens (DCD), three collections were madeper animal. Volume, filance, total motility, concentration, EPZ live, sperm membrane integrity and acrosomal integrity were evaluated. A Computer-assisted semen analysis (ISAS®) was used in the microscopic evaluations. The samples were diluted with Tris base to a concentration of 15 million EPZ per mL. It was added papain to samples collected by PC, followed by papain inhibitor. MCD was added in both methods, the cooled and thawed microscopic parameters were evaluated, a process in which dimethylformamide was used as a cryoprotectant. Macroscopic variables were evaluated using descriptive statistics, microscopic variables (mobility, live sperm, membrane functionality, and acrosomal integrity) were analyzed with a factorial arrangement in a randomized design. For all microscopic variables, significant differences (P<0.05) were found between the cooled stage in relation to thawing in both methods. For the PC method, no significant differences (P> 0.05) were found for mobility and vitality between treatments. However, EPZ treated with 1.5 mg of MCD presented higher (P<0.05) membrane functionality and acrosome integrity. In sperm from DCD, no significant differences (P> 0.05) were found between treatments for membrane mobility and functionality, but there was a significant difference (P< 0.05) for vitality and acrosomal integrity when 1.5 mg MCD relative to 3 mg MCD. In conclusion, the EPZ treated with 1.5 mg of MCD have better microscopic characteristics in cooled and thawed semen.

Resumen

El objetivo del estudio fue evaluar efecto de tres niveles (0, 1.5 y 3 mg) de colesterol cargado con metil-β-ciclodextrina (MCD) en la criopreservación de semen de alpaca. Se utilizaron seis alpacas machos de la raza Huacaya de 6 a 8 años, tres para la colección de semen por el método de poscópula (PC), y de tres se recuperaron los espermatozoides (EPZ) por desviación de los conductos deferentes (DCD), se hicieron 3 colectas por animal. Se evaluó el volumen, filancia, movilidad total, concentración, EPZ vivos, integridad de la membrana espermática e integridad acrosomal. Se empleó un sistema de análisis computarizado (ISAS®) en las evaluaciones microscópicas. Las muestras fueron diluidas con base Tris hasta una concentración de 15 millones de EPZ por mL a las muestras de PC se añadió papaína, después inhibidor de papaína. Se añadió MCD en ambos métodos, se evaluó los parámetros microscópicos en refrigerado y descongelado, proceso en el que se usó dimetilformamida como crioprotector. Las variables macroscópicas se evaluaron mediante la estadística descriptiva, las variables microscópicas (movilidad, EPZ vivos, funcionalidad de la membrana e integridad acrosomal) con un arreglo factorial en un diseño al azar. Para todas variables microscópicas en estudio se hallaron diferencias significativas (P< 0.05) entre la etapa de refrigerado e n relación al descongelado en ambos métodos. Para el método de PC, no se ha observado diferenciassignificativas

(P> 0.05) para la movilidad y vitalidad entre tratamientos. Sin embargo, EPZ tratados con 1.5 mg de MCD presentaron mayor (P< 0.05) funcionalidad de la membrana e integridad de acrosoma. En los espermas provenientes de la DCD, no se hallaron diferencias significativas (P> 0.05) entre tratamientos para la movilidad y la funcionalidad de la membrana, pero hubo diferencia significativa (P< 0.05) para la vitalidad e integridad acrosomal cuando se adicionó 1.5 mg de MCD en relación a 3 mg MCD. En conclusión, los EPZ tratados con 1.5 mg de MCD tienen mejores características microscópicas en al refrigerado y descongelado en ambos métodos.

Palabras clave:

Alpaca,
congelación,
espermatozoides,
methy-β-ciclodextrina,
membrana plasmática.

Introduction

Cryopreservation is a reproductive biotechnique that allows the long-term conservation of the genetic value of different species. In South American camelids (SAC), the cryopreservation process negatively affects sperm viability (EPZ). However, in recent years several studies have sought viable protocols, including the use of cryoprotectants and diluents that allow improving the survival of EPZ[1-3]. Events during the freezing/thawing of sperm cells can cause irreversible partial changes and damages, especially in the membrane, leading to a decrease in the fertilizing capacity of EPZ[4]. Sperm viability membranes are composed of lipids and proteins, with the predominant being phospholipids and cholesterol[5], when these are subjected to low temperatures they affect membrane fluidity, permeability and distribution of phospholipids[6]. The cholesterol/phospholipid ratio in EPZ is an essential factor for membrane fluidity. Several studies observed that higher cholesterol/phospholipid ratios are highly resistant to thermal shock, as in humans, rabbits and dogs[7,8] while others such as bulls, wild boars and sheep are susceptible[6].

The addition of cholesterol to the EPZ membrane increases the survival rate to cryopreservation, its incorporation into the strata of the sperm membrane is easily carried out by means of cyclodextrins[9,10]. Cyclodextrins (CDs) are cyclic oligosaccharides that can be hydrophilic external or hydrophobic internal cavity in character most commonly found as six to eight glycosidic units, designated α, β and γ[11]. Of these, the -β-cyclodextrins have a higher affinity with lipid compounds, particularly with cholesterol, even the addition of the methyl group to the DC molecule increases its solubility in water and the solubility capacity of hydrophobic compounds[12].

Based on these observations, there are studies that reveal better tolerance to cryopreservation in sheep[13], horses[14] cattle[10] and camels[15]. Therefore, the objective is to evaluate the effect of concentrations of 0, 1.5 and 3 mg of cholesterol loaded with methyl-β-cyclodextrins (MCD) in the cryopreservation of EPZ from alpacas collected by the post-copula method (PC) and recovered by diversion. of the vas deferens (DCD).

Materials and methods

The experimental procedures were approved by the Ethics and Animal Welfare Committee, as reflected in the regulations: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

Study place and animals. The study was carried out at the La Raya South American Camelid Research Center (CICAS), of the Faculty of Agricultural Sciences (FCA) of the National University of San Antonio Abad of Cusco (UNSAAC) at an altitude of 4130 meters, between February and March of 2018. The experimental group composed of 6 male alpacas aged

between 6 and 8 years, with an average body weight of 71.91±4.05 kg, and an electronic scale (Guindaste industrial LCD portable) were used. The animals were dewormed under the supervision of a veterinarian. The feeding was in a native meadow with a predominance of *Festuca* sp., *Muhlenbergia fastigiata, Scirpus rigidus and Alchemilla pinnata* and ad libitum water.

Semen collection/sperm retrieval and sperm evaluation.

Postcopula method (PC). Semen was collected from 3 male alpacas, for 3 occasions per animal, and at intervals of one week[3,16]. The animals were previously trained to handle them to facilitate the obtaining of the samples. The collected semen was kept at 37º C in a dry bath, the filance was calculated by the rupture of the sample thread, measured with a ruler, and the volume was visually evaluated[17]. Subsequently, papain (1:1) (v:v) (Sigma-Aldrich, St Louis, MO, USA) was added, mixed homogeneously and incubated for 30 min at 37º C in a water bath. Papain inhibitor (E64, Sigma -Aldrich, St Louis, MO, USA) was then added at a concentration of 20 µL/mL of semen diluted with papain for 5 min, maintaining the same temperature, centrifuged at 3000 rpm for 10 min[15]. The seminal plasma supernatant was eliminated, and finally, a Tris (fructose and citric acid)-based diluent was added to the semen samples at 37º C[18].

Deviation of the vas deferens (DCD). Three animals were used, on three occasions with intervals of one week[19]. The alpacas were previously surgically intervened and the vas deferens was diverted towards the internal face of the muscle[20] through a fistula, the drops of EPZ were absorbed and deposited in 0.3 mL of Tris base dilutor in 2 mL Eppendorf tubes immediately maintained at 37 ° C a dry bath[21].

Preparation of saturated cholesterol with cyclodextrin (MCD). Two solutions, A and B, were prepared. Solution A containing 1 g of CD was diluted in 2 mL of methanol, and solution B was diluted with 200 mg of cholesterol in 1 mL of chloroform[10]. Once prepared, 0.45 mL of solution B was added to solution A, mixed homogeneously and poured onto a glass Petri dish until completely dry (1.5 days) at 37° C in an oven. Once the solvent had evaporated, the remaining precipitate was collected and stored in amber glass bottles at room temperature until use. Subsequently, the MCD was mixed with a Tris base dilutor, at a concentration of 50 mg/mL[10].

Addition of cholesterol in the EPZ and cryopreservation. The samples of both PC and DCD methods were diluted to a final concentration of 15 million EPZ/mL, separated into 3 aliquots to be added with 0, 1.5 and 3 mg of MCD in the dark, at a temperature of 37° C for 15 min[15]. Immediately the samples were centrifuged (1750 rpm x 10 min), the supernatant was removed, the pellet was diluted in a cryoprotectant, which contained, Tris-based dilutor 10 mL, egg yolk 5 mL and dimethylformamide 3.125 µL[22]. The EPZ were loaded into 0.5 mL straws and sealed, for the cooling stage the temperature was gradually lowered from 37° C to 4° C in a period of 2.5 h, then, they were kept in liquid nitrogen vapor over 3 cm for 15 min. For freezing, the straws were immersed and kept in liquid nitrogen at -196º C until the subsequent evaluation and thawing was after 7 days in water at 37º C for 60 s[22].

Mobility, concentration, live EPZ, functional membrane integrity, and acrosomal integrity were evaluated in fresh, chilled, and thawed semen samples containing 0, 1.5, and 3 mg MCD using the Integrated Semen Analysis System (ISAS® v1.1). equipped with a UOP-UB200i microscope (Proiser R+D, Paterna,

Valencia, Spain). Mobility was evaluated on the stage tempered at 37° C of the microscope with the 10X negative phase contrast objective. 10 microvideos were evaluated, each video signal was acquired with a Proiser782C video camera, at a capture speed of 25 images per second, both for sperm mobility and concentration, using 5 µL sample.

The percentage of live EPZ was evaluated with the Vital Test® kit, while the functionality analysis of the sperm membrane (HOST) was performed with a hypoosmotic solution of 50 mOsm/L[19], acrosomal integrity analysis was performed using Coomasie blue[23].

Statistical analysis. Descriptive statistics were employed for macroscopic (volume, filance) and microscopic variables (total motility, concentration, live EPZ, sperm membrane functionality and acrosomal integrity of EPZ collected PC and recovered from vas deferens.

Normality and homoscedasticity assumptions were determined using the Shapiro-Wilks and Levene tests for microscopic variables in the cooling and thawing process, and data transformation of total motility percentage and live EPZ was performed using the Transreg procedure of SAS. These variables were analyzed with a factorial arrangement in a randomized design, the comparison of means was done with the LSD test (α=0.05). All statistical procedures were performed with SAS v 8.2 (NC State University, USA).

Results

Figure 1 Mobility of spermatozoa loaded with 1.5 mg cyclodextrin (MCD) classified according to their trajectory (red, green and blue) and static (yellow). (yellow)

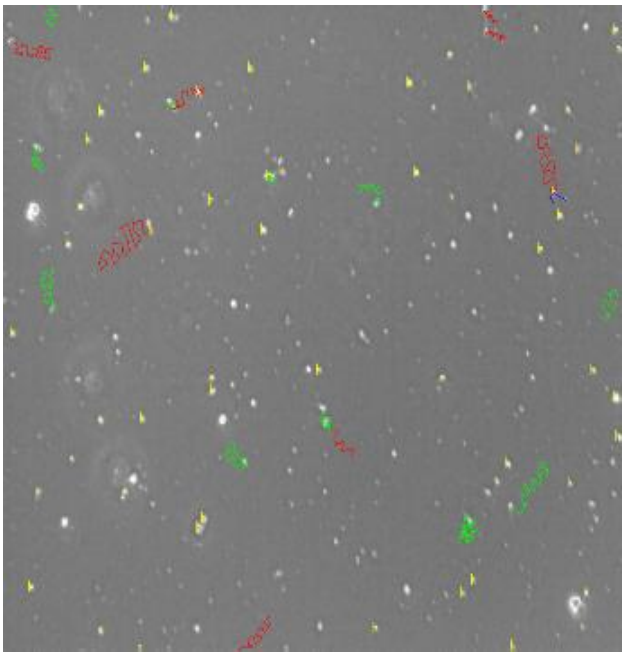


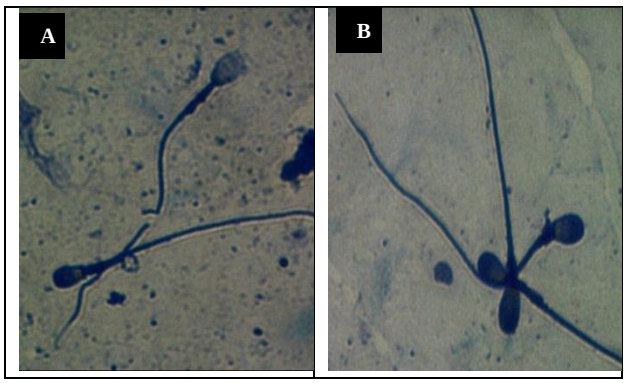
Table 1 Mean and standard deviation (S.D.) of the macroscopic and microscopic characteristics in fresh semen samples

Variable	PC	DCD
	Mean ± D.E.	Mean ± D.E.
Volume (mL)	3.73±1.60	
Filancy (mm)	23.8±0.70	
Predominant color (%)	44 % (rojizo)	44 % (blanco lechoso)
Total motility (%)	13.6±7.30	30.34±15.65
Concentration (10^6 /mL)	215.32±110.09	296.32±145.41
Vitality (%)	47.82±4.96	50.72±1.78
Sperm membrane functionality (%)	46.89±8.39	54.89±4.92
Acrosomal Integrity (%)	76.92±2.41	68.15±4.81

PC = post-crossover; DCD = vas deferens deviation. 3 animals, 3 replicates.

Discussion

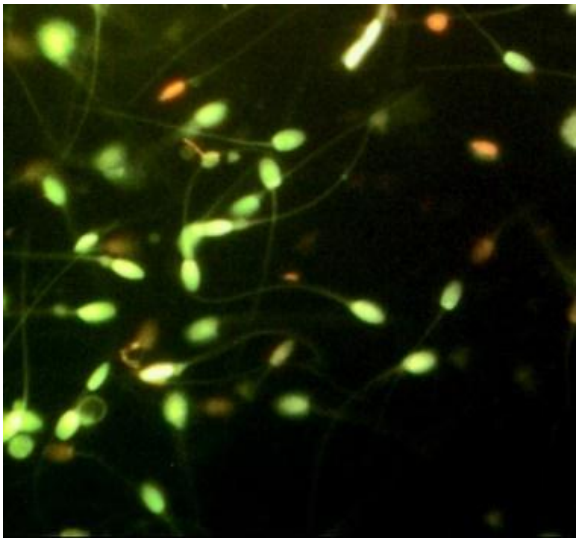
Figure 2 Acrosomal integrity with Coomasie blue staining. (A) EPZ with acrosome loss and (B) intact EPZ acrosome



The macroscopic characteristics of EPZ of alpacas obtained by two methods of post copula collection (PC) and deviation of the vas deferens (DCD) are shown in Table 1.

The seminal volume found is similar to those reported by Quispe et al.[19], and Ordoñez et al.[24], such variability is possibly due to the availability and nutritional value of the forage in the rainy season in relation to the dry season. The predominant color was light red with 44 % for the PC method, values that are within the range described by Huanca[25]. Filance is the ability to form thread and is a different characteristic of viscosity. In the work of Giuliano et al.[17] 23.8±0.70 mm was observed. Filance is mainly influenced by the species, breed, animal, forage quality and collection method Giuliano et al.[17] and Ciprian[26], in SAC the concentration of EPZ is directly influenced by the collection method. The EPZ recovered by DCD are devoid of seminal plasma, therefore, they show a higher concentration of EPZ in relation to the PC, electro ejaculation and artificial vagina methods due to the rheological characteristics (viscosity) of the whole semen, it is argued that the EPZ tend to agglomeration Ordoñez et al.[24], Huanca et al.[25]. This is reflected in the total mobility of fresh semen, which in the PC collection method was 13.6±7.30 %, lower than the DCD method of 30.34±15.65 %.

Figura 3 Vitality of spermatozoa analyzed with Vital test® EPZ live in green and dead in red (400X magnification)



Both for vitality, membrane functionality and acrosomal integrity, the values found are within the ranges described by Huanca[25], Quispe et al.[19] and Meza et al.[20], also, all these studies were carried out in the same experimental center, and with the same animals used for DCD.

Comparison of the addition of cholesterol-loaded methyl-β-cyclodextrin (MCD) on the microscopic characteristics of chilled and thawed spermatozoa.

The microscopic characteristics of chilled and thawed EPZ from alpacas, preloaded with MCD according to mobility, live EZP, sperm functionality and acrosomal integrity are shown in Table 2.

The ratio of phospholipids/cholesterol in EPZs varies between species, making some of them highly resistant to thermal shock, such as in humans, rabbits and dogs Oscheroff et al.[7]. Alpaca EPZs are thought to be especially sensitive to cold shock due to their

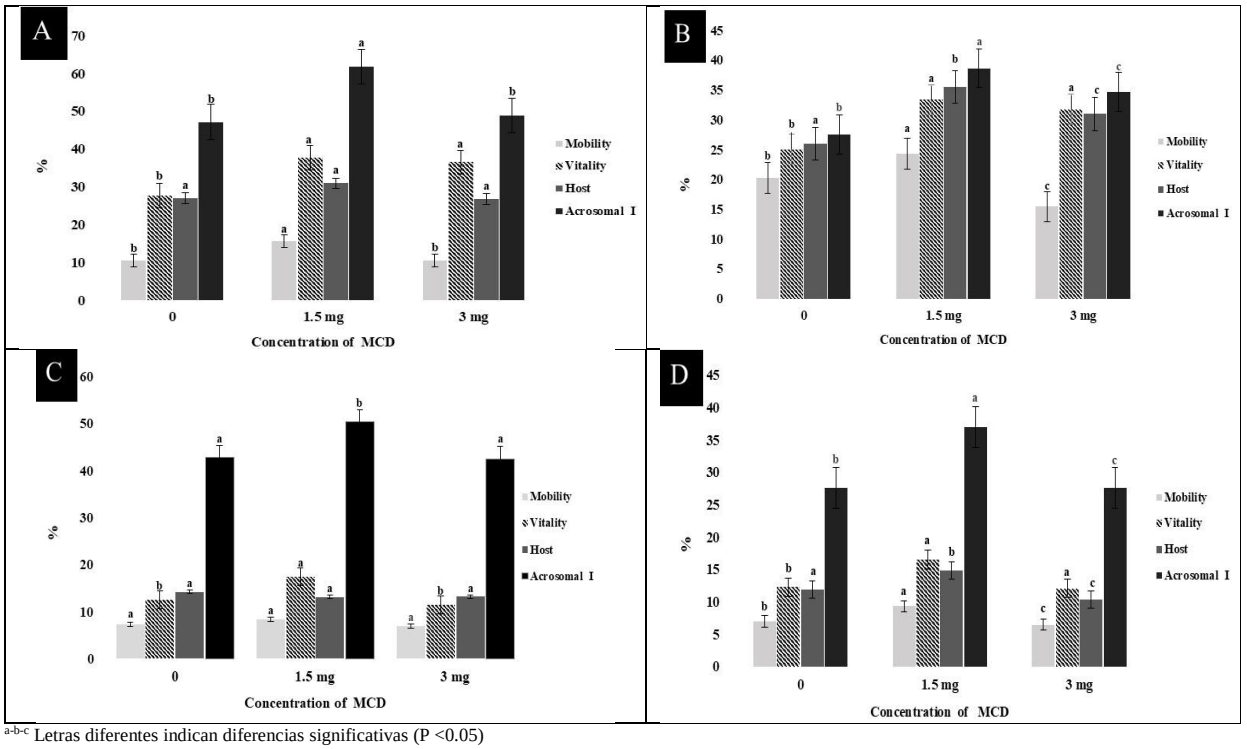
lower cholesterol/phospholipid molar ratio compared to the other species. For this reason, it is important to reduce the deleterious effect of cold shock, for example by increasing the cholesterol content in the cell membrane.

Table 2 Mean and standard deviation (SD) of the effect of the addition of cholesterol-loaded methyl-β-cyclodextrin (MCD) on the microscopic characteristics of chilled and frozen sperm in alpacas

Característics		Mean±D.E. (%)	
	Methods	**Refrigerated**	**Frozen**
Total motility (%)		12.28^{a} ±6.26	7.45^{b}±2.69
Spermatozoa live (%)	PC	34.03^{a} ±6.54	13.82^{b}±3.06
Sperm membrane functionality (%)		28.65^{a} ±5.58	13.46^{b}±1.67
Acrosomal integrity (%)		52.65^{a} ±8.42	44.97^{b}±5.47
Total motility (%)		20.02^{a} ±7.78	7.63^{b}±2.61
Spermatozoa live (%)	DCD	30.07^{a} ±9.63	13.65^{b}±2.54
Sperm membrane functionality (%)		30.82^{a} ±5.41	12.41^{b}±2.42
Acrosomal integrity (%)		33.62^{a} ±6.01	30.76^{b}±7.83

$^{a-b}$ Different letters within the same column represent significant differences (P<0.05) for each of the characteristics evaluated. PC= poscopula; DCD = vas deferens deviation.

Figure 4 Effect of the addition of cholesterol loaded with 0, 1.5 and 3 mg of methyl-β-cyclodextrin (MCD) in cryopreserved alpaca sperm. (A) Refrigerated PC method; (B) cooled DCD method; (C) Thawed PC method and (D) Thawed DCD method



$^{a-b-c}$ Letras diferentes indican diferencias significativas (P <0.05)

Generally, in this study, EPZs treated with refrigerated MCD had higher motility, live EZPs, sperm functionality, and acrosomal integrity than thawed EPZ samples. These results confirm that the addition of MCD improves the microscopic characteristics of the sperm, regardless of the species and collection method used, as opined by Ciprian[26] who used 2 and 4 mg of MCD in alpacas, Crichton et al.[15] added 1.5

mg of MCD in camels and Castillo et al.[13] used 2 and 4 mg of MCD in sheep.

In our study of the PC and DCD method in the refrigerated stage, the samples treated with 1.5 mg MCD showed greater mobility, live EZPs, plasma membrane functionality and acrosomal integrity in relation to the treatment with 3 mg MCD and control. Therefore, without considering the animal species, collection method and MCD concentration, an effect was observed in the microscopic characteristics, that agree with Crichton et al.[15], who added 1.5 mg of MCD in two incubation times 0 and 3 h and Castillo et al.[13] that added 2 mg of MCD Figure 4 (A) and (B). Post-thaw stage values for the PC and DCD method are shown in Figure 4 (C) and (D). In motility there was no significance (P>0.05) between treatments.

Then is speculated that may be due to the collection method, since the EPZ recovered by DCD are devoid of seminal plasma. The presence of seminal plasma can influence the quality of EPZ, being considered essential for the conservation and viability of EPZ because it is made up of antioxidants (vitamin C and E, urate, albumin, taurine, proteins) that is related to the viability of EPZ, Novak et al.[27]. In addition, Ciprian[26] with 2 mg of MCD using the electroejaculation collection method and Crichton et al.[15] with 1.5 mg of MCD using artificial vagina found greater mobility of EPZ, this studies that would support. However, for post-thaw sperm vitality, no significant difference (P >0.05) was found between treatments for the PC method, but for the DCD method there were statistically significant differences. Vitality determines the percentage of EPZ alive or dead, regardless of their viability Giuliano et al.[17]. The differences are possibly due to the seminal plasma, and therefore there is a higher viscosity of the semen that interferes with a better action of the extender on the EPZ Ciprian[26], despite the fact that in this work papain was added as a degeller. In the EPZ recovered by DCD, it was observed that they had a better affinity with 1.5 mg of MCD, probably because they were devoid of seminal plasma. On the other hand, Ciprian[26] found a higher percentage of live EPZ with 2 mg of MCD in alpacas, according to Viñán et al.[28] a higher percentage of live EPZ was observed for 1.5 mg of MCD compared to the control group in rams, averages that are higher than those found in this work, but follow the same pattern of improving vitality to EPZ.

The functionality of the sperm membrane was higher (P<0.05) for the samples treated with 1.5 mg of MCD in relation to the treatment with 3 mg and control for the PC method, however, there were no differences between treatments, the reason for the differences is not clear.

Post-thaw acrosomal integrity was higher (P>0.05) for EPZ treated with 1.5 mg MCD relative to the 3 mg treatment and control for both PC and DCD collection methods. Therefore, it is assumed that these differences would be associated with the concentration of the MCD addition. Crichton et al.[15] in dromedaries also reported that EPZs with the presence of 1.5 mg of MCD showed a higher percentage of EPZs with intact acrosomes, similar to that reported by Ciprian[26] who obtained higher averages with 2 mg of MCD.

The effect of the addition of MCD with different concentrations had the same tendency to improve mobility, viability and functionality of the membrane in the EPZ, fluidity and stability against osmotic and temperature changes to the cryopreserved as described by Mocé et al.[29].

In conclusion, the refrigerated spermatozoa collected by the PC and DCD method had greater mobility, vitality, membrane functionality and acrosomal integrity. In the refrigerated and thawed stage in both collection methods, EPZs treated with 1.5 mg of MCD had a positive effect on motility, percentage of live

EPZs, sperm membrane functionality, and acrosomal integrity.

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Conflicts of interest

The authors declare that this study has been carried out in accordance with the Code of Ethics for animal experiments, as reflected in the regulations: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

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Ethical considerations

The authors declare that this study has been carried out in accordance with the Code of Ethics for animal experiments, as reflected in the regulations: http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm

Authors' contribution to the article

Ccalta Hancco Ruth, preparation and execution. *Ordoñez-Rodríguez Cesar Domingo*, development of the methodology. C*calta Hancco Ada Luz*, conception and design, edition of the article. *Cucho Dolmos Hernán Carlos*, study supervision.

Research limitations

There were no limitations in the research

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