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NEW CRYOPROTECTANS SUITABLE FOR SLOW FREEZING PROCESS OF EPIDIDYMAL SPERMATOZOA OF ALPACA (*Lama pacos*)

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Abstract

Cryopreservation of sperm is a technique of assisted reproduction that cause changes and damage to mitochondria, acrosome and tail of sperm, for that reason, a suitable methodology of freezing is necessary. The objective of this study was to use new cryoprotectans suitable for freezing epididymal spermatozoa of Alpaca (Lama pacos) in a slow freezing method. The epididymides of alpaca were transported from Huancavelica city to our laboratory at 4ºC in physiological solution. The sperm cells were extracted from the epididymis fourteen hours later in HAMF10 medium. The YolK-Citrate medium with the cryoprotectants: Dimethyl sulfoxide (0,5M, 0,25M, 0,125M) and Dimethylacetamide (0,75M, 0,385M, 0,18M) were used for the cryopreservation, and were compared with a control group cryopreserved with Glycerol (0.6M). The stabilization period was one hour to 4ºC. The slow freezing was done with the use of a programmable biofreezer with the Cryogenesis 4.1 computer software until the temperature reached -80°C and finally the samples were transferred to liquid nitrogen to -196ºC. After the thawing process the differences between the results of viability and membrane integrity were significant; in the case of motility we found two groups: one group consisting by Me_2SO_4 0.25M, Me2SO4 0.5M, DMA 0.375M and DMA 0.75M in this group the highest motility values were obtained; and the second group consisted by Me2SO4 0.5M, 0.18M DMA and Glycerol 0.6M where values were the lowest. Cryoprotective agents DMA (0.375M and 0.75M) and Me_2SO_4 (0.25M and 0.5M) had an effective cryoprotectant effect compared to the control sample (Glycerol 0.6M).

Keywords: Alpaca, sperm cryopreservation, dimethylacetamide, dimethyl sulfoxide

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Introduction

Alpaca is an important South American camelid, most of these species live in the Peruvian Andes; the difficulties that confront alpaca breeders in the Andes are the lack of use of reproductive technologies. Semen cryopreservation of south American camelids is limited because the semen generally is poor quality, highly viscous making difficult its use, and the cryopreservation has not been well developed (Bravo et al. 2000c). The results generally of 6 – 20 % post-thaw motility (Vaughan et al. 2003a; Santiani et al. 2005); rarely exceed 40 %. Although, post thaw motility in excess of 45 % has been reported by Bravo et al. (1996) however, the details provided by the authors are not sufficient to allow replication of methods.

In recent years, there has been an interest in cryopreservation of epididymal sperm of alpaca from the epididymides of dead animals, whose handling in the laboratory is much more satisfactory and allowed to obtain samples with good

concentration of mature sperm. In a recent work (Morton et al, 2010) reported some interesting differences between medias used for cryopreservation depending on sample source (ejaculate or epididymis),in that work physiological differences were observed between these two groups, especially in their plasma membrane properties that affect sperm survival after cooling and freezing (Watson et al, 1995, Yu et al. 2002). consequently, the improvement of existing protocols of freezing for epididymal sperm of alpaca is necessary.

Generally, the low quality of frozen–thawed sperm is attributed to the cryoprotectant used; most of works about cryopreservation of alpaca sperm has employed glycerol with a concentration of 6 - 7 %,however, post thaw results show a poor recovery after thawing, accompanied by the loss of the initial motility (pre-freezing) during cryopreservation (Vaughan et al. 2003, Valdivia et al. 2005, Santiani et al. 2005), an efficient method of epididymal sperm cryopreservation has been developed recently which utilizes a final glycerol concentration of 3 % (Morton et al. 2007), but despite its benefits, glycerol is potentially cytotoxic to some concentrations (Almilid et al. 1988; Holt et al. 2000; Watson et al. 2000), and has a contraceptive effect in some species such as dogs (Hay et al. 1997).

Few studies have focused on alternative cryoprotectants for sperm cryopreservation of alpaca ; stimulating research about use of new cryoprotectant agents such as N,N-dimethylacetamide (DMA) and dimethylsulfoxide (Me_2SO_4) (Ball et al., 2001; Lovelock et al., 1959), they are good candidates due to its highly hydrophilic nature and low molecular weight, DMA has been used in the cryopreservation of sperm of birds (Blanco et al. 2000), mammals such as koala and kangaroo (McClean et al. 2008), and is the most common cryoprotectan agent in sperm cryopreservation of fishes.

The aim of this study was to evaluate the effectiveness of DMA and Me_2SO_4 such as cryoprotectants during the slow freezing process of epididymal sperm of alpaca.

Materials and methods

1. Animals

We used a total of thirty male alpacas, aged between 4 and 7 years old. These animals were slaughtered at the local slaughterhouse in Huancavelica city. Testicles were collected with their scrotum under hygienic conditions and transported to our laboratory within 14 hours in an ice tank with a temperature ranged between 4-8 °C using water bags.

2. Reagents and media

Chemicals used were of analytical grade and, as far as possible, cell culture tested by the manufacturer. All media components were purchased from Sigma-Aldrich (St Louis, MO, USA)

3.-Sample Collection

In laboratory, we proceeded to isolate the region of cauda from epididymis, which was cleaned and released of residual blood vessels, then was cut into small pieces to allow exit of sperm in saline solution (0.9% NaCl) at 37 ° C. Sperm sample were finally centrifuged and resuspended in Ham F 10 medium (SIGMA Catalogue number N6635) at 37° C, finally we recovered the proportion of the motile and morphologically normal sperm, then we evaluated the concentration, viability, motility and membrane integrity.

4.-Concentration, motility, viability and plasma membrane integrity

Concentration was evaluated by diluting 10 µL from fresh sperm sample with 90 µL of water, 10 µL of this solution was placed then on each side of a haemocytometer (Improved Naebauer, Weber, London U.K.).

Motility was evaluated subjectively by placing a 10-µLaliquot of resuspended sperm sample on a pre-warmed sheet and covered by pre-warmed lamella, as was described by Evans and Maxwell (1987) and then observed in a light microscopy

Viability was evaluated using a vital dye eosin Y, where a drop of sample was incubated with a drop of dye at 37 ° C for five minutes and then was proceeded to the counting of patterns presented, colorless sperm: sperm alive and orange sperm: dead sperm. Plasma membrane integrity was evaluated by the hypo-osmotic swelling test (Jeyendran et al. 1984), which consisted on incubating 100µl of the sample with 900µl of hypo-osmotic media (7.35g of sodium citrate and 13.51g of fructose 1000 ml, 150 mOsm/kg), and were incubated at 37°C for 30 min. It was evaluated by microscopy, sperm were classified according to the presence or absence of swelling in the tail region (Sperm with coiled flagellum: sperm with intact plasma membrane; sperm with straight flagellum, no curvature of any kind: sperm with altered plasma membrane).

5.-Experimental design:

We chose several concentrations of every cryoprotectants with the guidance on previous works of our laboratory (Valdivia et al., 2003). In the first tests we evaluated cryoprotectants such as: dimetylsulfoxide, propilenglicol, glycerol an dimethylacetamide on several concentrations, we obtained disappointing results because of in case of glycerol and propilenglycol (1.5M, 1M, 0.5M and 0.25M)was not found any motile sperm and the

viability and plasma membrane integrity were inferior to 30% (results not showed); in case of others such as cryoprotectants, dimetylsulfoxide and dimethylacetamide on concentrations of 2M, 1.5M y 1M found a post thawing motility of 10% and the others parameters (Viability and integrity plasma membrane) with values of 50%, for that reason we proceed to prove others concentrations such as: 0.5M, 0.25M and 0.125M to dimetylsulfoxide and 0.75M, 0.375M and 0.18M to dimethylacetamide .

For this work, all the samples were divided in three groups: One group was cryopreserved with Glycerol 3% (control group), other group was cryopreserved with dimethyl sulfoxide (0.5 M, 0.25 M, and 0.125 M) and the last was cryopreserved with Dimethylacetamide (0.75M, 0.375M and 0.18M), at the end, we obtained four straws of 0.25ml for every concentration of each cryoprotectans.

6. – Cryopreservation process

Media used for cryopreservation was Tes Tris Yolk egg which included sodium citrate 5% solution (SIGMA, code D1546), Test solution 2% (SIGMA code T1375), Trizma Base 8% solution (SIGMA code T6066), egg yolk 20%; the final solution was supplemented with streptomycin and penicillin (1:1000). This final solution was supplemented with cryoprotectants: Dimethylacetamide (DMA) at concentrations of 0.75M, 0.375M and 0.18M; Dimethylsulfoxide (Me_2SO_4) to concentrations of: 0.5 M, 0.25 M and 0.125 M and Glycerol (G) to concentration of 0.6M (≈ 3%).

For freezing, all samples were prepared to 20x106 sperm/ ml, methodology of freezing was slow. The washed sample was mixed with a proportion of 1:1 with Tes Tris Yolk supplemented with cryoprotectant agent. The final mixture was kept at 4 °C for one hour, then the samples were placed in cryopreservation straws of 250 µl, sealed and placed in programmable biofreezer, where the temperature and velocity of freezing were controlled by CryoGenesis 4.1 software.

The freezing protocol was as follows: The initial temperature was 4 °C for five minutes, the second period was the decrease in temperature of 4 °C to -2 °C at a rate of 3 °C/min and maintained in -2 °C for one minute. After we proceeded to do seeding by touching the straw away from the sample with a pre-cooled metal rod until it saw a slight change of color on the walls of the straws, a pause of 5 min was imposed to ensure that seeding was complete and equilibrium restored. The third period was from 2 °C to -30 °C with a freezing rate of 5 °C per minute and finally -30 °C to -80 °C with a freezing rate of 8 °C/minute. After this point, we put straws in liquid nitrogen at -196 °C.

The samples were thawed at 50°C for seven seconds after have been storage for 72 h in liquid nitrogen, and then the thawed sample was centrifuged (300 g; 10 min), the pellet obtained was resuspended in 0.5 mL media HAM F1 and maintained at 37°C for 10 minutes, after that we proceed to evaluate the motility, viability and plasma membrane integrity.

8.-Statistical analysis

The values found in routine evaluation of fresh and thawed sperm were analyzed by ANOVA after making a transformation of the data with arc-sen, and comparisons were rejected by the non-parametric statistical analysis (Tukey test), differences were considered significant when $p < 0.05$. Results were expressed as media ± standard deviation (SD). Analyses were performed using SPSS (version 17.00 for Windows).

Results and discussion

Cryopreservation of alpaca semen has had a poor success compared with other domestic livestock species (Vaughan et al. 2003a; Santiani et al. 2005); the recent use of epididymal sperm models has been used to further highlights in the physiology of sperm cell from semen. In the present work we evaluated possible new cryoprotectans Me2SO4 and DMA to use in the slow freezing process of epididymal sperm of alpaca; all the results were compared with a control sample, with glycerol to an adequate concentration (Morton et al., 2007).

We worked with epididymal sperm obtained after 14 hours post mortem and used Tes Tris Yolk for cryopreservation process, this media has been used in other species such as bull (Vishwanath and Shannon 2000), elk (Leboeuf et al. 2000), ram (Evans and Maxwell 1987), alpaca (Vaughan et al. 2003), camel (Niasari-Naslaji et al. 2006) and llama (Ratto et al. 1999).

In this study, the cooling rate was found between 4-7 ° C/min, the points during slow cooling were: +4 °C, -2 °C, -30 °C and -80 C (Willadsen et al. 1976, Curry et al. 1994), and finally stored at -196 °C (liquid nitrogen), thawing consisted on 50 ° C for 7 seconds.

Glycerol (at a concentration of 3%) is the most commonly used cryoprotectant (CPA) for alpaca sperm (Morton et al. 2007), but it produce toxic effects as well as contraceptive effects on cooled sperm from other species (Holt et al., 2000), its behavior during freezing process depends on cooling rate (Slow , fast or ultra-fast).

Our result post thaw survival (results not showed) with glycerol in several concentrations (1.5M, 1M, 0.5M and 0.25M) in slow freezing process were very deficient; a motility of 1% and the viability and plasma membrane integrity were inferior to 40%;for that reason we proceed to prove other cryoprotectans like N,N-dimethylacetamide (DMA) and dimethyl sulfoxide (Me_2SO_4), they are good candidates due to its highly hydrophilic nature, low molecular weight and their level of toxicity is minor compared with the level of glycerol; DMA reduces the formation of intracellular ice crystals and increases membrane permeability, thus decreasing osmotic damage (Ball et al., 2001); the penetration of Me_2SO_4 is also rapid due to its lower molecular weight relative to glycerol (Lovelock et al., 1959) and it has been used to successfully cryopreserved sperm in other species (Kundu et al., 2000).

Our initial sample showed a concentration average of 128.5 x 10^6 sperm, a motility of 77.25 ± 5%, a viability of 72.45 ± 4.2% and plasma membrane integrity of 63.15 ± 5.2%; after keeping the samples at 4 ° C with media Yolk Tes Tris supplemented with cryoprotectant agents at different concentrations, we proceeded to evaluate the different parameters of motility, viability and integrity of the plasma membrane, the table No. 1 shows the results obtained, which shows that the best results in the evaluated parameters were found when we used: DMA 0.75M, 0.375M, and 0.18M (P <0.05, Table 1).

Table 1: Results obtained after the stabilization period at 4 ° C for one hour using Tris tes egg Yolk media supplemented with cryoprotectants at different concentrations.

CRYOPROTECTANT	PARAMETERS		
	Progressive motility (%)	*Viability(%)*	*Intact plasma membrane(%)*
DMA 0.18M	56.36±4.33[a]	69.28±3.72[a]	64.02±3.52[a]
DMA 0.375M	55.09±3.21[a]	75.15±3.02[b]	72.81±3.21[b]
DMA 0.75M	52.69±5.68[a]	76.03±4.84[b]	67.28±2.95[a]
Me_2SO_4 0.125M	52.47±5.85[a]	68.47±4.51[a]	64.17±2.82[a]
Me_2SO_4 0.25M	42.77±4.53[b]	63.65±3.61[c]	61.75±2.54[a]
Me_2SO_4 0.5M	34.61±4.51[b]	60.69±3.42[c]	63.71±3.12[a]
Glycerol 0.6M	35.25%±3.07[b]	62.65±3.70[c]	61.75±3.64[a]

Data are presented as mean ± standard deviation .Values with different superscripts within a column indicate significant differences (p<0,05), Where DMA: Dimethylacetamide, Me_2SO_4: Dimethyl sulfoxide

After the cooling process, the samples were thawed at 50 °C for seven seconds, centrifuged and resuspended the pellet in HAM F1 medium and maintained at 37 ° C for 10 minutes, then, we proceeded to evaluate the different parameters. Our post thaw results showed a decrease in parameters of motility, viability and membrane integrity, being the most affected motility, as reported other authors (Watson 1995), our results are showed in the table No. 2. Our results of membrane integrity and viability were highest when the cryoprotectant agent used was DMA 0.375M; in the case of mobility variable, two groups were observed: a group formed by Me_2SO_4 0.5M, Me_2SO4 0.25M, DMA 0.75M and DMA 0.375M with which we obtained the highest mobility values ; and the second group formed by Me_2SO_4 0.125M, DMA 0.18M and glycerol 0.6M where the values were lower (p <0.05).

In this work, we used DMA in adequate concentrations, these concentrations were also tested in other species such rabbit (Dalimata and Graham 1997, Okuda et al., 2007), wild boar (Kim et al., 2011) turtle, fish (Tiersch et al. 2004 Vuthiphandchai et al. 2009, Yang et al. 2010), kangaroo (McClean et al. 2008), horse (Medeiros et al. 2002, Squires et al. 2004) and koala (Zee et al. 2008, Johnston et al . 2012) where they used concentrations between 0.25 M-1.25M, finding post thawing motility values was 45%, viability was 58%, and plasma membrane integrity was 60%.

Table 2: Results obtained after the process of freezing/thawing in the presence of different concentrations of cryoprotectants.

CRYOPROTECTANT	PARAMETERS		
	Progressive motility (%)	*Viability(%)*	*Intact plasma membrane(%)*
DMA 0.18M	26.99±1.42[a]	40.50±2.56[a]	45.44±11.99[a]
DMA 0.375M	34.44±1.41[b]	60.92±3.95[b]	64.38±1.66[b]
DMA 0.75M	28.63±4.70[b]	45.74±1.81[c]	52.54±8.19[c]
Me_2SO_4 0.125M	22.23±6.13[a]	46.88±6.76[d]	46.24±11.78[a]
Me_2SO_4 0.25M	33.33±4.85[b]	48.74±2.23[d]	52.24±1[d]
Me_2SO_4 0.5M	27.86±5.29[b]	50.30±4.98[d]	51.64±4.3[c]
Glycerol 0.6M	17.56±2.66[a]	35.52±3.78[e]	40.53±2.68[a]

Data are presented as mean ± standard deviation .Values with different superscripts within a column indicate significant differences (p<0,05), Where DMA: Dimethylacetamide, Me_2SO_4: Dimethyl sulfoxide.

In the case of Me_2SO4, this agent has been used in various species such as goats (Kundu et al. 2000), colorado Wolf (Johnson et al. 2014), fish (Anchordoguya et al. 1988 Thirumala et al. 2005 Thirumala et al. 2006 Vuthiphandchai et al. 2009, Sanches et al. 2013), pig (Hood et al. 1970), horse (Devireddy et al. 2002), and human (Serafini et al. 1986.) where they used concentrations between 0.5M-1.5M, finding post

thawing motility of 35%, viability 59%, and plasma membrane integrity 50%.

In the case of glycerol we didn't obtained satisfactory results, one possible reason for that is may be the molecular characteristics of glycerol during the freezing process, because glycerol is a good cryoprotectan in rapid freezing process due to the high viscosity that showed during the freezing process, similar behavior showed the sugars (high viscosity) and the optimal cooling rate with glycerol could be in the range of 10-50°C/min. These characteristics: toxicity ,contraceptive action and behavior during freezing process; have stimulated the study of alternative CPAs in slow freezing process of alpaca epididimary sperm cells.

The results that is showed in the present work represent the final result of several previous works and they are comparable with the results of similar works made by other researchers (Santiani et al. 2006, Valdivia et al. 2003 and Morton et al. 2010); this is the first work with slow freezing process with new cryoprotectants (DMA and Me_2SO_4) and represents a good alternative in the field of reproductive biotechnology in alpaca spermatozoa, there is no doubt that improvement of cryopreservation techniques of alpaca spermatozoa will contribute to the program development of genetic improvement in these species and probably may be applied in others South American camelids such as llama and vicuña.

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