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·*Original Article*·

Effects on the quality of frozen-thawed alpaca (*Lama pacos*) semen using two different cryoprotectants and extenders

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Abstract

Aim: To evaluate two extenders and two cryoprotectant agents (CPA) for alpaca semen cryopreservation. **Methods:** Semen samples were obtained from four adult alpacas (*Lama pacos*) and frozen using extender I (TRIS, citrate, egg yolk and glucose) or extender II (skim milk, egg yolk and fructose), each containing either glycerol (G) or ethylene glycol (EG) as CPA. Consequently, four groups were formed: 1) extender I-G; 2) extender I-EG; 3) extender II-G; and 4) extender II-EG. Semen was diluted in a two-step process: for cooling to 5 °C (extenders without CPA), and for freezing (extenders with CPA). Viability and acrosome integrity were assessed using trypan blue and Giemsa stains. **Results:** When compared, the motility after thawing was higher ($P < 0.05$) in groups II-EG (20.0 % ± 6.7 %) and II-G (15.3 % ± 4.1 %) than that in groups I-G (4.0 % ± 1.1 %) and I-EG (1.0 % ± 1.4 %). Viable spermatozoa with intact acrosomes in groups II-EG (18.7 % ± 2.9 %) and II-G (12.7 % ± 5.9 %) were higher than that in groups I-G (5.7 % ± 1.5 %) and I-EG (4.0 % ± 1.0 %). **Conclusion:** The skim milk- and egg yolk-based extenders containing ethylene glycol or glycerol to freeze alpaca semen seems to promote the survival of more sperm cells with intact acrosomes than the other extenders. *(Asian J Androl 2005 Sep; 7: 303–309)*

Keywords: cryopreservation; glycerol; ethylene glycol; extenders; alpaca spermatozoa

1 Introduction

South American camelids, alpacas (*Lama pacos*) and llamas (*Lama glama*), are species with great economic importance in Peru and Bolivia, as they provide fiber and meat for High-Andean people. Nevertheless, low rates of fecundity affect the development of camelids, an important animal resource in the altiplanic region of Sudamerica. In order to obtain a fast diffusion of individuals of higher genetic merit, artificial insemination (AI) has been used for many years in other species. Pregnancy rates higher than 60 % have been obtained by AI in alpacas using fresh semen, but this technique has not been widely applied to camelids because of difficulties in developing suitable protocols for dilution and conservation of alpaca semen [1, 2]. There are few reports concerning storage of South American camelid semen at low temperatures. More than 20 years ago, Graham *et al*. [3] froze llama semen, reporting 45 % of motility after thawing. Nevertheless, in other recent studies [4, 5],

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motility after thawing was lower than 25 % in llama semen.

In alpaca semen, motility after thawing was lower than 20 % [6] and Bravo *et al.* [7] reported between 30 % and 40 % after freezing semen from both llamas and alpacas. This indicates that the spermatozoa of camelids suffer considerable damage during cryopreservation with the protocols used.

The use of cryoprotectant agents is essential for the viability of spermatozoa after thawing as these agents minimize intracellular ice formation and restrict the solution effect [8]. The most frequently used cryoprotectant agent to freeze animal semen is glycerol [9], but ethylene glycol has also been applied to replace glycerol in many species. Some studies have shown better effects produced by the use of ethylene glycol (e.g. in human [10] and bovine [11]). So far the only cryoprotectant agent used for cryopreservation of semen in camelids has been glycerol [5, 6].

Most extenders used for the freezing of camelid semen have been prepared combining TRIS, citrate, glycerol and egg yolk [3, 5, 6] .

In the majority of previous reports, assessment of sperm function after storage has been limited to sperm motility [3, 6], with a single report describing the evaluation of viability using nigrosin-eosin stain [5]. However, the acrosome integrity has been a useful parameter to evaluate fertility [12]. Using an immunogold-labeling technique to determine the stability of sperm acrosomes, Valdivia *et al.* [6] indicated that only 5 % of spermatozoa were immunolabeled after freezing-thawing. Von Baer and Hellemann [4] utilized a spermatic stain to evaluate the acrosomal integrity, but viability was not measured.

There is no doubt that improvement of cryopreservation techniques of camelid spermatozoa will contribute to the development of programs of genetic improvement in these species. To that end, a 2 × 2 factorial experiment was designed to compare the efficiency of TRIS-citrate or skim milk-based extenders, containing either glycerol or ethylene glycol as cryoprotectant agents for the cryopreservation of alpaca semen. Trypan blue and Giemsa staining were used to assess the viability and acrosomal status.

2 Materials and methods

2.1 Animals

Four Huacaya-breed male alpacas from the National Institute for Agrarian Research (INIA-Puno) were used in this study. Males were maintained on natural pastures at the Quimsachata Experimental Center, which is located in Puno, Peru, at an altitude of 4025 m above sea level, 15°41´39" south latitude and 70°36´24" west longitude. This locality belongs to the zone of altiplano. Semen samples were collected during the period of February and March, which is the peak of the camelid breeding season in South America.

2.2 Semen collection

Semen was collected every second day, using a modified ovine artificial vagina, wrapped in an electric warming pad. The artificial vagina was mounted and fixed a dummy inside, with the external access through a hole. Samples from males who had copulated for more than 15 min were processed, as this amount of time is required to achieve camelids with semen good quality. Semen samples were transported to the laboratory for analysis. Due to the viscosity of the semen, samples were put under mechanical action, passing them through a small-gauge needle. They were assessed for volume, sperm concentration, motility, viability and acrosomal integrity. Only samples with normal seminal parameters (at least 60 % of motility and 80 million cells/mL) [13] were processed (n = 12).

2.3 Semen extenders

Two extenders were used in the experiments. Extender I (10 mL) was prepared in autoclaved double distilled water (prepared in house) containing 0.244 g TRIS (Merck, Darmstadt, Germany), 0.136 g citric acid (Mallinckrodt, Xalostoc, Mexico), 0.082 g glucose (Sigma-Aldrich, St. Louis, USA) and 20 % v/v egg yolk. Extender II (10 mL) consisted of skim cow milk (0.5 g/L fat, Gloria S.A., La Victoria Perú), 5 % v/v egg yolk and 0.485 g fructose (Sigma-Aldrich St. Louis, Mo, USA). Each extender was divided into two equal fractions: fraction A without the cryoprotectant used for cooling semen to 5 °C and fraction B containing 14 % v/v glycerol (G) (Ultra Pure Glycerol, Agtech, Manhattan, USA) or 14 % v/v ethylene glycol (EG) (Biolife Freeze Medium W/ 1.5 mol/L ethylene glycol, Agtech, Manhattan, USA) for freezing. Fraction B was added to an equal volume to semen samples diluted in fraction A at 5 °C. Therefore, the final percentage of cryoprotectant agents was 7 %, which means 1 mol/L glycerol and 0.1 mol/L ethylene glycol.

2.4 Semen freezing and thawing

After semen evaluation, ejaculates were divided into two groups in order to dilute them with equal volumes of extenders I or II (fraction A) at 35 °C. Tubes containing diluted semen were placed in a water bath at 35 °C. The cooling rate was approximately 1 °C /3 min, from 35 °C to 5 °C. Once at 5 °C, each fraction A dilution (I and II) was divided into two samples. Equal volumes of fraction B (with glycerol or ethylene glycol) at 5 °C were added in a second step, resulting in the following combinations of diluent and cryoprotectant: extender I and glycerol (I-G), extender I and ethylene glycol (I-EG), extender II and glycerol (II-G) and extender II and ethylene glycol (II-EG). After mixing fractions A and B in each group, tubes were equilibrated for 30 min at 5 °C. Before freezing, samples of cooled diluted semen were rewarmed and incubated at 37 °C for 5 min for motility analysis. Meanwhile, the extended semen was loaded into 0.25 mL plastic French straws and exposed to liquid nitrogen vapor for 15 min. Finally, straws (three straws per group) were plunged into liquid nitrogen. Straws were thawed in a water bath at 37 °C for 5 min to assess for motility, viability and acrosomal integrity.

2.5 Assessment of motility, viability and acrosomal integrity

Percentages of motile spermatozoa were assessed under a coverslip (18 mm × 18 mm) on a warm glass slide (37 °C) by a trained technician, using phase contrast microscopy (× 400 magnification) (CX31, Olympus, Tokyo, Japan). The double stain technique used to evaluate sperm viability and acrosomal integrity in spermatozoa from other species was carried out in alpaca semen, according to Didion *et al*. [14]. Due to the poor staining of the acrosomal membrane, Giemsa 20 % was used instead of 10 %. Briefly, 200 μL sperm suspension aliquots were mixed 1:1 with 2 % trypan blue (T-0887 Sigma, Sigma-Aldrich, St. Louis, Mo, USA) for 10 min at 37 °C. Then, aliquots were washed two or three times with SP-Talp medium [11] (100 mmol/L sodium chloride, 3.11 mmol/L potassium chloride, 25 mmol/L sodium bicarbonate, 0.3 mmol/L potassium phosphate monohydrate, 3 mL sodium lactate [60 % syrup], 2 mmol/L calcium chloride dehydrate, 4 mmol/L magnesium chloride hexahydrate, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 14 mmol/L penicillin G). Aliquots of 25 μL of the sperm suspension were placed on a slide, and air-dried at room temperature for 20 min. Thereafter, spermatozoa were stained for 40 min with Giemsa 20 % (Merck, Darmstadt, Germany) in distilled water prepared immediately before use. After staining, the slides were washed in distilled water, air-dried at room temperature for 30 min and mounted with permount and a coverslip. At least 200 cells were assessed according to the following staining patterns: 1) dead spermatozoa with intact acrosomes, showing a blue or dark-blue post-acrosomal region and a pink or purple acrosomal region; 2) dead spermatozoa with detached acrosomes, showing a blue or dark-blue post-acrosomal region and a white acrosomal region; 3) viable spermatozoa with intact acrosomes, showing a white post-acrosomal region and a purple acrosomal region; and 4) viable spermatozoa with detached acrosomes showing white color in both post-acrosomal and acrosomal region (Figure 1).

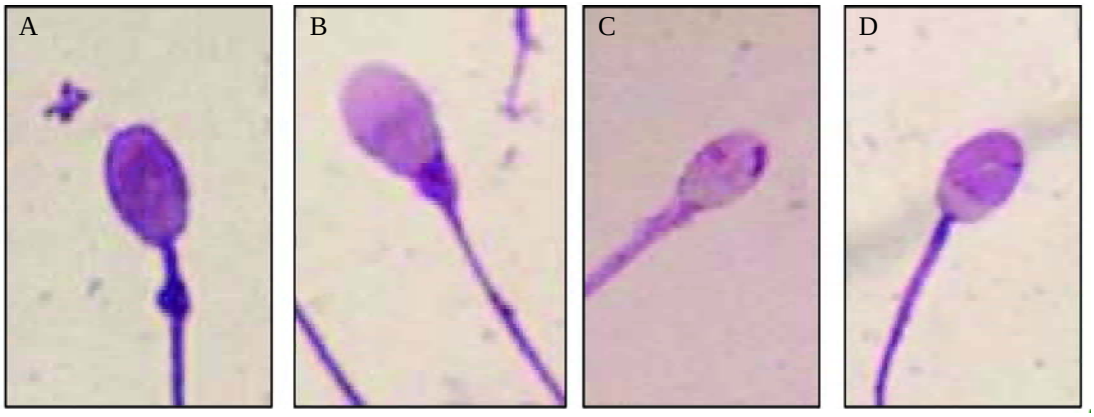

Figure 1. Double stain tripan blue-Giemsa 20 % in alpaca spermatozoa. (A): Dead spermatozoa with intact acrosome; (B): Dead spermatozoa with detached acrosome; (C): Viable spermatozoa with detached acrosome; (D): Viable spermatozoa with intact acrosome.

2.6 Statistical analysis

Data in percentage (motility, viability and acrosomal integrity) were transformed (arcsine-square root transformation) to approach a normal distribution. Comparison between the four groups (I-G, I-EG, II-G and II-EG) were analyzed using ANOVA (Prism version 3.0, San Diego, USA). Following a significant ANOVA, the Tukey multiple comparison test was used to determine differences between means. Data for motility and viability were compared using the Pearson correlation test in order to validate the double stain technique for alpaca spermatozoa. $P < 0.05$ was condidered as significant.

3 Results

3.1 Effect of cryoprotectant agents and extenders on sperm motility

The results of the present study suggested that sperm motility tended to decrease following incubation at 5 ºC for 30 min. The decrease was significant compared with the activity of fresh semen for extender II and glycerol (Table 1).

Sperm motility decreased further after freezing and thawing. Extender II with either glycerol or ethylene glycol was found to bring less influence to sperm motility than extender I.

3.2 Effect of cryoprotectant agents and extenders on sperm viability and acrosomal integrity

The percentages of viable spermatozoa and viable spermatozoa with intact acrosomes after freezing and thawing in extender II with glycerol or ethylene glycol were highest (Table 2). There was a significant difference ($P < 0.05$) between extenders but not between cryoprotectants. Viable spermatozoa also had intact acrosomes in both fresh and processed semen (Table 2).

3.3 Correlation between motility and viability

Motility and viability were strongly correlated in fresh and frozen-thawed semen ($P < 0.01$), with a correlation coefficient of $r = 0.96$ (Figure 2).

4 Discussion

In our study, the double stain technique (trypan blue and Giemsa 20 %) was reliable to evaluate both the vi-

Table 1. Mean percentages ± SD of motility of alpaca spermatozoa in fresh semen, after 30 min at 5 °C and after thawing in various extenders. ^{b,e} Data with different superscript letters within columns differ significantly ($P < 0.05$). Extender I: TRIS, citric acid + glucose + egg yolk; extender II: skim milk + fructose + egg yolk.

Extenders and cryoprotectant agents	Before thawing Motility (%)	After thawing Motility (%)
Fresh semen (before any dilution)	72.0 ± 13.0^{b}	
After 30 min of incubation at 5 °C:		
Extender I + glycerol	56.6 ± 7.6^{b}	4.0 ± 1.1^{b}
Extender I + ethylene glycol	66.5 ± 6.4^{b}	1.0 ± 1.4^{b}
Extender II + glycerol	49.2 ± 7.5^{e}	15.3 ± 4.1^{e}
Extender II + ethylene glycol	64.0 ± 13.9^{b}	20.0 ± 6.7^{e}

Table 2. Mean percentages ± SD of viable spermatozoa and viable spermatozoa with intact acrosome in fresh semen and after freezing-thawing in various extenders. ^{b,e,h} Data with different superscript letters within columns differ significantly ($P < 0.05$). Extender I: TRIS, citric acid + glucose + egg yolk; extender II: skim milk + fructose + egg yolk.

Extenders and cryoprotectant agents	Viable spermatozoa (%)	Viable with intact acrosome (%)
Fresh semen (before any dilution)	80.7 ± 10.1^{b}	78.3 ± 10.0^{b}
After freezing-thawing:		
Extender I + glycerol	6.2 ± 1.5^{e}	5.7 ± 1.5^{e}
Extender I + ethylene glycol	4.3 ± 4.3^{e}	4.0 ± 1.0^{e}
Extender II + glycerol	13.0 ± 6.1^{e,h}	12.7 ± 5.9^{e,h}
Extender II + ethylene glycol	21.0 ± 1.0^{h}	18.7 ± 2.9^{h}

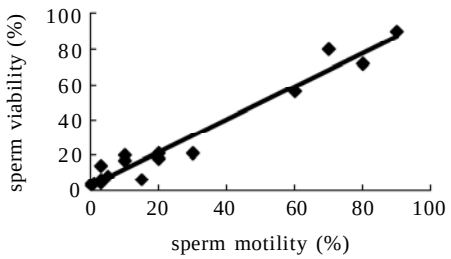

Figure 2. Correlation between percentages of motility and viable spermatozoa of alpacas (r = 0.96) (P < 0.0001).

ability and acrosomal integrity in alpaca spermatozoa. Preliminary trials using Didion's stain [14] produced only poor staining of the acrosomal membrane of alpaca spermatozoa. We therefore tried Giemsa 20 % which achieved good staining of the acrosomal membrane (Figure 1).

In an immunogold labeling technique for acrosomes with human anti-proacrosin/acrosin monoclonal antibody (C5F10) described by Valdivia *et al.* [6], only 5 % of thawed alpaca spermatozoa were labeled. It is probable that the results corresponded only to viable spermatozoa, because we found that most of the viable spermatozoa maintained intact acrosomes when examined by the trypan blue and Giemsa 20 % stain technique. Other authors [4] found that 50 % of llama spermatozoa had normal acrosomes after using the Spermac stain method, with percentages of motility between 20 % and 30 %.

In relation to the extenders used, the best viability results after thawing were observed in the groups diluted with extender II (skim milk, egg yolk and fructose), despite the fact that most extenders utilized to freeze camelid semen have been prepared based on TRIS, citrate, glucose or egg yolk [4–6]. We found that motility and viability in groups frozen in an extender containing TRIS, citric acid, glucose and egg yolk (I-G and I-EG) were lower than 10 % post-thawing.

Accordingly, Ratto *et al.* [15] reported better motility after conserving llama spermatozoa at 4 °C during 72 h using Kenney extender (skim milk and glucose) and Colorado extender (skim milk and salts) in comparison with TRIS- and egg yolk-based extender. Our results for sperm motility were similar after the cooling process, prior to freezing. Despite the fact that the percentages of motility were lower in group II-G (49.2 %) prior to freezing in comparison with groups I-G (56.6 %), I-EG (66.5 %) and II-EG (64.0 %), sperm motility rates post-thaw in groups II-G (15.3%) and II-EG (20.0 %) were better (P < 0.05) than that in groups I-G (4.0 %) and I-EG (1.0 %). It is possible that certain compounds of skim milk such as casein or lactose protects alpaca sperm better than TRIS-citric acid. It is concluded that the extender based on skim milk, egg yolk and fructose provides a higher protection of alpaca spermatozoa than the extender based on TRIS, citric acid, egg yolk and glucose as measured by results obtained post-thaw.

In relation to cryoprotectants, varying degrees of susceptibility and resistance in spermatozoa have been reported using different agents and concentrations of cryoprotectants. This can be attributed to differences between species with regard to the structure, permeability and function of the plasmatic membrane of spermatozoa [9,16].

The use of a final glycerol concentration of 7 % (1 mol/L) to freeze camelid semen has probably been adapted from protocols to freeze semen from rams or bulls. Holt [16] indicated that bovine spermatozoa are routinely cryopreserved with 4 %–8 % glycerol concentrations, and these concentrations have been successfully used to cryopreserve spermatozoa of some wild ruminants and primates. However, other species (pigs and mice) do not tolerate concentrations higher than 3 % and 1.75 %, respectively, and the effect seems to be species-specific instead of taxon-specific. For these reasons, alpaca and llama spermatozoa probably each require a specific concentration of cryoprotectant agent to obtain good post-thaw motility and viability rates, which are not necessarily the same optimal concentrations used for rams or bulls.

We utilized a final concentration of cryoprotectants of 1.0 mol/L glycerol in groups I-G and II-G and 0.1 mol/L ethylene glycol in groups I-EG and II-EG. Valdivia *et al.* [6] using 1 mol/L glycerol, obtained a similar motility recovery after thawing to that obtained in the present study. Using 6 % glycerol (approximately 0.86 mol/L), Aller *et al.* [5] found 32 % viability in thawed llama spermatozoa. On the other hand, using diluted semen with TRIS-extender and EDTA freezing extender, von Baer and Hellemann [4] obtained motility rates ranging from 20 % to 30 % after thawing. It seems that South American camelid spermatozoa cannot tolerate final cryoprotectant concentrations of 1 mol/L or more and that lower concentrations would have better cryoprotectant effects and/or less toxicity; similar results have been indicated in this

study but further research is required in this area.

Alpaca spermatozoa diluted in extender II were similarly cryoprotected by ethylene glycol and glycerol. Ethylene glycol has been successfully utilized as a cryoprotectant agent, replacing glycerol in many species. This is the first study to use ethylene glycol as a cryoprotectant in camelid semen. Ethylene glycol has shown better cryoprotectant effects than glycerol in human [10] and bovine [11] spermatozoa, as it permeates sperm membranes faster than glycerol [10]. In dogs, ethylene glycol can be used as a cryoprotectant agent at concentrations of 0.25, 0.5 and 1.0 mol/L as opposed to glycerol at 0.8 mol/L [17]. In addition, ethylene glycol could substitute glycerol as cryoprotectant agent if used at the same or lower concentrations to freeze horse semen [18]. Apparently, ethylene glycol fewer detrimental effects on the viability and motility of spermatozoa [19], providing a better protective effect to the acrosome than glycerol. However, in other species such as ovine, ethylene glycol is not as good as glycerol [20] for semen cryopreservation, but the best cryoprotectant effects of ethylene glycol have been obtained using lower concentrations (3 %) than glycerol (7 %) [20].

In conclusion, alpaca spermatozoa are highly susceptible to cryopreservation. Under our working conditions, this study suggests that ethylene glycol at lower concentrations with skim milk, fructose-based extender may have a better cryoprotectant effect than glycerol with TRIS-citric acid-based extender on alpaca spermatozoa. Because of the species-specific cryoprotectant effects, an optimization of specific concentrations of cryoprotectant agents for alpaca spermatozoa is necessary. Double stain with trypan blue and Giemsa 20 % turned out to be a valid technique for the concomitant evaluation of viability and acrosomal integrity in alpaca spermatozoa. Finally, our results in conjunction with those of others indicate the need for continued research into semen cryopreservation in alpacas.

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