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Vicugna pacos semen collection methods and review of semen parameters using Triladyl® diluent

Métodos de recolección del semen de *Vicugna pacos* y revisión de los parámetros seminales utilizando diluyente Triladyl®

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Abstract

To evaluate three methods of collecting *Vicugna pacos* semen and seminal parameters using Triladyl® extender, this research was carried out from April 25 to July 9, 2023, at the Technical University of Cotopaxi, Ecuador. 9 male alpacas were selected and the effect of semen collection was evaluated using a complete manikin, artificial vagina and urine use (A), rump manikin, artificial vagina and receptive female (B), and penile deviation, artificial vagina and receptive female (C), in libido and copulation. The effect of the Triladyl® extender on the characteristics of fresh and post-thawing semen was studied. Libido and acceptance of the method were compared by multiple comparison of proportions, mounts per hour with the Duncan test, the number of ejaculates with $\chi^2_{C\ de\ Yates}$ and semen characteristics using Student's t-test for paired samples. Libido and method acceptance did not differ between methods. The mounts per hour and the number of ejaculates were higher ($P< 0.05$) in method C. Concentration and motility in fresh semen were higher ($P < 0.05$), and mortality and morpho anomalies in post-thawing semen were higher ($P < 0.05$). It is concluded that method C is the most optimal for semen collection and that the use of Triladyl® diluent in the freezing protocol decreased seminal concentration and motility, while it increased mortality and sperm morpho anomalies after thawing.

Resumen

Para evaluar 3 métodos de recolección del semen de *Vicugna pacos* y los parámetros seminales utilizando diluyente Triladyl®, se desarrolló esta investigación. Del 25 de abril al 9 de julio de 2023, en la Universidad Técnica de Cotopaxi, Ecuador. Se seleccionaron 9 alpacas machos y se evaluó la colección de semen, mediante maniquí completo, vagina artificial y uso de orina (A), maniquí de grupa, vagina artificial y hembra receptiva (B) y desviación de pene, vagina artificial y hembra receptiva (C), en la libido y la cópula. Se estudió el efecto del *diluyente* Triladyl® en las características del semen fresco y post descongelación. Se compararon la libido y aceptación del método mediante comparación múltiple de proporciones, las montas por hora con la prueba de Duncan, la cantidad de eyaculados con $\chi^2_{C\ de\ Yates}$ y las características seminales mediante t-Student para muestras pareadas. La libido y la aceptación del método no

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difirieron entre los métodos. Las montas por hora y el número de eyaculados fueron mayores ($P< 0.05$) en el método C. Fueron superiores ($P< 0.05$) la concentración, la motilidad en el semen fresco, la mortalidad y las morfo anomalías en el en semen post descongelación. Se concluye que el método C es el más óptimo para la colección del semen y que el empleo del diluyente Triladyl® en el protocolo de congelación disminuyó la concentración y motilidad seminal, mientras que, aumentó la mortalidad y las morfo anomalías espermáticas posterior a la descongelación.

Introduction

To improve the genetics and bio productive and economic performance of the alpaca (*Vicugna pacos*), reproductive biotechnologies are used, which include, among others, the collection, evaluation, conservation of semen and artificial insemination (AI)[1].

Semen collection methods should not affect the libido of males, nor trigger inhibitory reflexes that reduce their acceptance, in addition, affect the collection and the quantity of ejaculates per male. They allow obtaining the maximum volume of ejaculate, with absolute purity of the seminal material[2].

Diluents influence the quality and fertility of alpaca sperm during freezing and post-thawing, because they contribute to maintaining isotonic osmotic pressure, provide nutrients for the life of sperm cells and supply lipoproteins or lecithin's that protect sperm from cold shock[3].

In Ecuador, studies on semen collection methods in alpacas are scarce and inconclusive, so research is required to develop a reliable technique for collecting semen that benefits the libido and performance of males during the collection.

Furthermore, there is little scientific information available on the diluents and freezing protocols used in the processing and conservation of alpaca semen in Ecuador[4], so scientific studies are needed on these aspects, which contribute to defining an effective methodology, for the optimization of the semen conservation process in this species and in this way, develop AI and other reproductive techniques.

The objective of this experiment was to evaluate 3 methods of collecting *V. pacos* semen and semen parameters using Triladyl® diluent.

Materials and methods

This research work was carried out between April 25 and July 9, 2023, in the facilities of the Salache Academic Experimental Center (CEASA) and the Reproduction Biotechnology Laboratory (LBR) of the degree in Veterinary Medicine of the Technical University of Cotopaxi (UTC), located in the Eloy Alfaro parish, 7.7 km from the city of Latacunga, province of Cotopaxi, Ecuador.

The research scenario is geographically located between 16º 31' 28" south latitude (LS) and 68º 20' 39" west longitude (LW), at 3990 m.a.s. with an average temperature of 12° C, average annual rainfall between 500 and 1500 mm and a clay soil[5].

9 male alpacas (*V. pacos*) were selected, provided by the UTC and the communities of Macas Chico and Apahua, 3 each, belonging to the province of Cotopaxi. All animals were of the Huacaya breed, aged

between 3 and 7 years, weighing 65±3.5 kg, clinically healthy and without reproductive problems or congenital defects.

Their diet was based on natural grasses and water ad libitum. The effect of semen collection methods was evaluated with a complete mannequin, artificial vagina (AV) and urine use (A), rump mannequin, AV and receptive female (B), and penis deviation with AV and receptive female (C) on libido, acceptance of the method, number of mounts and number of ejaculates. The effect of including the Triladyl® extender in the freezing protocol on the characteristics of fresh and post-thawing semen was studied.

The age of the animals was evaluated by inspecting their teeth using the gap technique[6,7]. The reproductive organs (Figure 1) were evaluated by the procedures described by Incahuanaco et al.[7].

Figure 1 Male reproductive tract evaluation

Raining the male alpaca for riding. Males were selected and kept for 10 days in a pen divided into 2 sections (Figure 2). In one section there were the males and in the other the receptive female, visual contact was facilitated between the animals of both sexes.

The training of the male alpacas for the 3 methods was carried out for 3 weeks at the CEASA facilities, 3 times a week, on alternate days, 2 h in the morning and 2 h in the afternoon.

Mounting procedures. The recognition of the male towards the dummies or the receptive female, the mounting and semen collection were carried out every 48 h, 3 times in a week for each method, the first daily mounting session was carried out between 6:00 and 8:00 in the morning and the second, 12 h after the first.

Figure 2 Separation of male and female alpacas

Before starting method, A (Figure 3A), this body fluid was placed so that the odor (pheromones) was impregnated for 48 h. For method B (Figure 3B), it was required to train the female with the same protocol described for males, so that her handling would be the least stressful. Both methods were executed according to the procedures described by Delgado *et al*.[8]. For method C (Figure 3C), the female was placed in the sternal position and the male in the copulating position, verifying that the penis was within the AV.

Semen collection. The semen was collected with the AV, according to the methodology described by Sumar[9]. A VA was used that consists of a rubber tube 21 cm long and 4 cm in diameter, to which a latex jacket was internally adapted. Externally, it was covered with an electric blanket to maintain a temperature of 40° C during collection.

The ejaculates were collected in falcon tubes (50 mL), protected by an external cover that maintained a temperature of 37° C, and were transported to the

CEASA LBR in less than 1 h from collection. Samples contaminated with urine were not processed.

Figure 3 Methods used for semen collection. A: Complete dummy plus artificial vagina and use of urine. B: Rump dummy, artificial vagina and receptive female. C: Penile detour with artificial vagina and receptive female

A B C C

Determination of semen parameters. The volume of the ejaculate was determined by direct observation using a graduated cylinder. Semen concentration was determined using the Neubauer chamber and motility using a DM4B microscope (Leica Microsystems AG, USA), at 10X. Both parameters were determined according to the procedures described by Allauca *et al*.[10].

Sperm vitality was determined by staining eosin-nigrosin, dead sperm do not They contain dye and appear pink, however, the vivid ones appear translucent[11].

Sperm morphology was evaluated by observing semen-eosin staining in a scanning electron microscope and the number of morphologically normal and

abnormal spermatozoa according to the recommendations of Pérez *et al*.[12].

Diluent used. The study was carried out with the Triladyl® CSS One-step diluent (Minitube International A.G., Germany), which is based on TRIS (Hydroxy-methyl aminomethane, a synthetic buffer, and also contains citric acid, sugars, buffer substances, ultrapurified water and antibiotics (Tylosin, Gentamicin, Spectinomycin, Lincomycin). This diluent used glycerol as well as the cryoprotector[13,14].

Preparation of the diluent. The diluent was prepared according to the manufacturer's recommendation and kept in a water bath at 37° C. 3 parts of sterile distilled water (60 %), one part of egg yolk in a water bath (20 %) and one part of the Triladyl® commercial concentrate (20 %), then centrifuged at 500 rpm.

Semen dilution. The ejaculate was measured in falcon tubes and kept warm in a water bath at 37º C. The dilution was carried out in a 1:1 ratio of semen and diluent.

Freezing semen with diluent. Once the diluent was mixed and the semen was cooled to 5° C with a cooling rate of 0.4° C/min, and kept at that temperature for 2 h, the sperm were in contact with the glycerol. After this time, the straws were packaged and placed horizontally in a rack at a distance of 4 cm above the surface of the liquid nitrogen, inside a polystyrene box or cooler. The straws were placed in liquid nitrogen vapors at -100° C for 20 min, and subsequently deposited directly in liquid nitrogen at -196° C. They were then deposited in the cryogenic tank.

Post-thawing of semen with diluent. Once the sample was frozen, the straw was removed from the cryogenic tank and placed in a water bath at a temperature of 37° C for subsequent use in sperm evaluation.

Statistical processing. Descriptive statistics were obtained for each quantitative variable. Between methods A, B and C, libido and acceptance of the method were compared through a multiple comparison of proportions, the number of matings per hour with the Duncan test[15] and the number of ejaculates with a χ^2 C Yachts test. The concentration of sperm in the ejaculate, and the motility, mortality and morph anomalies, between fresh semen without diluent and post-thawed semen, with diluent, using the t-Student test for paired samples. The statistical package Statgraphics Centurion Ver. XVII[16] was used in all processing.

Results

Table 1 shows that 22.22 % of the males had high libido when exposed to method A and accepted it, which occurred in 33.33 % of them when exposed to procedures B and C.

Table 1 Aspects evaluated with the three semen collection methods in alpacas

Method	High libido (%)	Method acceptance (%)	Mounts per hour ($\bar{X}$±DE)	Ejaculates (n)
A	22.22[a]	22.22[a]	.66±.27[b]	0[a]
B	33.33[a]	33.33[a]	1.33±.27[b]	0[a]
C	33.33[a]	33.33[a]	3.00±.27[a]	3.00[a]

Different letters in the same column indicate significant differences p< 0.05.
Multiple comparison of proportions was applied for libido and acceptance of the method.
Duncan15 to compare the number of mounts per hour and Yates' $\chi 2$ C to compare the number of ejaculates.

With method C, males performed a greater number (P< 0.05) of mounts per hour with procedures A and B (Table 1). With C, 3 ejaculates were obtained, which differed (P< 0.05) with A and B, with which no ejaculates were collected (Table 1). The volume of the ejaculates obtained was 1.30±0.20 mL, with a coefficient of variation of 15.38%.

Table 2 Sperm parameters ($\bar{X}$±DE) of fresh and post-thawed semen of alpacas

Parameters	Moments	
	Fresh without diluent	Post thawing, with diluent
Concentration, 10^6/mL.	1.80±.10[a]	1.30±.10[b]
Motility, %.	51.00±2.00[a]	13.00±1.00[b]
Mortality, %.	17.00±3.00[b]	24.33±.57[a]
Morpho anomalies, %.	29.00±2.64[b]	41.33±1.52[a]

[ab] Unequal letters in the same row indicate significant statistical differences at P< 0.01. (t-Student for paired samples)

Table 3 Morpho anomalies ($\bar{X}$±DE) of fresh and post-thawed semen of alpacas

Parameters (%)	Moments	
	Fresh without diluent	Post thawing, with diluent
Curved tails	9,33±.57[a]	8,33±.57[a]
Double tails	10.00±4.35[a]	13.33±.57[a]
Free heads	4.66±1.52[a]	2.66±.60[a]
Double heads	9.00±3.60[a]	13.00±1.00[a]
Presence of cytoplasmic droplet	3.66±3.05[a]	4.00±1.12[a]

Concentration and motility decreased (P< 0.05), while mortality and the percentage of sperm with abnormal shapes increased (P< 0.05) after semen thawing (Table 2).

The types of sperm anomalies did not differ statistically between fresh and post-thawing semen, however, numerically, in the latter the percentage of male gametes with curved tails and free heads was reduced, and the percentage of nemasperms with double tails, with double heads and with the presence of cytoplasmic drop (Table 3).

Discussion

The greater acceptance of methods B and C, in which the males had a higher sexual libido, suggests that they are more viable and accepted, which is due to the use of the receptive female, which helped to increase the libido of the males[8].

Obtaining semen with VA and deviation of the penis has the advantage that the entire ejaculate is collected, without it being contaminated with urine or etiological agents that are in the vagina of the female, without the need for expensive equipment and highly specialized personnel[8]. Its main disadvantage is the need for several days of training for males[6].

The order of acceptance of the methods in this investigation is similar to that obtained in llamas. However, in this species the acceptance percentages were 80 and 90 % in procedures B and C, respectively[8]. The cause of these differences may be motivated by differences in the temperament and reproductive physiology of each species[7].

No ejaculates were obtained with methods A and B,

with which, the males did not mate correctly and did not adapt to the mannequin, because A has a square shape and its corners hurt the male's abdomen, and in B the rump had a lot of mobility. Furthermore, the alpaca has persistent adhesions of the glans to the foreskin in some animals, which make extrusion of the penis impossible[17]. It also contributed that only 60 % of the males responded well to training with the mannequin, given the nervous nature of the species[18].

Obtaining ejaculates was associated with the semen collection method used ($\chi^2_{\text{C Yates}} = 4.0548$, P=.0440). The greater number of ejaculates with method C was due to the use of the combination of AV with the receptive female. These authors pointed out that the use of AV and a receptive female next to the mannequin increased the copulation time from 15.9 to 16.8 min and the quality of the ejaculate obtained with respect to the use of the mannequin alone.

The volume of the ejaculate in this experiment is lower than that published in alpacas of the Huacaya Breed, 3 to 5 years old, which ranged between 1.48 to 1.91 mL[20] and that, in alpacas of that breed, in which the extraction was performed seminal every 48 h[4].

The cause of the volume variability in this experiment and the lack of correspondence with published works is due to the fact that this parameter is influenced by race, age, diet, psychosexual separation and collection frequency[21,22]. However, there is consensus that this last factor is the most important cause of variation in ejaculate volume in alpacas[23].

Furthermore, alpacas have low ejaculate volume[24], which presents variability between individuals and between collections in the same male[20]. The evaluation of this parameter is made difficult by the presence of foam that forms during semen collection[21].

The decrease in the concentration of post-thawed semen is due to the addition of the Triladyl® extender. This and the freezing protocol used could increase the percentage of post-thawing sperm mortality in this experiment and, therefore, do not efficiently fulfill the function of preserving sperm survival after thawing, which is their main evaluation criterion[25].

The increase in mortality in post-thawing semen may be due to the cellular damage suffered by the spermatozoa, caused by temperature changes during the freezing process, mainly the dehydration of the sperm cells and the formation of intracellular ice crystals during this process[26].

The decrease in motility after semen thawing corroborates that approximately 50 % of sperm lose their motility during freezing and thawing, which constitutes a challenge in the sperm cryopreservation process[25].

The percentage of morpho-anomalies in fresh semen is lower than in works published by other authors[23,27], but those in post-freezing semen are similar to those published by the aforementioned researchers. The abnormal shapes in male gametes prior to dilution could be due to the semen collection method, which causes variation in sperm morphometry[27].

The increase in abnormal shapes in post-freezing semen compared to fresh semen may be due to the effect of the components of the Triladyl® extender and freezing. Also to the preparation of the sample, fixation method, staining technique, microscopic system and suggestive errors in its determination[28]. These factors can affect the repeatability of the analysis, its reproducibility and the comparison of results between laboratories[29]. However, more precise and conclusive studies are required on the effect of freezing on sperm morpho-anomalies in alpacas.

Sperm concentration, motility and mortality of sperm, and their morpho-anomalies are lower than those obtained in alpacas from Peru[30]. The results of this experiment confirm that the physical and biological characteristics of alpaca semen also vary depending on the collection conditions, including the collec-

tion method[31,32].

It is concluded that the method of penis deviation with AV and receptive female was more accepted, with males, they performed a greater number of mounts per hour and more ejaculates were obtained, making it the most optimal for semen collection. With the use of the Triladyl® diluent in the freezing protocol, semen concentration and motility decreased, while mortality and sperm morpho anomalies increased after thawing.

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Conflicts of interest

The authors declare that they have no potential conflicts of interest with respect to the authorship and/or publication of this article

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Ethical considerations

The protocols used in the study comply with the European standards indicated at (https://ec.europa.eu/environment/chemicals/lab_animals//legislation_en.htm) for the use of animals in research.

Authors' contribution to the article

*Rafael Alfonso Garzón Jarri*n, information search, study design, sample taking, writing and drafting of the document. *Luis Alonso Chicaiza Sánchez,* information search, study design, sample taking, writing and drafting of the document. *Blanca Jeaneth Villavicencio Villavicencio*, data collection, writing and drafting of the document. *Juan Ramón García Díaz,* data processing and analysis, editing and revision of the manuscript.

Research limitations

No conclusive results were achieved to establish and validate one of the methods used in this research to obtain semen in alpacas, and a diluent and semen freezing protocol, since the quantities needed for this were not processed.

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