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Motility and recovery of alpaca (*Vicugna pacos*) spermatozoa after centrifugation in a density gradient solution

Gómez-Quispe, O. E.[1, 2*]; Gutiérrez-Reynoso, G. A.[1]; Gallegos-Cardenas, A.[1]; Fumuso, F. G.[3]; Asparrin, M.[4]; Asparrin-Del Carpio, M.[4]; Jara, C. W.[4]; Ponce, D.[5]; Miguel, M.[5]; Youngs, C. R.[6] and Vivanco, H. W.[5]

[1]Department of Animal Production, Universidad Nacional Agraria La Molina, Lima, Perú; [2]Faculty of Veterinary Medicine and Zootechnics, Universidad Nacional Micaela Bastidas de Apurímac, Abancay, Perú; [3]Faculty of Veterinary Sciences, Universidad de Buenos Aires, CONICET, Argentina; [4]Fundo Mallkini de Michael & Cia S.A., Azángaro, Perú; [5]Vivanco International SAC, Lima, Perú; [6]Department of Animal Science, College of Agriculture and Life Science, Iowa State University, Ames, Iowa, 50011, USA

[*]***Correspondence:*** *O. E. Gómez-Quispe, Department of Animal Production, Universidad Nacional Agraria La Molina, Lima, Perú. E-mail: oegomezq@hotmail.com*

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Summary

Background: One of the factors limiting successful processing of alpaca (*Vicugna pacos*) semen is the viscosity of seminal plasma. The viscous nature of the collected ejaculate has hindered sperm cryopreservation as well as artificial insemination (AI) under field conditions. **Aims:** The objective of this investigation was to evaluate recovery, motility, and plasma membrane integrity of alpaca spermatozoa after centrifugation in one of two different solutions at one of three different combinations of speed and time. **Methods:** A total of 24 ejaculates was recovered from seven reproductively sound Huacaya males using a modified artificial vagina (AV) after training the animals for semen collection. A 2 × 3 factorial treatment arrangement was utilized for this study. Ejaculates were divided into fractions for centrifugation in one of two solutions (Tris extender or PureSperm®80 density gradient solution) at one of three combinations of speed and time (492 × g for 15 min, 1968 × g for 10 min, or 4448 × g for 7 min). The experiment was replicated eight times. **Results:** Analysis revealed that centrifugation at 4448 × g for 7 min in PureSperm®80 provided a high recovery rate of spermatozoa with the highest sperm motility and functional integrity of plasma membrane post-centrifugation. **Conclusion:** Results suggest that adoption of this procedure (centrifugation at 4448 × g for 7 min in PureSperm®80) in the initial processing of alpaca ejaculates may enhance subsequent ability to use semen for AI and other assisted reproductive biotechnologies in this species.

Key words: Camelids, Ejaculate, PureSperm®80, Seminal plasma, Viscosity

Introduction

In the alpaca (*Vicugna pacos*) and other South American camelids, field-practical artificial insemination (AI) procedures are not yet available. Development of AI has been limited by the highly viscous nature of the ejaculate (Fernandez-Baca and Calderon, 1965; Bravo *et al*., 1997a; Von Baer and Hellemann, 1998; Adams *et al*., 2009).

Various procedures have been investigated in an attempt to overcome obstacles associated with viscosity of ejaculates of South American camelids and camels (a species with similar ejaculate characteristics). These procedures include liquefaction of semen by enzymatic methods (Bravo *et al*., 1997a, 2000a; Giuliano *et al*., 2010), degelification either by culture at room temperature (Garnica *et al*., 1993) or incubation at body temperature (Wani *et al*., 2008), repeated pipetting of semen (Santiani *et al*., 2005; Raymundo *et al*., 2006), magnetic agitation (Mosaferi *et al*., 2005; Niasari-Naslaji *et al*., 2006, 2007), recovery of epididymal spermatozoa post-mortem (Morton *et al*., 2007) or acquisition of *in vivo* derived spermatozoa from the vas deferens (Pérez *et al*., 2014; Gómez-Quispe *et al*., 2016). None of these approaches however, has significantly improved the quality of spermatozoal parameters after processing.

Another potential approach to deal with the viscous nature of the ejaculate is to centrifuge spermatozoa at low speeds after dilution. No changes in motility of llama (*Lama glama*) spermatozoa were observed after centrifugation (Giuliano *et al*., 2010). The same was true for alpaca semen, although lower rates of spermatozoal recovery were observed (Morton *et al*., 2012). High-speed centrifugation of dromedary camel (*Camelus dromedarius*) semen, on the other hand, decreased sperm motility 12.7% (El-Bahrawy, 2010).

Using a gel-like solution as a density gradient during centrifugation is generally recognized as an effective way to separate motile from non-motile spermatozoa (Morrell *et al*., 2009) and to obtain spermatozoa free of seminal plasma (Ortega-Ferrusola *et al*., 2009). Low-speed centrifugation of goat semen in 240° Bloom gel improved motility (Salvador *et al*., 2006), and centrifugation of boar semen in iodixanol (a non-ionic polysucrose based gradient that acts like a cushion) decreased loss of motility compared with centrifugation in traditional extender (Matás *et al*., 2007).

One commercially available gel-like density gradient product (PureSperm®) was developed for centrifugation of human semen to obtain viable sperm for use in

assisted reproductive technologies (Söderlund and Lundin, 2000), and it reportedly does not produce changes in sperm parameters (Claassens *et al*., 1998). Low-speed centrifugation of canine spermatozoa in a single density gradient of this gel improved sperm parameters (Dorado *et al*., 2013). The use of two PureSperm® density gradients and low-speed centrifugation yielded good results with semen from brown bears (Nicolas *et al*., 2012; Gomes-Alves *et al*., 2014), bulls (Maxwell *et al*., 2007), sheep (Hollinshead *et al*., 2004), horses (Morell *et al*., 2009), and alpacas (Morton *et al*., 2008); in the latter case, they observed only partial recovery of sperm.

To our knowledge, there is no published information on centrifugation of alpaca semen in PureSperm®80 density gradient medium. Therefore, the objective of this research was to determine the effect of various centrifugation parameters on spermatozoal recovery rate, motility loss rate, functional membrane integrity, and acrosomal integrity of alpaca spermatozoa centrifuged in PureSperm®80 density gradient solution. This research also explored the potential of this method to overcome problems associated with the viscosity of alpaca semen.

Materials and Methods

Location

This research was conducted at Fundo Mallkini of Michael & Cia S.A., located at 4200 meters above sea level in the Peruvian Andes (district of Muñani, province of Azángaro, Department of Puno). In this locale, the average maximum and minimum temperatures are 15°C and -8°C, respectively, annual rainfall is 91.2 mm, and relative humidity is 54%.

Animals

Seven Huacaya male alpacas of proven fertility were used for this study. The feeding program was based on natural pastures with predominance of *Festuca dolichophylla*, *Mulenvergia fastigiata*, *Calamagrostis vicunarum* grasses and *Trifolium amabili* legume. During days of semen collection, animals were grazed in fenced paddocks with perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) supplemented with oat hay (*Avena sativa*) and *ad libitum* water.

Semen collection

For two weeks before initiation of the experiment, males were trained for semen collection via use of an artificial vagina (AV) with a sexually receptive female to the side (Dávalos and Olazával, 2002). Semen was collected using a modified AV (Vivanco *et al*., 2010). Briefly, the AV (Fig. 1) consisted of a 24 cm long rubber tube. The main opening of the rubber tube was 4 cm in diameter, and a straight latex holster and conical latex holster were placed inside the rubber tube. The opposite end of the rubber tube had a smaller opening (2 cm diameter) in the shape of an eccentric hole that simulated the cervix. The conical latex cone connected to a graduated semen collection tube. Before semen collection, 50°C water was added to the AV to produce an internal temperature of 38-40°C at the time of semen collection. After preparation, the AV was wrapped with an electric blanket to prevent excessive cooling of the AV.

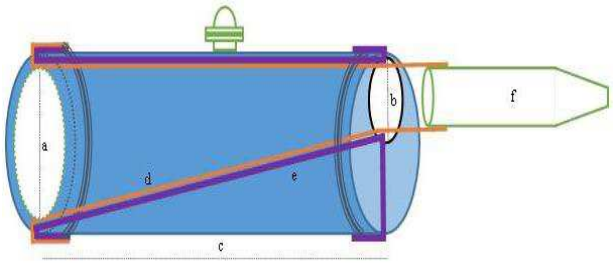

Fig. 1: Modified artificial vagina (Vivanco *et al*., 2010). **a**: Main opening of rubber tube (4 cm diameter), **b**: Eccentric tube opening (2 cm diameter), **c**: Length of rubber tube (24 cm), **d**: Conical latex holster (orange color), **e**: Straight latex holster (purple color), and **f**: Semen collection tube

The semen collection procedure consisted of pairing animals (male and female) to obtain the copula position (sternal recumbency) of both. Before intromission, the penis was diverted with the technician's hand into the AV where it was held until the end of copulation (approximately 15-25 min). The frequency of semen collection was 2 to 3 times per week during the reproductive season (February to March).

Ejaculate selection

Semen samples were taken to the laboratory for processing and evaluation. Gross motility or vibratory movement was assessed using the method developed for camel ejaculates (Niasari-Naslaji *et al*., 2007) and previously adapted for use in alpacas (Gómez-Quispe *et al*., 2016). Only those ejaculates with motility >60% and sperm concentration >80 million spermatozoa/ml (method of assessment described below) were accepted for the experiment.

Experimental design

A 2 × 3 factorial treatment arrangement was utilized for this study. Collected ejaculates were divided into fractions for centrifugation in one of two solutions (Tris base or PureSperm®80 density gradient solution [described below]) at one of three combinations of speed and time (492 × g for 15 min, 1968 × g for 10 min, or 4448 × g for 7 min). As speed of centrifugation was increased, time of centrifugation was purposefully decreased in an attempt to mitigate the potential deleterious effect of high speed centrifugation on spermatozoa. The speed-time combinations were considered as a single variable. The experiment was replicated eight times.

Preparation of semen for centrifugation

Tris extender and PureSperm®80 solutions were

prepared prior to semen collection. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Tris extender was prepared using 3.605 g of Tris (hydroxymetyl) amino-methane, 2.024 g of citric acid dihydrate, 1.488 g of fructose, and 50,000 IU of penicillin and bidistilled water of csp 100 ml. Tris extender was stored at room temperature until ready for use.

The PureSperm®80 density gradient solution was prepared by placing four parts of PureSperm®100 (Nidacon, Gothenburg, Sweden) in a test tube and diluting it with one part PureSperm® buffer. The resultant PureSperm®80 solution (300-310 mOsm) was stored at 4°C but warmed to room temperature prior to use in accordance with manufacturer's recommendations.

Preparation of the density gradient solutions consisted of placing 0.5 ml of PureSperm®80 or Tris extender in the bottom of a 15 ml conical centrifuge tube, onto which 0.5 ml of raw semen was carefully added on top. Sperm centrifugation was performed at 20°C using a standard centrifuge (Harmonic Series, model PLC-012E, Taiwan).

Recovery of spermatozoa after centrifugation

For spermatozoa centrifuged in Tris extender, supernatant (containing a mixture of Tris extender and seminal plasma) was removed using a pipette with a sterile tip, leaving the sperm pellet at the bottom of the centrifuge tube. The supernatant was placed into a petri dish to quantify the number of spermatozoa present.

In the case of spermatozoa centrifuged in PureSperm®80, approximately one-third of the supernatant (seminal plasma) was poured into another centrifuge tube and the remaining seminal plasma was removed using absorbent paper (Elite, Perú). The PureSperm®80 (together with impurities) was subsequently removed using a sterile pipette tip, allowing the sperm layer to fall toward the bottom of the centrifuge tube.

The sperm pellets formed in the Tris extender tube and the sperm layer near the bottom of the PureSperm®80 tube were both diluted with Tris extender in the ratio of 1:4 (spermatozoa: extender) to facilitate post-centrifugation sperm evaluations.

Evaluation of spermatozoa

All measurements were made according to validated published procedures. Sperm motility, sperm cell concentration, and membrane functionality were evaluated before and after centrifugation. Acrosomal integrity was assessed only after centrifugation due to the pre-centrifugation viscosity that hindered staining.

Sperm motility was evaluated in accordance with previously published procedures (Santiani *et al*., 2005; Morton *et al*., 2007; WHO, 2010; Kershaw-Young and Maxwell, 2011). Briefly, a 10 µL drop of semen was placed on a warmed glass microscope slide, covered with a coverslip, and placed on a heated microscope stage at 38°C. Sperm were observed at ×400 magnification using a phase contrast compound microscope (Olympus CX31, Hamburg, Germany). Motility of 200 spermatozoa was assessed in three replicates, and mean values were expressed in percentages.

Sperm cell concentration was measured using a hemocytometer (Neubauer chamber; Marienfeld, Germany) following methods standardized by WHO (2010).

Functional integrity of the sperm plasma membrane was evaluated by placing a drop containing spermatozoa cultured in hypo-osmotic swelling test (HOST) solution onto a warmed glass microscope slide and covering it with a coverslip. The endosmosis-positive sperm count was assessed via use of a phase contrast compound microscope at a magnification of ×400. The HOST solution was prepared by mixing 1351 mg of sodium citrate dihydrate and 735 mg of D-fructose, dissolved in 100 ml of bidistilled water (WHO, 2010). The HOST culture solution was made by mixing 0.01 ml of the sperm sample with 0.1 ml of HOST solution (Jeyendran *et al*., 1984), which was subsequently incubated for 15 min in a water bath at a temperature of 38°C (Vasquez *et al*., 2012).

Acrosomal integrity was assessed via use of a triple staining technique adapted from Kovács and Foote (1992) for use in llamas (Fumuso *et al*., 2015). Briefly, a 10 µL sample of semen and a 10 µL drop of trypan blue were placed adjacent to one another on a glass microscope slide, mixed, spread, and then air dried. To fix the sample, the slide was covered with neutral red dye for 2 min and subsequently rinsed with distilled water. Next, the slide was stained with giemsa for 1.5 h, rinsed by immersion in distilled water for 2 min, dried in open air, and observed at ×100 with immersion oil. Spermatozoa were categorized (Fumuso *et al*., 2015) as:

i) Alive with intact membranes and acrosome present (light blue post-acrosomal region and pink acrosome region)

ii) Alive with damaged or loose acrosome (light blue post-acrosomal region and clear lilac or violet acrosome region)

iii) Dead with acrosome present (dark blue post-acrosomal region and pink acrosome region)

iv) Dead with damaged or loose acrosome (dark blue post-acrosomal region and light or dark violet acrosome)

Sperm recovery rate, as well as sperm motility loss rate and functional membrane integrity loss rate after centrifugation, were determined in this study. To determine final sperm concentration, the supernatant from samples centrifuged in Tris extender (containing Tris extender and seminal plasma) and the supernatant from samples centrifuged in PureSperm®80 (containing PureSperm®80 and seminal plasma) were evaluated to quantify the number of spermatozoa present. Using this methodology, it was possible to also assess final values of the other spermatozoal parameters. Sperm recovery rate, motility loss rate and functional membrane integrity loss rates were calculated using the method previously reported by Campos *et al*. (2004) and Lima *et al*. (2013). This approach measures changes in semen parameters

between two different time points, but also uses absolute values of parameters in the following formula to facilitate interpretation:

$$|\text{Sperm parameter change}| = \left[\frac{(\text{Final sperm value} - \text{Initial sperm value})}{\text{Initial sperm value}}\right] X\ 100\%$$

Statistical analysis

Sperm parameters were evaluated under a completely randomized design with a factorial treatment arrangement. Parameter values were transformed to angular values (angle = arcsine $\sqrt{x}$) in an attempt to normalize data. Analysis of variance was performed using centrifugation solution (Tris extender or PureSperm®80), centrifugation speed-time combinations, and their two-way interaction as independent variables. Mean separation was performed using Tukey's honest significant difference. Differences were considered significant at a P-value less than or equal to 0.05. The processing and analysis of data were performed using the statistical software package R-3.3.2 for Windows.

Results

The location of alpaca spermatozoa after centrifugation of ejaculates in Tris or PureSperm®80 solution is shown in Fig. 2. A well-defined sperm pellet was found at the bottom of the tube when spermatozoa were centrifuged in Tris solution; impurities or contaminants were also localized near the bottom of the tube (Fig. 2, panel a). For semen centrifuged in PureSperm®80 solution, three different layers were commonly observed after centrifugation. PureSperm®80 containing impurities was the bottom layer, spermatozoa were in the middle layer, and the top layer consisted of seminal plasma. Low speed of centrifugation of semen in PureSperm®80 density gradient solution produced a diffuse layer of spermatozoa as well as highly dispersed locations of impurities. However, as the speed of centrifugation increased (and time of centrifugation was concomitantly reduced), a well-defined layer of spermatozoa formed with total separation of impurities (Fig. 2, panel b).

A speed-time X medium interaction was observed for post-centrifugation sperm recovery (P<0.001) and sperm motility loss (P<0.005) as shown in Table 1. In contrast, no speed-time X medium interaction was observed for post-centrifugation functional plasma membrane integrity (P<0.165) which was affected by speed-time (P<0.002) and medium (P<0.001).

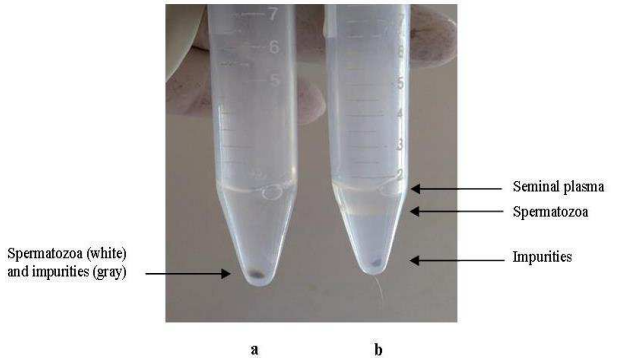

Fig. 2: Location of spermatozoa, seminal plasma and impurities in Tris (tube a) or PureSperm®80 (tube b) solution after centrifugation of alpaca semen at 4448 × g for 7 min

Rate of sperm recovery after centrifugation improved with an increase in the speed of centrifugation (Fig. 3). A speed-time combination of 4448 × g for 7 min enabled sperm recovery rates of 98.46% and 100% for ejaculates centrifuged in PureSperm®80 density gradient solution and Tris medium, respectively, although both were similar (Fig. 3; P=0.479). Grouped by medium of centrifugation, the rate of sperm recovery increased (P<0.001) as the speed of centrifugation increased (with a concomitant decrease in time) in both Tris and PureSperm®80 media (Fig. 4).

The loss in sperm motility, segregated by speed-time combination or centrifugation medium, is shown in Figs. 5 and 6, respectively. Within each speed-time combination, ejaculates centrifuged in PureSperm®80 exhibited a lower (P<0.001) loss of sperm motility than those centrifuged in Tris. Within each medium of centrifugation, loss of sperm motility was unaffected (P=0.084) by speed-time combinations. Centrifugation of ejaculates at 4448 × g for 7 min in PureSperm®80 yielded a sperm motility loss of only 3.5%.

Functional plasma membrane integrity decreased when ejaculates were centrifuged, being affected by speed-time combination (P<0.001; Fig. 7) and by medium of centrifugation (P<0.001; Fig. 8). Loss of membrane integrity was 8.8%, 7.6, and 5.8% for spermatozoa centrifuged at 492 × g for 15 min, 1968 × g for 10 min, and 4448 × g for 7 min, respectively (Fig. 7). Ejaculates centrifuged in PureSperm®80 averaged a 6.0% loss of plasma membrane integrity whereas an 8.8% loss was observed for ejaculates centrifuged in Tris medium (Fig. 8). These results suggest a potential protective effect of PureSperm®80 on the sperm plasma membrane when ejaculates are centrifuged at relatively high speeds.

Table 1: Rates of sperm recovery, sperm motility loss, and functional plasma membrane integrity of alpaca spermatozoa after centrifugation at various speed-time combinations in one of two different media

Variable	P-value		
	Speed-Time†	Medium‡	Speed-time X medium
Sperm recovery	<0.001	<0.001	<0.001
Sperm motility loss	<0.318	<0.001	<0.005
Functional plasma membrane integrity	<0.002	<0.001	<0.165

† Speed-time of centrifugation: 492 × g for 15 min, 1968 × g for 10 min, 4448 × g for 7 min, and ‡ Medium used for centrifugation of ejaculates: Tris or PureSperm®80

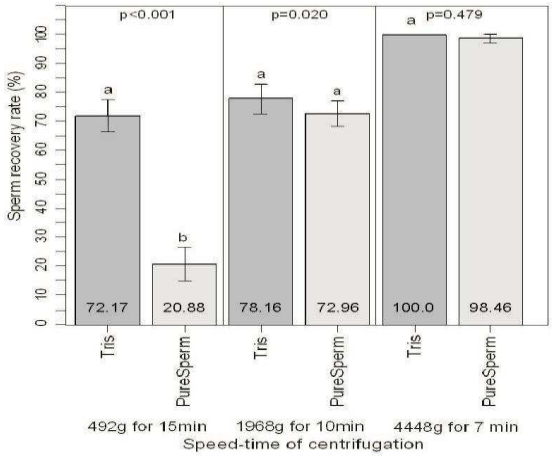

Fig. 3: Sperm recovery rate segregated by centrifugation speed-time combination. Numbers within each bar represent least squares means of sperm recovery

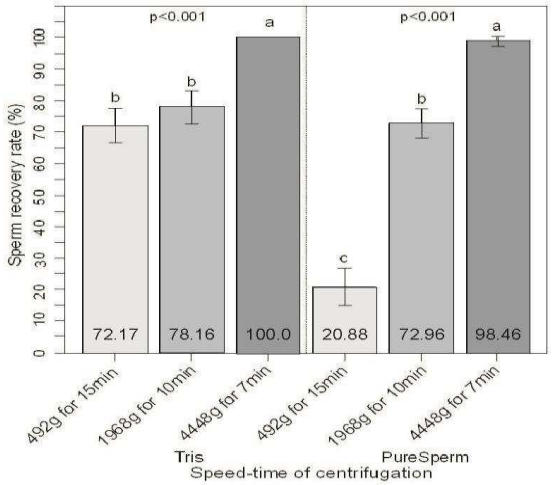

Fig. 4: Sperm recovery rate segregated by medium of centrifugation. Numbers within each bar represent least squares means of sperm recovery

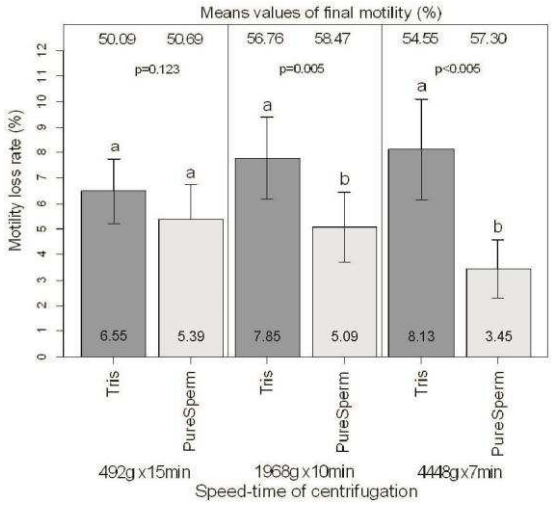

Fig. 5: Loss of sperm motility segregated by centrifugation speed-time combination. Numbers within each bar denote least square means of loss of motility, whereas numbers above the bars represent least square means of post-centrifugation motility

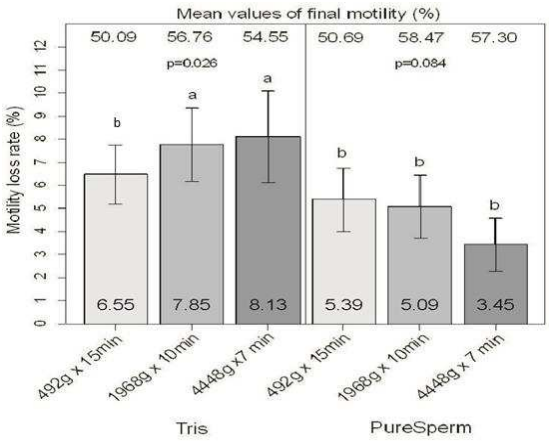

Fig. 6: Loss of sperm motility segregated by centrifugation medium. Numbers within each bar denote least square means of loss of motility, whereas numbers above the bars represent least square means of post-centrifugation motility

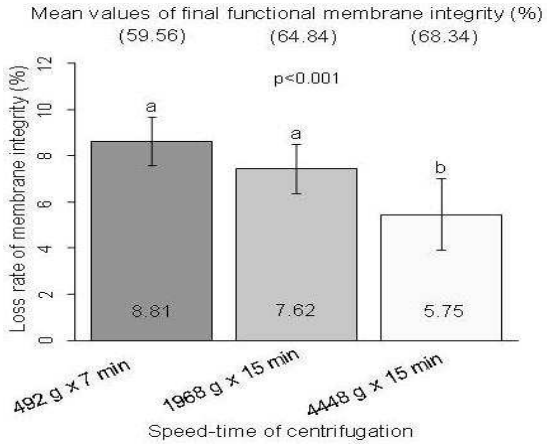

Fig. 7: Loss of functional sperm plasma membrane integrity segregated by centrifugation speed-time combination. Numbers within each bar denote least square means of loss of functional membrane integrity, whereas numbers above the bars (within parentheses) represent least square means of post-centrifugation functional plasma membrane integrity

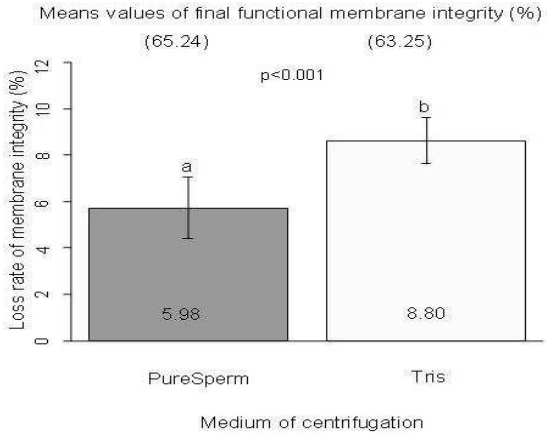

Fig. 8: Loss of functional sperm plasma membrane integrity segregated by centrifugation medium. Numbers within each bar denote least square means of loss of functional membrane integrity, whereas numbers above the bars (within parentheses) represent least square means of post-centrifugation functional plasma membrane integrity

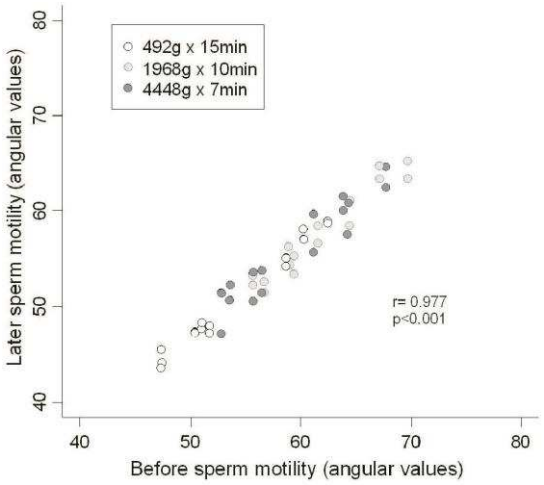

Fig. 9: Correlation between sperm motility before and after centrifugation of alpaca ejaculates in Tris medium or PureSperm®80 density gradient solution at one of three different speed-time combinations. White dots represent centrifugation at 492 × g for 15 min, gray dots represent centrifugation at 1968 × g for 10 min, and black dots represent centrifugation at 4448 g × for 7 min

A strong correlation (r=0.977; P<0.001) was observed between sperm motility before and after centrifugation (Fig. 9), providing evidence that the speed-time combinations chosen for this study were appropriate for alpaca spermatozoa.

Discussion

For a species which produces a very viscous ejaculate (such as the alpaca), implementation of field-practical methods of AI is extremely difficult. In an attempt to overcome obstacles created by the viscous nature of the seminal plasma, researchers have investigated centrifugation as a means to isolate spermatozoa for subsequent use in AI. The medium in which spermatozoa are suspended during centrifugation can potentially influence sperm recovery rates. In our study, we compared a traditional diluent (Tris) with a single-layer density gradient solution (PureSperm®80) as a centrifugation medium. Alpaca ejaculates in these media were centrifuged at one of three different combinations of centrifugation speed-time, and centrifugation produced either a sperm pellet in the bottom of the centrifuge tube (Tris medium) or a stratified layer of spermatozoa (PureSperm®80).

For alpaca spermatozoa centrifuged in Tris medium, separation of the sperm pellet from the seminal plasma and Tris mixture was carried out quite easily using a pipette. On the other hand, difficulties were encountered in isolating the sperm layer formed after centrifugation in PureSperm®80 density gradient solution, a result similar to that reported by Morton *et al*. (2008). To circumvent this problem, we used sterile absorbent paper to remove seminal plasma from the centrifuge tube. The absorbent paper was introduced perpendicular to the centrifuge tube and inserted into the seminal plasma layer (top layer) where it remained until nearly all seminal plasma had been absorbed. Although at the end of this procedure there were still remnants of seminal plasma (we did not want to inadvertently remove spermatozoa) and PureSperm®80 on both sides of the sperm layer, these remnants apparently did not affect the spermatozoa.

Centrifugation of alpaca semen at low speed (600 × g for 20 min) in a dual-layer (45%:22.5%) density gradient solution of PureSperm® (Morton *et al*., 2008) led to a 70% sperm recovery rate accompanied by a low loss of sperm motility (3.6%). This low loss of sperm motility was similar to that observed in the present study (3.5% loss) with high speed centrifugation (4,448 × g for 7 min) in PureSperm®80; however, in the present study we achieved a sperm recovery rate exceeding 98%. To our knowledge, this is the first report of a method where spermatozoa were nearly completely recovered from alpaca ejaculates with minimal changes in sperm motility and sperm membrane functional integrity (data described below). However, we acknowledge that it is necessary to conduct additional research to confirm the consistency and repeatability of this finding.

Sperm motility is the parameter more susceptible to harmful microenvironmental conditions than any other characteristic associated with viability of spermatozoa (Soler *et al*., 2003; Monteiro *et al*., 2013). In the alpaca, sperm motility is directly related (r=0.96) to the viability of spermatozoa (Santiani *et al*., 2005). We observed that centrifugation of alpaca spermatozoa in a gel-like density gradient solution (PureSperm®80) allowed for a homogeneous distribution of spermatozoa in the layer formed during centrifugation, and furthermore that high speed centrifugation facilitated an almost complete extraction of spermatozoa from the viscous seminal plasma without substantially affecting either sperm motility or sperm membrane integrity. This observation is supported by the minimal changes in sperm motility observed after centrifugation in PureSperm® at 300 × g for 20 min in the dog (Dorado *et al*., 2013), and at 600 × g for 7 min in the alpaca (Morton *et al*., 2008; Morton *et al*., 2012). Similar responses were obtained with respect to motility and acrosomal integrity of goat spermatozoa after centrifugation in a gelatin (240° Bloom) at 900 × g for 10 min (Salvador *et al*., 2006). Recent results in the dromedary camel (Malo *et al*., 2017; Malo *et al*., 2018) also revealed that single layer centrifugation in a colloid solution improved sperm motility in both fresh and frozen-thawed sperm.

Consistent with the above result in camels, Kershaw-Young and Maxwell (2011) observed that incubation of alpaca spermatozoa in various concentrations of seminal plasma produced cell damage, but incubation in 10% seminal plasma actually improved sperm motility. Other researchers found no changes between the initial and final sperm motility after incubation in PureSperm® in marmots (Hernández-Lopez *et al*., 2005) or humans (Boomsma *et al*., 2007). A high sperm recovery rate was observed after centrifugation of boar spermatozoa at 1000 × g for 20 min in iodixanol medium (Matas *et al*., 2007).

Ejaculates of several species have been centrifuged in various media at different speeds. Decreased sperm motility was observed when boar semen was centrifuged at 800 × g for 10 min in BTS (Matas *et al*., 2007) and when goat semen was centrifuged at 900 × g for 10 min in skim milk (Salvador *et al*., 2006). Similar adverse effects on sperm motility and functionality of the sperm plasma membrane were obtained with llama spermatozoa centrifuged at 800 × g for 8 min in H-TALP-BSA medium, although exposure of the ejaculate to various concentrations of collagenase may have negatively influenced those results (Giuliano *et al*., 2010). In contrast, no change in alpaca sperm motility was observed after centrifugation at 600 × g for 7 min in Androhep® medium, but the recovery rate of spermatozoa reached only 63% and it was not possible to remove the seminal plasma due to the absence of semen liquefaction (Morton *et al*., 2008). In another study (Kershaw-Young and Maxwell, 2011) with alpaca semen centrifuged at 1000 × g for 10 min in PBS, a 56% sperm motility was obtained (although initial sperm motility was not reported and any loss of motility could not be determined).

When camel ejaculates were centrifuged in Tris medium at extreme velocities (18,000 × g for 15 min), sperm motility decreased to 63.3% with a loss rate of 12.7% (El-Bahrawy, 2010). Centrifugation of alpaca ejaculates in Tris medium at 4,448 × g for 7 min in the present study produced sperm motility of 54.6% with a loss rate less than 8.2%. Comparing results of high speed centrifugation (4,448 × g for 7 min) in either Tris or PureSperm®80 gel revealed a potential protective effect of the PureSperm®80 density gradient solution on sperm motility and plasma membrane integrity of alpaca spermatozoa. This hypothesized protective effect is consistent with a reduction of sperm DNA fragmentation observed in dromedary camel ejaculates subjected to single layer centrifugation in a colloid solution (Malo *et al*., 2018).

In our study where semen was collected with a modified AV, initial values of sperm motility (53.6 to 61.6%), functional integrity of the membrane (65.5 to 72.6%) and sperm concentration (102.1 to 130.4 million/ml) were in range of previously published reports that used a semen collection dummy-AV (*motility:* Bravo *et al*., 1997a, 2000b; Santiani *et al*., 2005; Raymundo *et al*., 2006; Alarcon *et al*., 2012; *concentration:* Bravo *et al*., 1997a; Alarcon *et al*., 2012; *plasma membrane functional integrity:* Garcia *et al*., 2017; Pacheco *et al*., 2017). Unpublished work in alpacas by Vivanco (personal communication) revealed similar sperm motility (57.1 ± 17.6%), with a variation in sperm concentration (39.8 ± 34.4 × 10^6 sperm/ml) using the same semen technique used in this research. Nevertheless, one can not overlook the fact that sperm parameters could vary with the type of diet (Juyena *et al*., 2013), frequency of ejaculate collection (Bravo *et al*., 1997b), type of semen collection (Alarcon *et al*., 2012), and/or presence of receptive female during copulation (Dávalos and Olazával, 2002) among other factors.

In the present research, the high initial sperm motility observed before centrifugation was also observed after centrifugation (r=0.977), suggesting that our methods are not causing major harm to the spermatozoa. Subsequent data analysis with ANCOVA confirmed the influence of the initial sperm values on the results of the parameters after centrifugation. It is necessary to take into account the initial values of sperm parameters to fully understand changes that occur over time in an experiment using rate (Campos *et al*., 2004; Lima *et al*., 2013) or change analysis (Barati *et al*., 2009; Scott *et al*., 2016). In camelids, unfortunately, many publications do not report initial spermatozoal values. In the alpaca, ejaculation is intermittent (Lichtenwalnerl *et al*., 1996) and yields a highly viscous ejaculate (Garnica *et al*., 1993; Bravo *et al*., 1997a). The duration of copulation lasts from 12 to 47 min (Fernandez-Baca and Calderon, 1965; Raymundo *et al*., 2006), so the values of the initial parameters of experimentation are not the same as those during ejaculation. In sheep and cattle, however, those values are similar due to their rapid ejaculation time. Therefore, camelid semen should be evaluated not only using final values, but also using percentages of change (Gomez-Quispe *et al*., 2016), variation rate (Morton *et al*., 2008) or other suitable methodologies to correlate final values with initial values.

During the conduct of this investigation, it was not possible to effectively use the triple-staining technique (Kovács and Foote, 1992) to determine acrosomal integrity that was previously adapted by Fumuso *et al*. (2015) for use with llama spermatozoa. Although we applied the Fumuso *et al*. (2015) technique, we were unable to clearly differentiate spermatozoa into the categories indicated for this test. Perhaps this was due to the use of the giemsa dye which lacks full capacity to impregnate itself with the sperm acrosome (Morton *et al*., 2008). Further adapation of this technique for use with alpaca spermatozoa is needed to accurately assess acrosomal status.

Centrifugation of alpaca ejaculates at 4448 × g for 7 min in PureSperm®80 density gradient solution provided an extremely high rate of post-centrifugation sperm recovery with minimal loss of sperm motility and plasma membrane functional integrity. These results provide strong encouragement for routine adoption of this method for processing of alpaca spermatozoa for subsequent use in AI, sperm cryopreservation, and other assisted reproductive biotechnologies.

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Conflict of interest

The authors declare no conflicts of interest.

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