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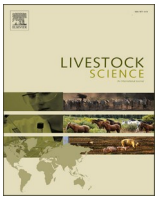

Non-enzymatic extraction of spermatozoa from alpaca ejaculates by pipetting followed by colloid centrifugation

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HIGHLIGHTS

- Alpaca ejaculates were liquified by gentle pipetting in Tris-citrate-fructose buffer.
- Sperm were separated from seminal plasma with or without colloid centrifugation.
- Motility and membrane integrity were greater after colloid centrifugation than in controls.
- Sperm quality in colloid centrifuged samples was maintained during 24h cold storage.
- Sperm quality in thawed samples was also greater after colloid centrifugation but the freezing protocol requires optimization.

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ABSTRACT

Viscous camelid ejaculates present problems for sperm handling and sperm preservation. In the present study, a technique that had been used for dromedary camel semen was tested with alpaca semen. Ejaculates (n=9) were collected by artificial vagina at San Marcos University, Lima, and were liquefied by gentle pipetting in tris-citrate-fructose. Half of the sample was prepared by Single Layer Centrifugation (SLC) through a colloid; the other half was centrifuged without colloid as a control. Each control and SLC sample was then split into two parts; one part was stored cooled for 24 h at 5 °C and the other part was frozen, resulting in 4 treatments for each ejaculate. All samples were evaluated for sperm motility, hypoosmotic swelling test (HOST), plasma membrane integrity, and morphology, immediately after centrifugation and again after storage Total motility and plasma membrane integrity were greater in samples prepared by SLC than controls (motility 72±13% vs. 57±7%; plasma membrane integrity 63±13% vs. 54±8%, for SLC and controls respectively). Normal morphology and HOST were not different between treatments (65±13 vs. 61±13% and 42±6 vs. 39±10%, for SLC and controls respectively). After 24 h cooled storage, motility and plasma membrane integrity were greater for SLC samples (motility: 51±16 vs. 34±15%; $p<0.001$; membrane integrity: 51±15 vs. 40±18%; $P < 0.05$ for SLC and controls, respectively); HOST (40±14 vs. 34±11%) and normal morphology (67±13 vs. 63±14%) were not different between treatments. Sperm quality decreased considerably after cryopreservation ($P<0.001$ for all parameters); however, motility ($P<0.01$), plasma membrane integrity ($P<0.05$) and morphology ($P<0.05$) were higher for SLC than for controls. These results indicate that alpaca spermatozoa can be extracted from semen using a combination of pipetting and SLC, potentially with a beneficial effect on sperm quality. Samples could be stored cooled for 24 h, retaining better motility than controls; motility and plasma membrane integrity were greater in SLC samples than controls after freezing and thawing but the freezing protocol requires improvement.

1. Introduction

Alpacas originate from South America, particularly the high Andes, where they are kept mainly for their fleece ("fiber") and for meat (Fernández-Baca, 1977). They have now become popular in many countries around the world, where they are kept as novelty companion

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animals and for fiber (Abraham et al., 2017). Peruvian alpacas previously produced fine quality fiber, but a reduction in fiber quality has occurred due to export of superior males to other countries, indiscriminate breeding between llamas and alpacas, and a focus on white coat color (Montes et al., 2013). Selective breeding to improve fiber quality is theoretically possible, if males and females could be segregated and kept separately from llamas. However, such segregation is not often feasible in the Andes where alpacas and llamas are herded together in mixed sex groups (Fernández-Baca, 1975).

In some other countries, it is customary to move animals around for mating - so-called mobile matings (Morton et al., 2008), but this poses a risk of disease transmission, and welfare could be compromised (Vaughan et al, 2003). Use of artificial insemination (AI) instead of natural mating allows more efficient use of genetically superior males (Miragaya et al., 2006; Huanca and Adams, 2007), which could result in improved fiber quality (Reyna, 2005). Since the need to move animals around for mating would be decreased, animal welfare would be improved and disease transmission would be reduced. Therefore, development of a technique for AI is a priority, both in the alpacas' native homelands for poverty alleviation and in other countries where the species is gaining popularity (Abraham et al., 2017). However, there is still a need for further research before this technology can be applied in a commercial setting, for example in developing methods for semen storage (Morton & Maxwell, 2018).

One of the major problems hindering the development of AI is the viscous nature of the camelid ejaculate, making semen handling difficult (Santiani et al., 2005; Huanca and Adams, 2007). The spermatozoa are trapped within the viscous seminal plasma, and move with a slow oscillatory movement (Garnica et al., 1993; Deen et al., 2003). The seminal plasma impedes measurement of sperm concentration as well as attempts to divide the ejaculate into aliquots. Moreover, it creates difficulties when making wet smears for sperm evaluation, and might inhibit penetration of cryoprotectants (Kershaw-Young and Maxwell, 2012). Gentle aspiration through a needle ("needling") was tried by some researchers in an attempt to break up the ejaculate (Santiani et al., 2005; Raymundo et al., 2006), but this technique can damage spermatozoa. Gentle pipetting with a plastic pipette is a recent modification of this technique (Zampini et al., 2020). Some authors recommended using enzymes to break down the viscous ejaculate (e.g. Bravo et al., 2000a; El Bahrawy et al., 2006; Giuliano et al. 2010) but others consider that they might have deleterious effects on spermatozoa. Alpaca sperm motility was affected by collagenase but not by trypsin or papain, whereas acrosome integrity was affected by papain at all the concentrations tested and by trypsin at 4 mg/mL but not by collagenase (Morton et al. 2012). Stuart and Bathgate (2015) considered that the spermatozoa were not damaged but they did not survive freezing. Recent reports recommend the use of papain (Kershaw-Young et al. 2013, Kershaw et al. 2016) or catalase (Morton & Maxwell, 2018).

Colloid centrifugation of semen from other species (Morrell and Rodriguez-Martinez, 2009) allows motile, viable, morphologically normal spermatozoa with intact chromatin to be separated from seminal plasma and other cells in the ejaculate. Colloid centrifugation can be used as a density gradient, using two or more layers of colloid of different densities, or as Single Layer Centrifugation (SLC) using one layer of colloid. The latter has been used on llama semen after a brief enzyme treatment (Trasorras et al., 2012; Santa Cruz et al., 2016) and also with dromedary camel semen that was not treated with enzymes but had been pipetted gently in an excess of buffered extender (Malo et al., 2017), or with llama semen without enzyme treatment (Bertuzzi et al., 2020). The llama spermatozoa were fertile when used for *in vitro* fertilization; the dromedary spermatozoa were fertile when used fresh for AI and generated offspring by AI after cryopreservation.

The objective of the present study was to investigate if spermatozoa could be extracted from alpaca semen using the method developed for dromedary camel semen, and whether these spermatozoa could be cooled and stored for 24 h, or cryopreserved.

2. Materials and methods

2.1. Tris buffer

A buffered formulation for ram semen (Aisen et al., 2000) was used for these experiments, modified for alpaca semen by the addition of glycine (Guerrero et al., 2009) and 100 mg/l gentamycin. Fraction A consisted of 171 mM Tris (N-Tris hydroxymethyl aminomethane), 66.7 mM anhydrous citric acid, 55.5 mM Fructose, 133.2 mM Glycine and 10% (v/v) egg yolk. Fraction B was fraction A supplemented with 250.8 mM trehalose, 6% glycerol and an additional 10% (v/v) egg yolk.

Colloid: the silane-coated silica colloid formulation used for dromedary camel semen (Colloid 1; Malo et al., 2017, supplied by JMM) was diluted with Buffer B (patent applied for, JMM).

2.2. Animals

Four male alpacas, held at the Faculty of Veterinary Medicine, San Marcos University, were available for semen collection during September 2018. They were kept separately from females and were 4 to 12 years old. They were housed and cared for according to national and international guidelines on the husbandry and care of animals; the study was approved by the university ethics committee (San Marcos University, Lima). The males were accustomed to semen collection with an artificial vagina (Bravo et al., 1997) after mounting a receptive female; semen collection was carried out for a maximum of 10 minutes to avoid negative effects on sperm quality from a prolonged collection time (WH, unpublished observations). Semen was collected twice a week over a three week period. Some ejaculates could not be included in the study because of insufficient volume for the treatments. As a result, three ejaculates were available for this study from each of two males, two ejaculates from one male and one ejaculate from the remaining male. The experimental design is shown in Fig. 1.

2.3. Initial semen evaluation

2.3.1. Physical characteristics

The volume and thread formation were determined directly after collection. The volume of the ejaculate was measured in a graduated falcon tube. Thread formation was measured i.e. the height of a thread produced from a drop of semen on a slide when the pipette is lifted up slowly from the drop.

2.3.2. Sperm motility

Total motility (Giuliano et al., 2008) was assessed subjectively in a 10 μL drop of the sperm sample placed on a pre-warmed slide and covered with a cover slip on a warm plate (37 °C). The sample was observed using a phase microscope at 40X; motility was evaluated in 10 fields.

2.3.3. Sperm concentration

Sperm concentration was determined using a Neubauer improved counting chamber, after mixing semen and water (0.1:9.9 mL v/v).

2.4. Semen preparation

The semen was extended 1:3 with Fraction A and was incubated at 37°C for 30 min, repeatedly aspirating it with a pasteur pipette every 5 min to help liquefy the sample. After 30 min, an aliquot was evaluated by microscopy to confirm that the spermatozoa could move freely. Once liquefied, the sample was split: one portion was pipetted over colloid (diluted 1:1 v/v) in a 15 mL centrifuge tube (called SLC), and the other aliquot was centrifuged without colloid as a control (CON). The tubes were centrifuged at 300 g for 20 min, before retrieving the sperm pellet and resuspending in Fraction A (0.5 mL). Aliquots of the sperm suspension were then removed for evaluation. Thereafter, the samples were

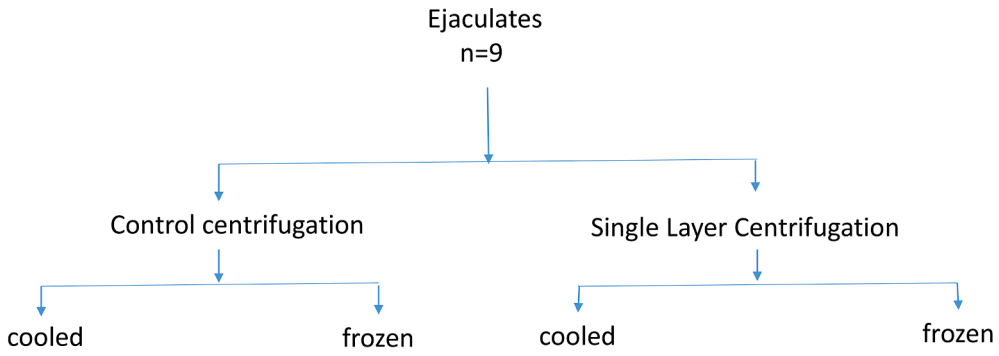

Fig. 1. Experimental design.

transferred into glass tubes and placed in a beaker containing water at 37 °C in a refrigerator, to cool the samples gradually to 5 °C over a period of 2 h. The cooling rate was approximately 1°C/min. Each sample was then divided into two again, one aliquot for continued cooled storage and the other for freezing. The samples for cooled storage were kept in the refrigerator for 24 h.

2.5. Cryopreservation

Fraction B at 5°C was added 1:1 (v:v) to the samples for freezing. The samples were loaded into 0.5 mL straws and cooled stepwise as follows; the straws were placed in a rack 10 cm above the surface of liquid nitrogen for 5 min, 5 cm above for 5 min, and 1 cm above for 2 min, before transferring to liquid nitrogen for storage. The theoretical cooling rates are approximately 23°C/ min for 5 min, followed by 15°C/min for 5 min and then 10°C/min for 2 min.

2.6. Thawing

The semen samples were stored in liquid nitrogen until evaluation, approximately 2 days later. For thawing, the straws were warmed in a water bath at 37 °C for 45 s and then placed in an incubator at the same temperature for 5 min before evaluation. Post-thawing, the samples were evaluated for motility, membrane integrity, sperm membrane functionality and morphological abnormalities.

2.7. Sperm evaluation

The following evaluations were carried out on the sperm samples after centrifugation, and again after cooled storage for 24 h or freezing and thawing.

2.7.1. Membrane integrity and morphology

Eosin-nigrosin staining was used to assess membrane integrity (vitality) and morphology (Bamba, 1988). In total, 200 spermatozoa were assessed per sample. Eosin/nigrosin stain solution (10 μL) was mixed with an equal volume of sperm sample; a drop of the mixture was immediately smeared on a glass slide. After air drying, the proportions of spermatozoa that had acquired stain (indicating damaged membranes) and unstained spermatozoa (intact membranes) were determined.

The slides were transported to the Swedish University of Agricultural Sciences (SLU) for evaluation of sperm morphology using phase contrast microscopy and 100X objective.

Abnormal spermatozoa were classified according to the location of the abnormality (tail or head; Dascanio, 2014). Morphology was assessed using the parameters; normal, abnormal head (narrow, narrow base, pear-shaped, abnormal contour) or abnormal tail (simple, coiled, double folded). It was not possible to count droplets in these preparations due to difficulties in visualizing them.

2.7.2. Hypo-osmotic swelling test (HOST)

The functional integrity of the sperm plasma membrane was evaluated using HOST. An aliquot (100 μL of sperm sample) was mixed with 500 μL sodium citrate adjusted to 150 mOsm. The mixture was incubated at 37 °C for 25 min and thereafter the reaction was halted by adding 4 % formalin in PBS. A 10 μL drop of the mixture was placed on a slide, covered with a cover slip; the spermatozoa were evaluated for coiling of the tail, which indicates an intact sperm membrane. A total of 200 spermatozoa was assessed per sample. Any spermatozoa where the tail could not be assessed fully were excluded. The number of spermatozoa with a coiled tail was counted and used to calculate the proportion of spermatozoa with a functioning membrane.

2.8. Statistical analysis

Treatment means for each parameter were compared using a general mixed model, which allows comparisons of mean values from different groups. Here we use treatment (Control, SLC) and time (0, 24), as well as their interaction, as fixed factors in the model. The experimental design was taken into account by including a random factor animal and a random factor sample within animal. Post-hoc tests were made using Tukey's adjustment. Statistical Analysis Software (SAS; version 9.4) was used to fit the model and compute post-hoc tests. Residuals plots were checked to verify that assumptions for this analysis were fulfilled.

Statistical significance was set at $P < 0.05$. Values shown are mean ± standard deviation (SD).

3. Results

3.1. Cooled storage

The initial evaluation of the samples is shown in Table 1. After incubating the samples in Tris buffer with gentle pipetting every 5 min, spermatozoa were seen to be moving freely.

Sperm quality evaluation in the samples after centrifugation and resuspension of the sperm pellet is shown in Table 2. Total motility was not different between the raw ejaculate and the control samples but was significantly greater in the SLC samples than the controls ($P < 0.001$ for

Table 1
Initial semen quality in raw alpaca ejaculates collected by artificial vagina.

Male and Sample	Time for collection (min)	Volume (mL)	Thread height (cm)	Initial motility (%)	Concentration (millions/mL)
Male 1, ejac 1	7	0.5	1.5	75	40.5
Male 2; ejac 1	10	1.5	3.0	75	148
Male 4, ejac 1	5	0.5	1.5	55	351
Male 3, ejac 1	10	3.5	1.0	52	47.5
Male 3, ejac 2	10	2.0	2.0	34	39.5
Male 2, ejac 2	10	1.0	3.0	50	59
Male 4, ejac 2	7	1.0	1.0	54	101
Male 3, 3jac 3	7	2.5	3.0	61	38.5
Male 2, ejac 3	5	1.5	4.5	52	146.5
Mean±SD	7.9±2.1	1.4±0.9	2.3±1.1	60±9	113.5±92.9

Note: Total motility was estimated subjectively in 10 fields per sample.

Table 2
Alpaca sperm quality (total motility, hypoosmotic swelling test, plasma membrane integrity and normal morphology) in controls and SLC samples before and after cooled storage for 24 h (n = 9).

Parameter	Time (h)	Control	SLC
Total motility	0	57±7 $^{a\ A}$	77±7 $^{a\ A}$
	24	34±15 $^{a\ A}$	51±16 $^{a\ A}$
HOST	0	39±12	44±10
	24	34±11	40±14
Membrane Integrity	0	54±8 $^{a\ B}$	63±13 $^{a\ B}$
	24	40±16 $^{a\ B}$	51±15 $^{a\ B}$
Normal morphology	0	62±4	64±17
	24	64±14	67±13

Notes: SLC = Single Layer Centrifugation. HOST = hypoosmotic swelling test. Time 0 h = immediately after centrifugation. Same superscript lower case letter within a parameter indicates statistical significance within treatment a P=0.0001. Same superscript upper case letters indicates statistical significance or a trend towards statistical significance between treatments A $P < 0.001$; B $P < 0.05$.

both). The residual (random) variance was by far the largest (147.6), while variation between samples within an animal (30) and between animals (~0) were much smaller. Motility decreased in both treatment groups during storage ($P< 0.001$). The treatment effect was independent of time. Plasma membrane integrity was higher in SLC samples than controls ($P < 0.05$) but decreased during cooled storage in all samples ($P < 0.01$). Again, the residual (random) variance was by far the largest (137.0), while variation between samples within an animal (43.4) and between animals (~0) were much smaller. The effect of treatment was independent of time.

Values for HOST did not differ between controls and SLC at either 0 h or 24 h.

Normal morphology did not differ between the two treatments and did not change during cooled storage. The morphological abnormalities observed (Table 3) were narrow heads, heads that were narrow at the base, pyriform heads, heads with an abnormal contour, and coiled tails (simple and double). The most frequently encountered sperm abnormalities were heads that were narrow at the base, pear-shaped heads, and coiled tails. There were fewer coiled tails in the SLC samples than in control (9.9±4.6 versus 5.4±3.6; p<0.05).

3.2. Cryopreservation

Motility, HOST and membrane integrity were significantly decreased in all samples ($P<0.001$) after cryopreservation compared to pre-freeze

Table 4
Alpaca sperm quality (total motility, hypoosmotic swelling test, plasma membrane integrity and normal morphology) in control and SLC samples before and after cryopreservation (n=9).

Parameter	Time point	Control	SLC
Total motility	Before freezing	57±7 $^{a\ A}$	77±12 $^{a\ A}$
	After thawing	12±6 $^{a\ A}$	17±17 $^{a\ A}$
HOST	Before freezing	39±12 ^{a}	44±10 ^{a}
	After thawing	19±6 ^{a}	20±5 ^{a}
Plasma membrane Integrity	Before freezing	54±8 $^{a\ B}$	63±13 $^{a\ B}$
	After thawing	16±5 $^{a\ B}$	20±7 $^{a\ B}$
Normal morphology	Before freezing	62±12	64±13
	After thawing	56±14 ^{C}	67±13 ^{C}

Notes: SLC = Single Layer Centrifugation. HOST = hypoosmotic swelling test. Same superscript lower case letter within a parameter indicates statistical significance within treatment: a P<0.0001. Same superscript upper case letter within a parameter indicates statistical significance between treatments: A $P < 0.01$; B $P < 0.05$; C $P = 0.05$.

Table 3
Breakdown of morphological defects per sample and treatment (0 h).

Sample	Treatment	Narrow head	Narrow at base	Pear-shaped head	Abnormal contour	Single bent tail	Coiled tail	Double bent tail
Male 1, ejac 1	Control	1	2	3	0	5.5	8	0
	SLC	1	9	2.5	0	2	10	0
Male 2, ejac 1	Control	2	16.5	5.5	3	1	6.5	1
	SLC	9	8.5	6	1	1	1.5	1
Male 4, ejac 1	Control	3	13	11	1	7.5	10.5	0
	SLC	3	9	10	0	10.5	10.5	1
Male 3, ejac 1	Control	2	12.5	11	4.5	5	5	1.5
	SLC	1	2	2	12	6.5	2.5	3
Male 3, ejac 2	Control	1	5	2	15	3	7	1
	SLC	2	6.5	3	16	4	2	2
Male 2, ejac 2	Control	2.5	3	3	12	4	7	3
	SLC	5	14	4	7	3	6.5	0
Male 4, ejac 2	Control	1	13.5	21.5	4	9	14.5	0
	SLC	0	16	24.5	4.5	6	9	0
Male 3, ejac 3	Control	1	16	0	2	4	4	1
	SLC	1	9.5	4	5	3.5	1	1
Male 2, ejac 3	Control	2	5.5	1	1	3	8	0
	SLC	1	5	1	4	3.5	0	0
Total	Control	2.8±2.5	8.9±5.4	7.8±9.7	3.8±2.2	3.7±2.2	9.9±4.6*	0.9±0.9
	SLC	2.5±2.3	8.7±4.7	5.7±7.0	5.2±3.8	2.4±2.0	5.4±3.6*	0.4±0.5

values (Table 4), although SLC values for motility and membrane integrity were higher in SLC samples than controls ($P<0.001$). There was no treatment and time interaction (Note: "time" here refers to pre-freeze versus post-thaw). Normal morphology was decreased in all samples after cryopreservation ($P<0.001$) but was higher in the SLC samples than in controls ($P<0.05$).

4. Discussion

The purpose of this study was to determine whether alpaca spermatozoa could be extracted from viscous seminal plasma using a similar method to the one used for dromedary camel spermatozoa, and whether these spermatozoa could be cooled for 24 h or cryopreserved. The results showed that spermatozoa could be liberated from the seminal plasma by incubation and gentle pipetting. Sperm motility, plasma membrane integrity and morphology were seen to be improved after SLC, particularly a decrease in coiled tails. Therefore, although not directly compared in this study, this method of extracting alpaca spermatozoa from seminal plasma appears to be advantageous compared to needling (Santiani et al., 2005) or enzyme extraction (Morton et al., 2012). The deleterious effect of needling might be due to an increased production of reactive oxygen species (ROS) from mechanical damage, or by an increased exposure of the spermatozoa to oxygen in the environment during the manipulation as previously indicated for stallion spermatozoa (Katila, 1997). We assume that gentle pipetting of the semen using a plastic pipette presents lower mechanical force than aspirating the semen through a needle, which would therefore be less damaging to the spermatozoa. The effect of enzyme extraction on sperm quality is the subject of considerable debate, as mentioned previously; Morton et al. (2012) reported sperm membrane and acrosome damage after treatment with some enzymes, whereas Stuart and Bathgate (2015) did not detect damage apart from poor cryosurvival.

The results of the present study are consistent with the study on dromedary camel semen (Malo et al., 2017) where improved motility and membrane functionality were seen after extraction of the spermatozoa by pipetting and selecting them by SLC with a high density colloid. Similar results were obtained with llama spermatozoa (Trasorras et al, 2012), although in the latter case, the semen was treated with enzymes to release the spermatozoa before selection with SLC. Morton & Maxwell (2018) also reported some success using a colloid formulation designed for human spermatozoa with alpaca semen. The difference between the studies with the dromedary and llama spermatozoa, compared to the two studies with alpaca semen, is that a low density colloid was used for the alpaca spermatozoa. The efficiency of sperm selection during colloid centrifugation is dependent on the density of the colloid (Morrell et al., 2011). Therefore, we can assume that using the low density colloid with alpaca semen provided minimal selection for robust spermatozoa. In the present study, a higher density colloid (1.104 g/mL) had been tested with alpaca semen initially but few spermatozoa were able to pass through the colloid. Therefore, we used the colloid diluted 1:1 (v/v), which would have resulted in a density of 1.052 g/mL, similar to the formulation used by Morton et al. (2008), and by Sabés-Alsina et al. (2020) for bull spermatozoa. There were fewer spermatozoa with coiled tails in the SLC samples compared to the controls, implying that it was difficult for the spermatozoa with coiled tails to pass through the low density colloid and therefore there were fewer of them in the sperm pellet..

The SLC samples showed better motility than control samples immediately after centrifugation and also after cooled storage for 24 h. Furthermore, membrane integrity was maintained in the SLC samples during cooled storage, whereas there was a deterioration in the control group during storage. Morton et al. (2008) reported a rapid loss of motility in cooled alpaca semen unless the extender contained egg yolk. Our results showed a loss of motility in control samples stored overnight, despite the inclusion of egg yolk. Therefore, maintenance of motility and membrane integrity in the SLC samples during cooled storage is encouraging. In contrast to our results, Morton et al (2008) reported lower motility after density gradient centrifugation with PureSperm® (a colloid for human spermatozoa) than in controls. Their results suggests that the colloid formulation used was not optimal for alpaca spermatozoa and emphasizes the need to take into consideration the physical characteristics of the semen when designing colloids for animal spermatozoa (Morrell et al., 2011).

Although in the present study SLC samples showed better motility after 24 h cooled storage than controls, it was disappointing that post-thaw motility was not different to controls. In fact, most of the improvements in sperm quality in fresh spermatozoa achieved by SLC disappeared during cryopreservation, in contrast to results with dromedary camel semen (Malo et al., 2018, 2019a). In non-camelid species, SLC had a beneficial effect on cryosurvival of stallion spermatozoa (Hoogewijs et al., 2011), boar spermatozoa (Martinez-Alborcia et al., 2012, 2103) and bull spermatozoa (Nongbua et al., 2017). In a study comparing the efficiency of selecting goat spermatozoa with SLC before or after freezing, it was concluded that SLC-selection should be performed after thawing for greatest efficiency (Jiménez-Rabadán et al., 2012). They concluded that if the selection was performed prior to freezing, some of the selected spermatozoa died during cryopreservation. Since damaged spermatozoa might release ROS, an unfavorable environment for survival of the remaining spermatozoa could be created. The results of the present study with alpaca semen suggest that the cryopreservation protocol for alpaca spermatozoa was not optimal, since the control spermatozoa showed poor survival, in agreement with previous attempts to freeze alpaca spermatozoa (Santiani et al., 2005, Morton et al., 2008; Adams et al., 2009; Partyka *et al.*, 2012). Even so, in the present study, the SLC samples showed higher motility and membrane integrity than controls.

The reason for the apparent low cryosurvival of spermatozoa from South American camelids compared to other camelid species is not known. Thus, although it was possible to produce offspring after AI or *in vitro* fertilization (IVF) with thawed dromedary camel spermatozoa, and embryos and live young were obtained after IVF with thawed llama spermatozoa, artificial insemination of thawed llama spermatozoa tends to result in a low success rate (Fumuso et al., 2019). A clue to this enigma may have been found in a recent study, where extensive chromatin fragmentation was observed in thawed llama sperm samples (Fumuso et al., 2019). Zampini et al (2020) reported considerable ultrastructural damage to llama spermatozoa as a consequence of freezing and thawing, with injury to the plasma and acrosomal membranes, disruptions to the mitochondria and the appearance of nuclear vacuoles. The damage appeared first during cooling prior to freezing but intensified during freezing. Since integrity of all of these structures is essential for sperm plasma membrane integrity and fertilizing capability, future efforts should be directed towards minimizing such damage induced by cryopreservation.

One cause of excessive DNA damage during cryopreservation is believed to be attack by ROS. Antioxidants are found in seminal plasma, but in the present study the spermatozoa were separated from seminal plasma, and therefore from potential antioxidative effects, before freezing. Adding seminal plasma in various concentrations before freezing had a beneficial effect on llama sperm motility but did not improve membrane integrity or prevent chromatin damage (Fumuso et al., 2019). Furthermore, the antioxidant tempol, added at 5mM to alpaca spermatozoa extracted from the vas deferens (and therefore without prior exposure to seminal plasma), was shown to have a beneficial effect on sperm motility and membrane integrity (Gómez-Quispe et al., 2016). Similarly, addition of catalase (an antioxidant) to dromedary camel spermatozoa after thawing improved sperm motility although there was no effect on fertility following artificial insemination (Malo et al., 2019b). These results suggest promising avenues of research to improve cryosurvival of alpaca sperm samples by adding various antioxidants.

The results of this study should be treated with caution because the

sample size is small and the subjective methods used to evaluate sperm quality are prone to operator error and bias due to the small number of spermatozoa evaluated. It would be interesting to use flow cytometry on these sperm samples (Santiani et al., 2016). Computer Assisted Sperm Analysis was not available but could permit greater objectivity in the evaluation of sperm motility than the subjective motility used here. The low volume of the ejaculate (mean 1.4 mL; range 0.5 to 3.5 mL) was a limitation to the number of evaluations that could be performed. However, these volumes were in accordance with previously reported values for alpaca semen e.g. 0.8 to 3.1 mL (Bravo et al., 2000b; Villanueva et al., 2018). Since evaluation of the concentration required 0.1 mL of sample, it was only possible to measure it once. If it had been possible to evaluate the sperm concentration after SLC, it would have been possible to calculate the recovery rate.

5. Conclusions

It was possible to extract alpaca spermatozoa from ejaculates by gentle pipetting in the presence of Tris buffer, followed by SLC using a low density colloid. The SLC samples showed greater motility and plasma membrane integrity than controls and maintained these characteristics better during cooled storage. Although sperm motility and membrane integrity were greater in SLC samples than controls after freezing and thawing, sperm quality was generally low. Therefore, although this method is promising for extracting alpaca spermatozoa and for maintaining sperm quality during cooled storage, more research is required to improve cryopreservation protocols.

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CRediT authorship contribution statement

Jane M Morrell: Conceptualization, Project administration, Methodology, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing. **Sofia Karlsson Warring:** Investigation, Data curation, Writing – original draft. **Emma Norrestam:** Investigation, Data curation, Writing – original draft. **Clara Malo:** Methodology, Writing – original draft. **Wilfredo Huanca:** Validation, Resources, Supervision, Project administration, Writing – original draft.

Declaration of Competing Interest

JMM is the inventor and one of the patent holders of the colloid formulation used in this study

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