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Comparison of two cooling protocols for llama semen: with and without collagenase and seminal plasma in the medium

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Summary

Seminal plasma (SP) of South American Camelids could interfere with the interaction of spermatozoa with the extenders; therefore it becomes necessary to improve semen management using enzymatic treatment. Our objective was to compare two cooling protocols for llama semen. Twelve ejaculates were incubated in 0.1% collagenase and then were divided into two aliquots. One was extended in lactose and egg yolk (LEY) (Protocol A: collagenase and SP present). The other aliquot was centrifuged, and the pellet was resuspended in LEY (Protocol B: collagenase and SP absent). Both samples were maintained at 5°C during 24 hr. Routine and DNA evaluations were carried out in raw and cooled semen. Both cooling protocols maintained sperm viability, membrane function and DNA fragmentation, with Protocol A showing a significantly lowered total and progressive motility ($p < .05$) and Protocol B showing a significant increase in chromatin decondensation ($p < .05$). Protocol A avoids centrifugation, reducing processing times and making application in the field simpler. However, as neither protocol showed a significant superiority over the other, studies should be carried out in vivo to evaluate the effect on pregnancy rates of the presence of collagenase and SP in semen samples prior to either cooling or freeze-thawing.

KEYWORDS

collagenase, cooling, *Lama glama* (llama), semen, seminal plasma

1 | INTRODUCTION

South American Camelid (SAC) semen preservation has not had the same success as that of bovine semen. Even when inseminating the same number of live motile spermatozoa, very low pregnancy rates are obtained (0%–26%) both in llamas and alpacas (Adams, Ratto, Collins, & Bergfelt, 2009; Aller, Rebuffi, Cancino, & Alberio, 2003; Bravo, Skidmore, & Zhao, 2000; Giuliano et al., 2012; Huanca, Cordero, Huanca, & Adams, 2007; Vaughan, Galloway, & Hopkins, 2003). As a consequence, there are no massive artificial insemination (AI) campaigns with preserved (cooled or frozen-thawed) semen. The particular characteristics of SAC ejaculates should be taken into account when trying to develop semen preservation protocols to produce acceptable pregnancy rates with AI (Apichela et al., 2014; Giuliano et al., 2010; Kershaw-Young, Stuart, Evans, & Maxwell, 2013). Two of the most important seminal plasma (SP) are its capacity for thread formation when pipetted and its high structural viscosity (Casaretto et al., 2012). In contrast to other species, semen liquefaction in SAC takes various hours (24–48 hr; Garnica, Achata, & Bravo, 1993) or directly does not occur (Apichela et al., 2006; authors' personal observations). With regard to semen manipulation, these rheological characteristics make it difficult to separate the spermatozoa from SP, dilute the ejaculate, homogenise samples and load straws (Carretero et al., 2014; Kershaw-Young et al., 2013; Tibary & Vaughan, 2006). Other seminal characteristics of SAC ejaculates to be taken into account are the low sperm concentration and low seminal volume, both of which limit the number of doses that can be obtained. In addition, SAC spermatozoa do not present progressive motility, only oscillatory motility in their

ejaculates (Carretero, Fumuso, Miragaya, Herrera, & Giuliano, 2015; Giuliano et al., 2010; Lichtenwalner, Woods, & Weber, 1996; Vaughan et al., 2003). Previous studies have demonstrated that thread formation can be avoided by incubating raw llama semen with collagenase diluted in HEPES-TALP-BSA, thus obtaining progressive motility while maintaining sperm viability and chromatin condensation (Carretero, Giuliano, Casaretto, Gambarotta, & Neild, 2012; Conde et al., 2008; Giuliano et al., 2010) and also obtaining the first pregnancy from embryos produced with in vitro fertilisation (Trasorras et al., 2014). Additionally, when working with semen for freeze-thawing, to maintain sample homogeneity and allow loading in the straws, it was necessary to incubate samples with 0.1% collagenase prior to diluting in the extenders (Carretero et al., 2014).

For all the above-mentioned reasons, it becomes necessary to enzymatically treat SAC semen samples prior to cooling because the presence of SP makes sample handling difficult and interferes with the sperm interaction with the extenders used. The objective of this study was to compare two cooling protocols for llama semen, with and without collagenase and SP in the medium.

2 | MATERIALS AND METHODS

2.1 | Reagents

All the components used, unless otherwise specified, were purchased from Sigma Chemicals, Argentina. Type I collagenase (Clostridium peptidase A from Clostridium histolyticum) was used. TALP medium (Parrish et al., 1986) was supplemented with 15 mmol/L HEPES (H-TALP).

The LEY extender contained lactose (11% in Milli-Q deionised water) and fresh egg yolk in the following proportions: 80 ml of 11% lactose with 20 ml of egg yolk. Lactose was purchased from Carlo Erba (Milan, Italy).

2.2 | Animals and location

The study was carried out at the Faculty of Veterinary Sciences of the University of Buenos Aires, in Buenos Aires, Argentina. The city is situated at sea level, latitude 34° 36′ and longitude 58° 26′.

For the study, six male *Lama glama* ranging between 6 and 10 years of age and weighing 133.97 ± 14.12 kg (mean ± *SD*) were used. Animals were kept out at pasture in pens and supplemented with bales of alfalfa; they also had free access to fresh water throughout the study. All males were shorn during the month of November.

2.3 | Semen collection

Semen collections were carried out between the months of April and October using electroejaculation (EE) under general anaesthesia according to the technique described by Director et al. (2007). The frequency of collection for each male was determined randomly. As EE requires general anaesthesia, this method was not used on the same male at an interval of <15 days. The Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires approved all procedures (protocol 2010/24).

2.4 | Cooling of llama semen

To compare two cooling protocols for llama semen, with and without collagenase and SP in the medium, 12 ejaculates were obtained from six male *Lama glama* (n = 6; r = 2). Raw semen was evaluated, and the rest of the ejaculate was immediately cooled according to Giuliano et al. (2012) with some modifications. Briefly, each ejaculate was diluted 4:1 in 0.1% collagenase in H-TALP medium and incubated 4 min at 37°C (four parts semen to one part collagenase, final concentration of collagenase 0.02%; Giuliano et al., 2010). After that, the sample was divided into two aliquots. One was extended at 37°C in LEY extender (one part semen in two parts extender) and cooled to 5°C (Protocol A: collagenase and SP present). The other aliquot was centrifuged at 800 g for 8 min, the supernatant (SP plus collagenase solution) was discarded, the pellet was resuspended in 2 ml of LEY and finally cooled to 5°C (Protocol B: collagenase and SP absent). For cooling, all samples were placed in a 37°C water bath which was then placed in a box refrigerator and the temperature descent was monitored, verifying that the temperature of 5°C was reached in 2.5 hr. The samples were then maintained at 5°C for 24 hr. After this period, the cooled semen (Protocols A and B) was warmed to 37°C to carry out routine seminal characteristics and DNA evaluations. Routine evaluations were carried out in raw and cooled semen samples (Protocols A and B). DNA evaluations were carried out in the following samples: (i) raw semen, (ii) raw semen incubated with collagenase, (iii) semen diluted with LEY in the presence of collagenase and SP (Diluted semen – Protocol A), (iv) semen diluted with LEY in the absence of collagenase and SP (Diluted semen – Protocol B), (v) cooled semen in the presence of collagenase and SP (Cooled semen – Protocol A) and (vi) cooled semen in the absence of collagenase and SP (Cooled semen – Protocol B).

2.5 | Routine seminal characteristics evaluation

The following semen characteristics were evaluated: ejaculate volume, sperm motility and concentration. Sperm motility (oscillatory, circular and progressive) was evaluated using a phase contrast microscope and a warm stage (37°C). Sperm count was carried out in duplicate, using a Neubauer hemocytometer (20 µl of semen in 1980 µl of water; dilution 1:100) averaging both chambers.

2.6 | Evaluation of sperm membrane function and integrity

The HOS test for evaluating membrane function and the stain using fluorochromes 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) for evaluating membrane integrity (viability)

were conducted according to Giuliano, Director, Gambarotta, Trasorras, & Miragaya (2008). For the HOS test, semen (50 µl) was incubated (37°C) 20 min in 200 µl of hypo-osmotic solution: fructose (2.45 mg/ml)–sodium citrate (4.5 mg/ml) in Milli-Q deionised water, adjusted to 50 mOsm. After incubation, a minimum of 200 spermatozoa were evaluated using a phase contrast microscope (400×). Spermatozoa showing the characteristic swelling of the tail were classified as HOS positive, having a functional plasma membrane. Osmolarity of the solutions was measured using an automatic cryoscopic osmometer (Osmomat® 030; Gonotec, Berlin, Germany).

For evaluating membrane integrity, samples of semen (50 µl) were incubated at 37°C for 10 min in 510 µl of staining medium. This medium contained 10 µl of a solution of CFDA (0.5 mg/ml in dimethylsulphoxide) and 500 µl of saline medium (described by Harrison & Vickers, 1990). After the first 10 min of incubation, 10 µl of a solution of PI (0.5 mg/ml in isotonic saline) were added and incubated for another 10 min at 37°C. A minimum of 200 spermatozoa were evaluated per sample using an epifluorescence microscope with a rhodamine and standard fluorescein filter set. Spermatozoa that fluoresced green throughout their length were classified as being viable (intact membrane) while sperm nuclei that fluoresced red were classified as nonviable (damaged membrane).

2.7 | Sperm DNA evaluation

2.7.1 | DNA fragmentation

The sperm chromatin dispersion (SCD) assay was carried out according to Carretero, et al. (2012) to evaluate the degree of DNA fragmentation. Briefly, each spermatozoon suspension was mixed with low-melting-point aqueous agarose and pipetted onto a glass slide. Each slide was incubated sequentially in mercaptoethanol/SDS and SDS lysing solutions, dehydrated in sequential ethanol baths and stained with 6% Giemsa in distilled water. Images of spermatozoa heads were captured by a Leica DC180 camera (Leica Microsystems Co., Wetzlar, Germany), obtaining 200 images per sample. Based on the description reported by Fernández et al. (2003), spermatozoa were classified into four patterns according to the size of the halo: (i) nuclei with large DNA dispersion halos (LH); (ii) nuclei with medium halos (MH); (iii) nuclei with small halos (SH) and (iv) nuclei with no halo (NH). The first two patterns (LH and MH) were considered spermatozoa without DNA fragmentation, and the other two patterns (SH and NH) spermatozoa with DNA fragmentation. A random semen sample incubated at 100°C during 30 min was used as a positive control for the SCD technique.

2.7.2 | DNA condensation

The Toluidine blue (TB) stain was carried out according Carretero, Giuliano, Casaretto, Gambarotta, & Neild (2009) to evaluate the degree of chromatin condensation. Briefly, each sample was smeared on clean, nongreasy slides and once dry, fixed with ethanol 96% and stained with a working solution of 0.02% TB. Preparations were observed directly under immersion oil (1,000×) evaluating a minimum of 200 spermatozoa per smear. Spermatozoa were classified into three groups according to the degree of chromatin condensation: light blue (negative, no chromatin decondensation), light violet (intermediate, some degree of decondensation) and dark blue-violet (positive, high degree of decondensation). A random semen sample incubated with dithiothreitol (DTT) 1% in distilled water was used as a positive control for the TB stain.

2.8 | Statistical analysis

For routine seminal characteristics, an analysis of variance (factorial design) was used to compare raw semen with cooled semen (Protocols A and B) taking the male as a blocking factor. For DNA evaluation, two factorial designs were used. The first factorial design was made to compare raw semen; raw semen incubated with collagenase and diluted semen (Protocols A and B). The second design was made to compare diluted semen (Protocols A and B) with cooled semen (Protocols A and B). Therefore, the second design has two factors with two levels each (first factor with levels: diluted and cooled; second factor with levels: presence or absence of collagenase and SP) taking the male as a blocking factor. Normal distribution of the variables was tested in all cases using the Shapiro – Wilk test. The logarithmic and the square root transformation were appropriate for sperm motility and for DNA evaluation (SCD and TB). All statistical analysis was conducted using the R 2.2.1. Program (2005).

3 | RESULTS

3.1 | Routine seminal characteristics

Semen volume was 4.3 ± 2.2 ml and sperm concentration was $35.3 \pm 32.0 \times 10^6$ sperm/ml (mean ± *SD*), with a range of 17.0–127.0 sperm/ml.

Results showed that oscillatory motility was greater ($p < .05$) in raw semen than in cooled semen (both Protocols A and B). Progressive motility in raw semen improved after collagenase treatment. However, values for total and progressive motility were reduced ($p < .05$) only after storage at 5°C in samples with Protocol A, remaining unchanged in Protocol B. Results for the evaluation of the seminal parameters can be found in Table 1.

3.2 | Sperm membrane function and integrity

Percentages of spermatozoa with functional membranes increased in semen cooled with Protocol A with respect to raw semen and semen cooled with Protocol B ($p < .05$) while viability was not affected by the cooling process in either protocol ($p > .05$; Table 1).

TABLE 1 Percentages of motility (total, oscillatory, progressive and circular), membrane function and viability in raw llama semen, raw semen incubated with collagenase, semen diluted with lactose and egg yolk (LEY) in the presence of collagenase and seminal plasma (SP Protocol A), semen diluted with LEY in the absence of collagenase and SP (Protocol B), semen cooled in the presence of collagenase and SP (Protocol A) and semen cooled in the absence of collagenase and SP (Protocol B)

	Motility					
	Total	Oscillatory	Progressive	Circular	Membrane function	Membrane integrity (viability)
Raw semen	30.8 ± 18.8[a]	30.8 ± 18.8[a]	0.0 ± 0.0	0.0 ± 0.0	35.3 ± 11.0[a]	54.2 ± 17.5[a]
Collagenase	45.5 ± 22.1[a]	15.5 ± 18.9[a,b]	30.0 ± 27.4[a]	0.0 ± 0.0	–	–
Diluted semen – Protocol A	46.8 ± 16.7[a]	11.8 ± 7.8[a,b]	35.0 ± 18.5[a]	0.0 ± 0.0	–	–
Diluted semen – Protocol B	44.0 ± 20.3[a]	9.1 ± 7.0[b]	34.6 ± 20.9[a]	0.3 ± 0.0[a]	–	–
Cooled semen – Protocol A	12.8 ± 13.5[b]	6.2 ± 7.5[b]	3.4 ± 4.9[b]	3.2 ± 4.5[a]	51.6 ± 12.3[b]	55.1 ± 10.6[a]
Cooled semen – Protocol B	38.4 ± 21.0[a]	9.1 ± 6.3[b]	19.6 ± 16.3[a,b]	9.7 ± 9.5[a]	36.3 ± 13.5[a]	59.8 ± 14.6[a]

Different letters between column indicate significant differences ($p \leq .05$).
The values of motility that were 0 were not included in the statistical analysis. The values are expressed as mean ± *SD* ($n = 6$, r = 2).

TABLE 2 Percentages of sperm chromatin dispersion (SCD) patterns (large, medium, small and no halos) observed in llama spermatozoa from raw semen, raw semen incubated with collagenase, diluted semen in the presence of collagenase and seminal plasma (SP Protocol A) and diluted semen in the absence of collagenase and SP (Protocol B). Large and medium halos (LH + MH) correspond to spermatozoa with undamaged DNA, and small or no halos (SH + NH) correspond to spermatozoa with fragmented DNA

SCD patterns	Raw semen (%)	Collagenase (%)	Diluted semen – Protocol A (%)	Diluted semen – Protocol B (%)
LH	2.4 ± 3.8[a]	1.5 ± 1.5[a]	2.2 ± 3.0[a]	2.6 ± 3.9[a]
MH	81.1 ± 7.3[a]	77.6 ± 5.4[a]	79.1 ± 6.6[a]	77.4 ± 6.3[a]
LH + MH	83.5 ± 8.3[a]	79.1 ± 6.0[a]	81.3 ± 7.6[a]	80.0 ± 6.3[a]
SH	14.9 ± 7.7[a]	19.6 ± 6.6[a]	17.7 ± 7.3[a]	18.7 ± 6.6[a]
NH	1.6 ± 1.2[a]	1.3 ± 0.7[a]	1.0 ± 1.0[a]	1.3 ± 1.2[a]
SH + NH	16.5 ± 8.3[a]	20.9 ± 6.0[a]	18.7 ± 6.7[a]	20.0 ± 6.7[a]

Different letters between columns indicate significant differences ($p \leq .05$).
The values are expressed as mean ± *SD* ($n = 6$, r = 2)

3.3 | Evaluation of sperm DNA fragmentation

No significant differences ($p > .05$) were observed in each of the patterns of SCD (large, medium, small and no halos) and in the total fragmentation (small halos + no halos) between raw semen, raw semen incubated with collagenase and diluted semen (Protocols A and B; Table 2).

No significant differences ($p > .05$) were observed in each of the patterns of SCD and in the total fragmentation between diluted semen (Protocols A and B) and cooled semen (Protocols A and B). See Fig. 1 for Protocol A (diluted and cooled semen in the presence of collagenase and SP) and Fig. 2 for Protocol B (diluted and cooled semen in the absence of collagenase and SP).

3.4 | Evaluation of sperm DNA condensation

No significant differences ($p > .05$) were observed in each of the patterns of TB (negative, intermediate and positive) and in the total DNA decondensation (intermediate + positive) between raw semen, raw semen incubated with collagenase and diluted semen (in both protocols; Table 3).

In all cooled semen samples, the percentages of TB-positive llama spermatozoa increased with respect to all samples prior to the process and were higher in semen cooled with Protocol B with respect to semen cooled with Protocol A ($p < .05$). The percentages of TB intermediate and total DNA decondensation increased in semen cooled in Protocol B with respect to diluted semen ($p < .05$), but this difference was not observed with semen cooled in Protocol A ($p > .05$; Table 3).

4 | DISCUSSION

Owing to the particular characteristics of SAC semen, various authors have suggested that it is necessary to enzymatically treat samples to decrease viscosity and thread formation, both of which interfere with the interaction of spermatozoa with semen extenders and hinder sample homogeneity (Bravo, Callo, & Garnica, 2000; Giuliano et al., 2010; Kershaw-Young & Maxwell, 2012; Tibary & Vaughan, 2006). Therefore, in this study, the effect of the presence of 0.1% collagenase and of SP in the cooling extender for llama spermatozoa was evaluated.

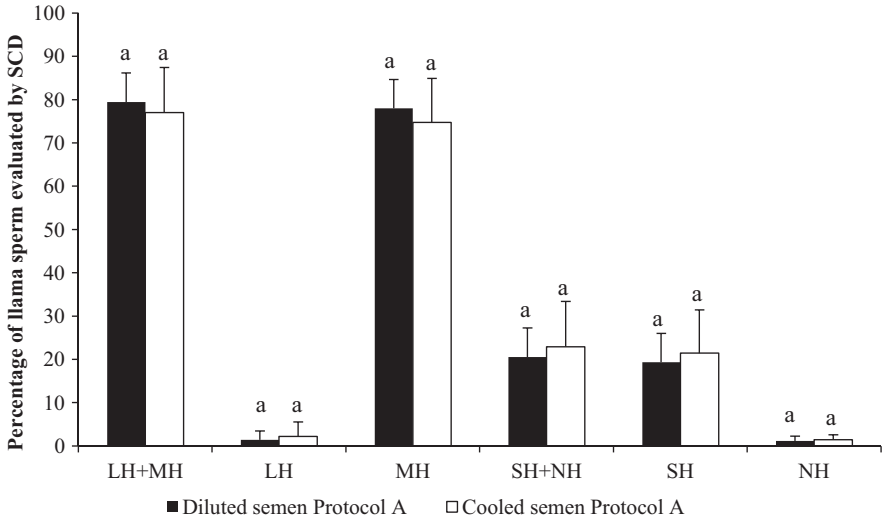

FIGURE 1 Percentages of sperm chromatin dispersion (SCD) patterns (large, medium, small and no halos) observed in llama spermatozoa from diluted semen and samples cooled with Protocol A (presence of collagenase and seminal plasma). The total DNA integrity (LH + MH) and total DNA fragmentation (SH + NH) are shown (n = 6, r = 2). LH + MH: large halos + medium halos, LH: large halos, MH: medium halos, SH + NH: small halos + no halos, SH: small halos, NH: no halos. Different letters (a, b) indicate significant differences within each SCD patterns ($p \leq .05$)

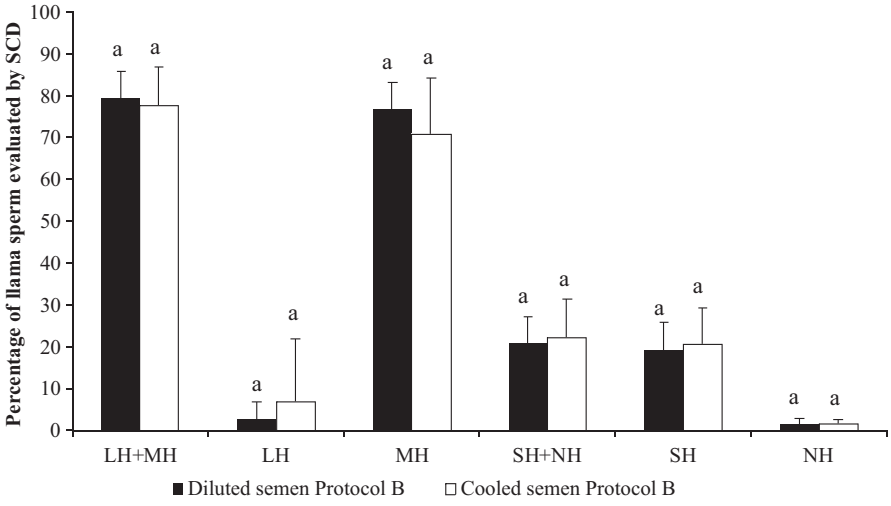

FIGURE 2 Percentages of sperm chromatin dispersion (SCD) patterns (large, medium, small and no halos) observed in llama spermatozoa from diluted semen and samples cooled with Protocol B (absence of collagenase and seminal plasma). The total DNA integrity (LH + MH) and total DNA fragmentation (SH + NH) are shown (n = 6, r = 2). LH + MH: large halos + medium halos, LH: large halos, MH: medium halos, SH + NH: small halos + no halos, SH: small halos, NH: no halos. Different letters (a, b) indicate significant differences within each SCD patterns ($p \leq .05$)

TABLE 3 Percentages of Toluidine blue (TB) patterns (positive, intermediate, positive + intermediate and negative) observed in llama spermatozoa from raw semen, raw semen incubated with collagenase, diluted semen (Protocols A and B) and cooled semen (Protocols A and B)

TB stain patterns	Raw semen	Collagenase	Diluted semen – Protocol A	Diluted semen – Protocol B	Cooled semen – Protocol A	Cooled semen – Protocol B
Positive (%)	2.4 ± 2.0^{a}	2.1 ± 1.4^{a}	2.5 ± 1.3^{a}	2.9 ± 1.5^{a}	4.3 ± 1.8^{b}	6.2 ± 2.4^{c}
Intermediate (%)	22.8 ± 6.4^{a}	23.5 ± 5.2^{a}	24.0 ± 5.0^{a}	23.6 ± 6.9^{a}	28.0 ± 4.3^{a,b}	31.5 ± 5.5^{b}
Positive + intermediate (%)	25.2 ± 7.7^{a}	25.6 ± 5.9^{a}	26.5 ± 4.6^{a}	26.5 ± 7.1^{a}	32.3 ± 5.1^{a,b}	37.7 ± 6.7^{b}
Negative (%)	74.8 ± 7.7^{a}	74.4 ± 5.9^{a}	73.5 ± 4.6^{a}	73.5 ± 7.4^{a}	67.7 ± 5.1^{a,b}	62.3 ± 6.7^{b}

Different letters between columns indicate significant differences for each TB pattern ($p \leq .05$).
The values are expressed as mean ± *SD* (n = 6, r = 2).
Diluted semen – Protocol A: raw semen diluted with LEY in the presence of collagenase and seminal plasma.
Diluted semen – Protocol B: raw semen diluted with lactose and egg yolk (LEY) in the absence of collagenase and seminal plasma.
Cooled semen – Protocol A: cooled semen in the presence of collagenase and seminal plasma.
Cooled semen – Protocol B: cooled semen in the absence of collagenase and seminal plasma.

The results for all raw semen parameters were within the normal range reported for raw ejaculates in this species (Aller et al., 2003; Apichela et al., 2014; Bravo, Skidmore, Zhao, 2000; Carretero et al., 2014; Casaretto et al., 2012; Giuliano et al., 2010). In the present study, the percentage of progressive motility increased from a mean of 0.0% in raw semen to 30%–35% in

samples incubated with collagenase and diluted samples (protocols A and B prior to cooling) respectively. In addition, the percentage of oscillatory motility greatly decreased from a mean of 30.8% in raw semen to 9%–15% in the samples incubated with collagenase and those diluted in the protocols prior to cooling. These results were similar to those reported by Giuliano et al. (2010) who observed an increase in progressive motility from 4.4% in raw semen to 45% in samples treated with collagenase and to those published by Trasorras et al. (2012) showing an analogous increase from 0% to 37%. Both in our study and in those mentioned previously, SP lost its capacity for thread formation after enzymatic treatment; thus, the increase in progressive motility could be attributed to an indirect effect of collagenase that of modifying semen rheological characteristics. A mechanical impediment to the progression of spermatozoa has been attributed to SP as SAC-ejaculated spermatozoa only present oscillatory movement (Bravo, Callo, et al., 2000; Brown, 2000; Lichtenwalner et al., 1996; von Baer & Helleman, 1998). Nevertheless, recent studies have suggested that the lack of progressive motility is not only due to a mechanical action but to a more complex effect, because the simple dilution of SP does not induce an increase in progressive motility (Carretero et al., 2014; Giuliano et al., 2010, 2012).

In samples cooled using Protocol B, sperm progressive motility was higher when compared to raw semen (19.6% versus 0.0%) and to samples cooled with Protocol A (19.6% versus 3.4%). Interestingly, we observed that viable spermatozoa and spermatozoa with functional membranes did not significantly decrease using either of the cooling protocols, indicating that although spermatozoa cooled using Protocol A had lower progressive motility, most were viable and had functional membranes. These results would seem to indicate that the absence of SP in cooled samples (Protocol B) could maintain progressive motility in llama spermatozoa without seeming to have a significant effect on viability and membrane function. This apparent effect would be supported by the results published by Carretero et al. (2015), who reported the effects of raw SP on sperm movement patterns (oscillatory, progressive and hyperactive). They determined that progressive sperm motility was observed after collagenase treatment, similar to what was observed in this study, but disappeared immediately after the addition of SP, changing to only oscillatory movements. In contrast, samples without SP incubated in H-TALP maintained progressive motility and became hyperactive. Also, similar to our study, sperm viability was not different between samples incubated with H-TALP and those incubated with SP ($p > .05$). This effect of SP on sperm progressive motility is similar to that observed in vivo, as SAC epididymal spermatozoa show progressive motility while ejaculated ones do not; highlighting the importance of the effect SP has on progressive motility in these species. In addition, it is yet unknown whether it is beneficial for SAC spermatozoa to acquire progressive motility in a cooled or frozen-thawed protocol, because as seen in raw semen samples, low or inexistent progressive motility is inherent to these species (Giuliano et al., 2008) and does not prevent females from becoming pregnant.

With regard to membrane function, it was interesting to note that semen cooled with Protocol A (presence of collagenase and SP) showed a significantly higher percentage of spermatozoa with functional membranes than raw semen and semen samples cooled with Protocol B (absence of collagenase and SP). Similarly, Giuliano et al. (2010) observed a greater percentage of spermatozoa with functional membranes in semen samples incubated with collagenase when compared to the raw ejaculates. Although collagenase has been shown to decrease SP thread formation (Bravo, Callo, et al., 2000; Carretero et al., 2015; Giuliano et al., 2010), the mechanism by which it does so is still unknown. It has been reported in SAC that mucin 5B is probably the protein responsible for the thread formation and viscosity of SP (Kershaw-Young & Maxwell, 2012). Hence, it is possible that one of the mechanisms of decreasing thread formation could be by modifying SP proteins and conceivably this modification could produce the positive effect on the function of sperm membranes observed in both these studies.

In this study, both methods of cooling (in the presence or absence of collagenase and SP) did not alter the degree of DNA fragmentation compared to raw and diluted semen. However, chromatin decondensation was higher in cooled samples with respect to raw and diluted semen samples and was even higher in cooled semen preserved in Protocol B (absence of collagenase and SP) when compared to semen cooled in Protocol A (presence of collagenase and SP). The higher percentage of spermatozoa with chromatin decondensation in samples cooled with the Protocol B could be due to a greater oxidative stress in these samples. Henkel et al. (2010) reported a positive association between oxidative stress and alterations of chromatin condensation in human spermatozoa. Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant capacity of SP and is reported to be involved in the damage to DNA (Sharma, Said, & Agarwal, 2004). Spermatozoa would be particularly susceptible to the damage induced by ROS because their plasma membrane has large quantities of unsaturated fatty acids and their cytoplasm has low concentrations of antioxidant enzymes (Agarwal & Allamaneni, 2004); hence, the enzymes in SP would be the ones that protect spermatozoa. It is possible that the absence of SP in the samples cooled using Protocol B generates greater oxidative damage and as a result a higher percentage of spermatozoa with chromatin decondensation. Furthermore, as no significant differences in chromatin decondensation were observed between either diluted semen samples (Protocols A and B), these results could not be attributed to the centrifugation applied in Protocol B; rather, the greater chromatin decondensation observed in these samples should be attributed to the absence of collagenase and SP in the media.

Despite the variability in total number of spermatozoa in the ejaculates used (range: $51.0–141.6 \times 10^6$ spermatozoa), we verified that the presence of collagenase in the cooled samples did not have a deleterious effect on sperm viability, membrane function and DNA (either condensation or fragmentation). Therefore, 0.1% collagenase in H-TALP can be added to raw semen to aid its manipulation prior

to implementing protocols of semen preservation (either cooled or frozen-thawed).

5 | CONCLUSIONS

Both cooling protocols adequately maintained sperm viability, membrane function and DNA fragmentation, with Protocol A showing a lowered total and progressive motility and Protocol B showing an increase in chromatin decondensation. Protocol A avoids centrifugation, reducing processing times and making application in the field simpler. However, as neither protocol showed a significant superiority over the other, studies should be carried out in vivo to evaluate the effect on pregnancy rates of the presence of collagenase and SP in semen samples prior to either cooling or freeze-thawing.

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