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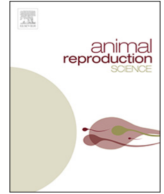

Seminal plasma affects the survival rate and motility pattern of raw llama spermatozoa

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ABSTRACT

The objectives of this study were to evaluate the effect over time of different percentages of seminal plasma (SP) on llama sperm characteristics in raw semen and correlate the techniques routinely used to evaluate sperm viability and acrosome status with the Fluorescein Isothiocyanate –*Arachis hypogea* agglutinin/Propidium Iodide (FITC-PNA/PI). Eighteen ejaculates, obtained from 6 male llamas using electroejaculation, were incubated in 0.1% collagenase in HEPES-TALP (HT), centrifuged and resuspended with SP and HT: 0, 10, 50 and 100% SP. Samples were incubated (37 °C) until evaluation at 0; 1.5 and 3 h. Split plot and factorial designs were used to analyze sperm motility, viability, membrane function and acrosome status and Spearman's test was used for correlation. At 0 h, samples with 100% SP showed oscillatory motility; whereas in samples with 0 and 10% SP, progressive motility was predominant. Viability, membrane function and total motility decreased significantly at 3 h of incubation in samples with 100% SP. Sperm with intact acrosomes were fewer in 0% SP media at all times. FITC-PNA/PI correlated with 6-Carboxyfluorescein Diacetate and Propidium Iodide (CFDA/PI) and with Coomassie Blue (CB) stains ($r = 0.8$; $p = 0.0$ and $r = 0.5$; $p = 0.0$ respectively). Conclusions: the motility pattern of llama sperm is influenced by the concentration of SP. The use of SP as the only medium is not able to maintain sperm motility, viability and membrane function for 3 h. A certain percentage of SP is necessary in the medium to avoid spontaneous acrosome reactions. The correlations observed could help to shorten evaluation times and reduce costs in sperm laboratories.

1. Introduction

The effect of seminal plasma on sperm physiology has been studied in various species, both in raw and cryopreserved semen, evidencing beneficial and adverse results according to the species (bovine: Graham, 1994; ovine: Graham, 1994; Maxwell et al., 1999; Domínguez et al., 2008; Leahy et al., 2010; equine: Aurich et al., 1996; Moore et al., 2005; Morrell et al., 2010; alpaca: Kershaw-

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Young and Maxwell, 2011 and llama: Carretero et al., 2015a, 2017).

South American Camelid (SAC) SP possesses very particular rheological characteristics. Among these characteristics one should mention the high structural viscosity (Casaretto et al., 2012) and the capacity to form a thread, which diminishes after incubation with proteolytic enzymes (Bravo et al., 2000; Giuliano et al., 2010; Kershaw-Young and Maxwell, 2011; Kershaw-Young et al., 2013, 2016). Another unusual characteristic of the semen from these species is that, only oscillatory motility is observed. Enzymatic treatment of the samples not only reduces thread formation, it also allows sperm to acquire progressive motility (Giuliano et al., 2010). In a study carried out on raw semen by Carretero et al. (2015a), it was shown that SP is involved in the pattern of motility exhibited by spermatozoa. These authors observed that when the pellet of sperm was resuspended with SP, after they had acquired progressive motility due to enzymatic incubation, the cells returned to an oscillatory pattern of movement; whereas if a different medium was used to resuspend them, sperm motility remained mainly progressive. A mechanical impediment to the progression of sperm has been attributed to SP as SAC ejaculated sperm only present oscillatory movement (Lichtenwalner et al., 1996; Von Baer and Helleman, 1998; Brown, 2000). Nevertheless, recent studies have suggested that the lack of progressive motility in SAC raw semen is not only due to a mechanical effect of SP but would seem to be more complex, because the simple dilution of SP does not induce progressive motility (Giuliano et al., 2010, 2012a; Carretero et al., 2015b). Other studies carried out in alpacas (Kershaw-Young and Maxwell, 2011) determined that incubation of raw semen with a final concentration of 10% SP was the most adequate for preserving sperm motility, viability and acrosomes over time when compared to concentrations of 25, 50 and 100% SP. However, in this study no distinction was made between oscillatory and progressive motility. These effects of SP on SAC spermatozoa could be showing that dilution or enzymatic treatment might be important factors to be taken into account. It would be interesting to study the effects other concentrations of SP have on llama spermatozoa. These studies would increase current knowledge regarding sperm physiology and the interaction between spermatozoa and SP.

Validated semen evaluation techniques which are practical and reliable are now available. Sperm motility, viability and acrosome status are some of the characteristics that are currently evaluated in SACs and are considered to reflect sperm quality. In our laboratory, CFDA together with PI is the fluorescent stain routinely used to evaluate sperm viability and the CB stain for light microscopy is used to determine the presence or absence of the acrosome in llama spermatozoa (Fumuso et al., 2014; Carretero et al., 2015c). In recent years, new techniques to simultaneously evaluate more than one sperm parameter have begun to become popular and to replace the more simple methods used before. The fluorescent stain with FITC-PNA/PI jointly evaluates sperm viability and acrosome status, permitting the evaluation times to be shortened as more than one characteristic is evaluated simultaneously. This stain has been used to evaluate llama sperm and has correlated positively with the simpler CB stain (Carretero et al., 2015c); however, it has not yet been validated with other techniques used routinely for the evaluation of llama sperm viability.

The objectives of this study were: 1) evaluate the effect over time of different percentages of seminal plasma on llama sperm characteristics in raw semen and 2) correlate the techniques routinely used to evaluate llama sperm viability and acrosome status (CFDA/PI and CB) with the FITC-PNA/PI fluorescent stain.

2. Materials and methods

2.1. Reagents

All the components used 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 mM HEPES-TALP (HT).

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, 6 male *Lama glama* ranging between 8 and 10 years of age and weighing 136.5 ± 14.1 kg (mean ± SD) were used. The same males were used to obtain SP for the assay according to the experimental design. 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 anesthesia according to the technique described by Director et al. (2007). As EE requires general anesthesia, this method was not used on the same male at an interval of less than 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 2015/27).

2.4. Seminal plasma

To obtain SP, a total of 12 ejaculates were collected from 4 male llamas (n = 4; r = 3) using electroejaculation under general anesthesia (Director et al., 2007). Prior to separating SP, each ejaculate was evaluated for thread formation, sperm motility, acrosome status and membrane integrity and function. To separate SP, ejaculates were subjected to successive centrifugations at 12,000 g for

30 min each, until the absence of sperm in the supernatant was confirmed using a phase contrast microscope (400 ×). Seminal plasma from the different males was pooled and kept at −20 °C until use.

2.5. Experimental design

A total of 18 ejaculates were obtained from 6 male llamas (n = 6; r = 3). The volume of the ejaculates was measured with a micropipette, sperm motility was analyzed in a phase contrast microscope (100 ×) with a warm stage and thread formation was evaluated with a pipette. Afterwards, each ejaculate was incubated in a 0.1% collagenase solution in HT for 8 min at 37 °C (Giuliano et al., 2010), with the objective of reducing thread formation and aid manipulation of the samples for the separation of SP. Then, the samples were subdivided into four aliquots and were centrifuged for 10 min at 800 g. The pellets obtained were resuspended in four treatments of different percentages of SP in HT: 1) 0% SP (100% HT, control); 2) 10% SP (90% HT, 10% SP); 3) 50% SP (50% HT, 50% SP) and 4) 100% SP (SP without HT, control). The samples were maintained at 37 °C until evaluation at time 0; 1.5 and 3 h of incubation. At each evaluation time the following sperm parameters were assessed: motility, viability, acrosome status and membrane function.

2.6. Seminal characteristics evaluation

The following seminal characteristics were evaluated in the different treatments of the experimental design (0, 10, 50 and 100% SP): sperm motility, membrane function, membrane integrity and acrosome status.

Sperm motility was evaluated using a phase contrast microscope (100 ×) and a warm stage (37 °C). Every evaluation was carried out by two independent observers and an average was calculated for each sample and treatment. The patterns observed were: oscillatory motility (OM) and progressive motility (PM). In addition, total sperm motility (TM = OM + PM) was determined.

Thread formation was evaluated indirectly according to the capacity of the samples to form a thread when 20 μl was pipetted onto a slide using a micropipette. This parameter was classified as either present or absent.

The hypoosmotic swelling (HOS) test was used for assessing sperm membrane function, and the CFDA/PI stain was used for assessing membrane integrity (viability). These techniques were carried out according to Giuliano et al. (2008). For the HOS test, semen (50 μl) was incubated at 37 °C for 20 min in 200 μl of hypoosmotic solution: fructose (2.45 mg ml-1) – sodium citrate (4.5 mg ml-1) in Mili Q deionized water, adjusted to 50 mOsm. After incubation, a minimum of 200 spermatozoa were evaluated using a phase contrast microscope (400 ×). Sperm 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 sperm membrane integrity, 50 μl of semen 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-1 in dimethylsulphoxide) and 500 μl of saline medium (described by Harrison and Vickers (1990)). After the first 10 min of incubation, 10 μl of a solution of PI (0.5 mg ml-1 in isotonic saline) were added and the samples were 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 (400 ×). Spermatozoa that fluoresced green throughout their length were classified as being viable (intact membrane) while sperm nuclei that fluoresced red were classified as non-viable (damaged membrane).

Acrosome status was evaluated by using two different techniques: CB stain and FITC-PNA/PI stain (the latter which simultaneously evaluates viability). The presence or absence of the acrosomal cap can be established with the CB stain. This technique was carried out according to Giuliano et al. (2012b). Briefly, smears were made with the different samples, air dried and fixed with 4% paraformaldehyde in PBS for 15 min. The fixed smears were then washed with PBS, air dried once again and stained with 0.22% CB for 5 min. After rinsing with distilled water, evaluation was carried out using bright field microscopy (1000 ×). Sperm were classified into two categories according to the color of the acrosomal region: i) violet/blue acrosome cap (acrosome present) and ii) lack of color in the acrosomal region (acrosome absent).

The FITC-PNA is a lectin that selectively binds the outer acrosomal membrane; the staining technique that combines this lectin with PI for viability, was carried out according to Carretero et al. (2015c). Briefly, samples of semen (50 μl) were incubated at 37 °C for 10 min in 510 μl of staining medium. This medium contained 2 μl of a solution of FITC-PNA (2 mg ml-1 in PBS) and 500 μl of saline medium (NaCl 140 mM ml-1, glucose 10 mM ml-1, ClK 2.5 mM ml-1, polyvinyl-pyrrolidone 0.5 mM ml-1 and HEPES 20 mM ml-1). After the first 10 min of incubation, 2 μl of a solution of PI (0.5 mg ml-1 in isotonic saline) were added and incubated for another 10 min at 37 °C. Immediately after the second incubation, 5 μl of stained sperm was placed onto a glass slide and covered with a coverslip and was evaluated using an image analyzer (Leica QwinV4) with bright field and epifluorescence microscopy (Leica® Microsystems Co., Wetzlar, Germany). Sperm were classified into 6 categories: i) live spermatozoa with intact acrosomes (no fluorescence in either head or acrosome cap); ii) live, acrosome reacting spermatozoa (no fluorescence in the head and intense green fluorescence in the acrosome cap); iii) live, acrosome reacted spermatozoa (no fluorescence in the head and spotted or half-moon green fluorescence in the acrosome cap or green fluorescent equatorial segment); iv) dead spermatozoa with intact acrosome (red fluorescent head with no fluorescence in the acrosome cap); v) dead, acrosome reacting spermatozoa (red fluorescent head with intense green fluorescence in the acrosome cap) and vi) dead, acrosome reacted spermatozoa (red fluorescent head with spotted or half-moon green fluorescence in the acrosome cap or green fluorescent equatorial segment). To aid statistical analysis, the categories of acrosome reacting and acrosome reacted sperm (either viable or dead) were grouped together: live AR sperm (live acrosome reacting spermatozoa + live, acrosome reacted) and dead AR sperm (dead, acrosome reacting spermatozoa + dead, acrosome

reacted).

2.7. Statistical analysis

Statistical analysis was carried out using the R 2.2.1 program. In all cases normality of the data was corroborated using the Kolmogorov test. The level of significance was set at 0.05 for all analysis. To analyze sperm motility, sperm viability, membrane function and acrosome status, split plot and factorial designs were used, blocking by the males. Two factors were used in the designs: i) time of incubation, with three levels (0, 1.5 and 3 h) and ii) treatments, with four levels (0, 10, 50 and 100% SP). A Spearman's test was used to evaluate correlation between the two viability assays: FITC-PNA/PI and CFDA/PI and to evaluate the correlation between the assays for evaluating acrosome status: FITC-PNA/PI and CB stain.

3. Results

The seminal characteristics of the ejaculates that were used to separate SP were normal for the species (mean ± SD): volume 3.9 ± 2.7 ml; oscillatory movement: 26.1 ± 16.5%; concentration: $98 \pm 75 \times 10^6$ spermatozoa/ml; membrane function: 34.0 ± 10.5%; membrane integrity (viability): 62.1 ± 11.6% and acrosome integrity (CB): 96.8 ± 3.6%. All ejaculates evidenced thread formation.

Mean values of the ejaculates used in the experiment were: volume 4.3 ± 2.2 ml and sperm with oscillatory movement 30.3 ± 16.4% (mean ± SD). None of the ejaculates presented progressive motility and all ejaculates presented thread formation prior to incubation with collagenase.

3.1. Sperm motility

At time 0 h, samples that were incubated with 100% SP showed statistically similar values of oscillatory motility to those of raw semen (26.7 ± 12.8 *vs.* 30.3 ± 16.4% respectively; $p > 0.05$) and were higher than the other treatments. Although not statistically different, samples with 50% SP showed half the OM of those with 100% SP (15.3 ± 13.4%) and maintained this motility throughout the incubation time, whereas in the samples with 100% SP, OM decreased significantly at 3 h (7.7 ± 7.3%; $p < 0.05$) (Table 1).

Progressive motility was almost inexistent in samples with 100% SP (only 1–3 %), being significantly lower than the samples incubated with 0 and 10% SP at time 0 h (16.3 ± 11.7 and 19.7 ± 11.0% respectively; $p < 0.05$) (Table 1).

With regard to total motility, no significant changes were observed over time in samples with 0, 10 and 50% SP ($p > 0.05$) whereas samples with 100% SP showed a significant decrease of TM at 3 h of incubation when compared to time 0 h (Table 1).

3.2. Thread formation

None of the samples treated with 0 and 10% SP presented thread formation when this parameter was assayed at each of the incubation times. In samples treated with 50% SP, only 7/18 (around 40%) of them presented thread formation at 0 h and, at 3 h this characteristic was absent in all samples. In samples treated with 100% SP, thread formation was present in all of them at 0 h and remained present at 3 h in 11/18 (around 60%).

3.3. Sperm viability (CFDA/PI) and membrane function (HOS test)

Samples incubated with 0, 10 and 50% SP, conserved both parameters (viability and membrane function) throughout the 3 h of

Table 1
Percentages of llama sperm with oscillatory movement (OM) and progressive motility (PM) and total motility (TM) in raw semen samples treated with collagenase and incubated at 37 °C for 0, 1.5 and 3 h with different concentrations of seminal plasma (SP): 0, 10, 50 and 100%. Values are mean ± standard deviations.

Time (h)	SP (%)	OM (%)	PM (%)	TM (%)
0	*0*	10.1 ± 8.7 [ab]	16.3 ± 11.7 [a]	26.4 ± 12.6 [ab]
	10	6.6 ± 6.0 [b]	19.7 ± 11.0 [a]	26.3 ± 8.9 [ab]
	50	15.3 ± 13.4 [ab]	11.6 ± 11.2 [abc]	26.8 ± 8.9 [ab]
	100	26.7 ± 12.8 [a]	1.0 ± 0.8 [c]	27.7 ± 14.0 [ab]
1.5	*0*	8.8 ± 7.8 [b]	9.1 ± 9.0 [abc]	17.9 ± 10.9 [abc]
	10	5.8 ± 5.4 [b]	16.7 ± 9.9 [a]	22.5 ± 11.8 [abc]
	50	14.9 ± 9.7 [ab]	9.3 ± 8.9 [abc]	24.2 ± 11.1 [abc]
	100	13.1 ± 13.0 [ab]	3.0 ± 2.9 [bc]	16.1 ± 16.0 [bc]
3	*0*	9.3 ± 7.3 [ab]	8.9 ± 8.5 [abc]	18.2 ± 11.2 [abc]
	10	7.2 ± 5.1 [b]	12.3 ± 9.9 [ab]	19.5 ± 13.1 [abc]
	50	11.7 ± 11.6 [ab]	7.0 ± 6.6 [abc]	18.7 ± 14.2 [abc]
	100	7.7 ± 7.3 [b]	1.2 ± 0.8 [b]	8.9 ± 8.2 [c]

[a,b,c] Within columns, different letters between rows indicate significant differences for each of the types of motility ($p < 0.05$).

Table 2
Percentages of viable llama sperm, sperm with functional membranes (HOS positive) and intact acrosomes (CB positive) in raw semen treated with collagenase and incubated at 37 °C for 0, 1.5 and 3 h with different concentrations of seminal plasma (SP): 0, 10, 50 and 100%. Values are mean ± standard deviations.

Time (h)	SP (%)	Viability (%)	Membrane function (%)	Intact acrosomes (%)
0	*0*	53.5 ± 22.2 [a]	43.2 ± 13.0 [a]	74.7 ± 17.6 [a]
	10	57.9 ± 18.0 [a]	43.5 ± 16.6 [a]	86.0 ± 12.0 [a]
	50	56.5 ± 18.0 [a]	43.9 ± 13.0 [a]	86.2 ± 12.3 [a]
	100	55.4 ± 16.5 [a]	41.4 ± 15.3 [a]	86.5 ± 12.3 [a]
1.5	*0*	47.1 ± 22.3 [a]	45.2 ± 14.2 [a]	70.0 ± 22.3 [a]
	10	53.2 ± 19.5 [a]	43.6 ± 15.9 [a]	84.4 ± 11.5 [a]
	50	52.1 ± 20.0 [a]	43.9 ± 14.0 [a]	85.4 ± 12.1 [a]
	100	40.3 ± 21.5 [a]	34.3 ± 13.0 [a]	83.1 ± 13.3 [a]
3	*0*	47.3 ± 21.2 [a]	41.3 ± 12.9 [a]	64.7 ± 20.4 [b]
	10	49.5 ± 22.5 [a]	41.1 ± 14.5 [a]	79.0 ± 15.6 [a]
	50	47.9 ± 19.8 [a]	39.6 ± 13.4 [a]	82.9 ± 12.8 [a]
	100	29.1 ± 19.1 [b]	26.9 ± 15.0 [b]	80.8 ± 13.3 [a]

[a,b,c] Within columns, different letters between rows indicate significant differences for each of the sperm characteristic ($p < 0.05$).

incubation; however, samples incubated with 100% SP showed a significant decrease both in viability and membrane function at 3 h of incubation when compared to 0 h ($p < 0.05$) (Table 2).

3.4. Acrosomal status

Sperm acrosome status, evaluated with the CB stain, presented a significant decrease in the percentage of sperm with acrosomes in the samples incubated without SP (0%) at 3 h of incubation ($p < 0.05$). The rest of the treatments (10, 50 and 100% SP) maintained the acrosome status of the sperm throughout the incubation ($p > 0.05$) (Table 2).

3.5. Viability and acrosomal status

With the FITC-PNA/PI stain, a significantly higher percentage of total (live and dead) acrosome intact sperm was observed in the samples incubated with 50 and 100% SP when compared to those incubated with 0% SP at all incubation times ($p < 0.05$) (Table 3).

At 3 h of incubation, although not statistically significant, samples incubated with 50% SP showed the highest percentages of live acrosome intact sperm ($p > 0.05$). On the other hand, samples incubated with 100% SP showed the highest percentages of dead sperm with an intact acrosome, being in this case significantly higher than the samples with 0 and 10% SP at all incubation times ($p < 0.05$) (Table 3).

At all incubation times, samples with 0 and 10% SP showed higher percentages of live sperm with reacted acrosomes when compared to the samples with 50 and 100% SP; however, this was only significant between the samples with 0 and those with 100% SP ($p < 0.05$). In addition, samples incubated 3 h with 100% SP showed a significant increase ($p < 0.05$) in dead sperm with reacted acrosomes when compared to time 0 h (Table 3).

Table 3
Llama sperm stained with FITC-PNA/PI in raw semen treated with collagenase and incubated at 37 °C for 0, 1.5 and 3 h with different concentrations of seminal plasma (SP): 0, 10, 50 and 100%. Values are mean ± standard deviation (n = 6, r = 3).

Tim (h)	*SP (%)*	Live, acrosome intact sperm (%)	Live, acrosome reacted sperm (%)	Total live sperm (%)	Dead, acrosome intact sperm (%)	Dead, acrosome reacted sperm (%)	Total sperm with intact acrosomes (%)
0	*0*	30.6 ± 17.7 [abc]	16.5 ± 13.6 [a]	47.1 ± 17.7[ab]	13.8 ± 10.1 [d]	39.1 ± 18.4 [ab]	44.4 ± 21.6 [a]
	10	39.8 ± 17.6 [abc]	12.7 ± 11.5 [abc]	52.6 ± 15.1 [a]	18.1 ± 8.6 [cd]	29.3 ± 14.0 [abcd]	57.9 ± 21.4 [ab]
	50	49.1 ± 16.8 [a]	4.1 ± 5.5 [abc]	53.2 ± 17.4 [a]	29.8 ± 11.4 [abc]	17.1 ± 9.7 [cd]	78.9 ± 10.9 [bc]
	100	48.6 ± 17.7 [ab]	3.0 ± 6.5 [c]	51.7 ± 18.9 [a]	37.2 ± 15.5 [ab]	11.1 ± 7.5 [d]	85.8 ± 9.7 [c]
1.5	*0*	27.9 ± 15.7 [bc]	15.2 ± 13.7[ab]	43.1 ± 15.1[ab]	17.9 ± 10.6 [cd]	39.0 ± 14.8 [ab]	45.8 ± 21.4 [a]
	10	31.2 ± 17.2 [abc]	16.8 ± 16.0 [ab]	48.0 ± 16.9 [a]	20.5 ± 14.9 [bcd]	31.5 ± 15.0 [abc]	51.7 ± 24.8 [ab]
	50	38.6 ± 15.7 [abc]	5.7 ± 6.7 [abc]	44.3 ± 17.9 [ab]	31.3 ± 11.4 [abc]	24.4 ± 13.6 [abcd]	69.9 ± 15.2 [bc]
	100	29.3 ± 17.4 [abc]	3.3 ± 5.1 [c]	32.8 ± 19.2 [ab]	45.9 ± 20.2 [a]	19.9 ± 16.1 [bcd]	75.2 ± 25.9 [c]
3	*0*	21.2 ± 11.9 [c]	16.7 ± 14.4 [a]	37.9 ± 15.4[ab]	18.0 ± 8.4 [cd]	44.1 ± 14.1 [a]	39.2 ± 21.3 [a]
	10	25.8 ± 16.5 [c]	15.9 ± 17.5 [ab]	41.8 ± 18.0 [ab]	18.4 ± 12.5 [cd]	39.8 ± 13.8 [a]	44.2 ± 20.7 [a]
	50	31.5 ± 16.2 [abc]	5.5 ± 6.2 [abc]	37.1 ± 16.4 [ab]	33.2 ± 13.8 [abc]	29.7 ± 13.2 [abc]	64.7 ± 16.0 [c]
	100	23.5 ± 15.2 [c]	3.0 ± 4.5 [c]	26.5 ± 17.5 [b]	43.2 ± 15.9 [a]	30.4 ± 13.3 [abc]	66.7 ± 14.3 [bc]

Total live spermatozoa: Live acrosome intact sperm + live acrosome reacted sperm; Total sperm with intact acrosomes: live acrosome intact sperm + dead acrosome intact sperm.
[a,b,c] Within columns, different letters between rows indicate significant differences for each of the types of pattern ($p < 0.05$).

A significant decrease of total live spermatozoa (live acrosome intact + live acrosome reacted) was observed at 3 h of incubation in the samples with 100% SP when compared to time 0 h ($p < 0.05$). Samples incubated with 0, 10 and 50% SP presented higher sperm viability (Table 3).

3.6. Correlation between techniques evaluating sperm viability and sperm acrosome status

A highly positive correlation ($r = 0.8$; $p = 0.0$) was observed between the percentages of live sperm observed with CFDA/PI and FITC-PNA/PI stains and a moderate correlation ($r = 0.5$; $p = 0.0$) was observed between the percentages of sperm with an acrosome observed with the FITC-PNA/PI (intact acrosome) and the CB (presence of an acrosome) stains.

4. Discussion

Seminal plasma in SACs carries out different roles in the reproductive physiology of these species: sperm nutrition, protection, transport through the female reproductive tract and transport of protein factors involved in the process of ovulation (Adams and Ratto, 2013; Silva et al., 2015; Berland et al., 2016). It is also involved in the interaction of sperm with the oviduct epithelial cells, thus participating in the formation of sperm reservoirs (Apichela et al., 2014). However, despite the different functions attributed to SP, there are still many physiological roles that remain unknown. As mentioned previously, the effect of SP on sperm differs according to the species and even between individuals of a same species. In this study, we observed that the sperm motility pattern that predominates differs according to the percentage of SP added, for example, samples incubated with higher percentages of SP show higher percentages of sperm with OM. In contrast, samples with lower percentages of SP showed statistically higher percentages of PM than those with 100% SP.

It is interesting to highlight that in a previous study, progressive motility was only observed in spermatozoa extended in HT media without SP, while samples with 100% SP only showed oscillatory movement (Carretero et al., 2015a). This evidences that the absence of SP produces a change in the motility pattern of llama spermatozoa. Some authors propose that this response or behavior is due to a mechanical effect that SP from these species exerts on spermatozoa through its rheological properties (Lichtenwalner et al., 1996; Von Baer and Helleman, 1998; Brown, 2000). However, the interaction between SP and SAC spermatozoa could be more complex. It has been observed that the simple dilution of raw ejaculates with media containing egg yolk, allows llama sperm to acquire progressive motility, even while maintaining the characteristics of thread formation and high structural viscosity (Giuliano, personal observation). This observation is further supported by our results in the present study, where sperm oscillatory motility was present in samples even though thread formation was absent (50% SP at 3 h), reinforcing a non-mechanical hypothesis for the action of SP. The only other study on the effect of the addition of SP to spermatozoa performed in SACs, was carried out in alpacas by Kershaw-Young and Maxwell (2011). However, these authors did not evaluate thread formation nor did they differentiate sperm motility patterns, thus they could not establish a relationship between these two parameters.

In our study, we observed that total sperm motility was maintained throughout the 3 h of incubation in the samples with 0, 10 and 50% SP, while the samples with 100% SP showed a significant decrease in total motility. Similar work by Kershaw-Young and Maxwell (2011) reported greater percentages of motility in alpaca raw semen samples with 10% SP as compared to those with 100% SP, also at 3 h of incubation. They also compared other percentages of SP (10, 25, 50 and 100% PS in PBS) and found that at 3 h of incubation, samples with 10 and 25% SP showed higher motility than samples with 50 and 100% SP, with no significant differences between either 10 and 25% SP or between 50 and 100% SP. On the other hand, in contrast to our study, Castellani et al. (2000) observed a significant decrease in motility in rabbit sperm incubated without, or with low, percentages of SP (0 to 1%) at 1 and 3 h of incubation, while with 100% SP, motility was maintained for 6 h. The differences observed between these two studies and ours, could be due to species differences both in sperm physiology and in SP composition (Kershaw-Young and Maxwell, 2012). It has been established that the different protein composition of the SP of different males is linked to the percentage of motile sperm observed in raw semen (Rodrigues et al., 2013). In addition, the media used to dilute sperm and SP also varies between studies and this could be another factor contributing to the different results reported. In our study, we used HT as the medium to extend sperm and SP while Kershaw-Young and Maxwell (2011) used PBS and Castellini et al. (2000) used a Tris-based medium. As mentioned before, our study used HT as the medium to resuspend sperm based on previous studies where sperm parameters were not altered by this treatment (Giuliano et al., 2010). In addition, *in-vitro* fertilization embryos were obtained with the same protocol we applied to separate llama spermatozoa from SP (enzymatic treatment, centrifugation and resuspension of the sperm in HT) (Trasorras et al., 2014). This protocol was applied because to date there are no standardized selection methods that allow separation of spermatozoa from SP in SAC and it had worked well in our lab conditions.

In addition, in the incubation with 100% SP not only was a decrease in sperm motility observed after 3 h, but also a significant decrease in both sperm membrane function and viability (observed both with CFDA/PI and FITC-PNA/PI), while the samples with lower concentrations of SP (0, 10 and 50%) preserved both these sperm characteristics over time. Similar results were reported by Kershaw-Young and Maxwell (2011) in alpaca sperm, where the viability of spermatozoa incubated with 100% SP decreased significantly as compared to samples with 10% SP after 3 h of incubation (100% SP: 7.0 ± 0.7% *versus* 10% SP: 23.1 ± 4.0%). Thus, these authors reported that the addition of 10% SP was the best treatment for preserving alpaca sperm viability. However, in their study 10% SP showed a significant decrease in viability (around 50%) at 3 h, whereas in our study, viability was not affected over time. This difference in results could be due to the different medium used to dilute the sperm (PBS *vs*. HT). HEPES-TALP is a buffered saline solution with a similar osmolarity to the ejaculate and it also contains metabolic substrates for the spermatozoa, while PBS only contains buffered salts. In other species such as rabbit, different results were reported. In this case, raw semen samples incubated with

low percentages of SP, a decrease in viability was observed (Castellini et al., 2000). Again, these contrasts in results could be attributable to the same reasons hypothesized for the differences found in sperm motility (Juyena and Stelletta, 2012; Kershaw-Young and Maxwell, 2012). In addition, different studies have also reported dissimilar results regarding the effect of SP on sperm, even between individuals of the same species (Leahy and de Graff, 2012; Morrell et al., 2014). Hence, this aspect deserves further studies to further elucidate the underlying physiology.

Concerning membrane function, there are no reports that have evaluated this sperm characteristic in raw semen treated with different final concentrations of SP, so we were unable to compare our study with that of other species. The loss of membrane function could be due to the death of spermatozoa, as evidenced by the decrease in the percentage of live sperm.

Regarding acrosome integrity, samples incubated with 0% SP showed the highest percentages of acrosome reacted sperm with FITC-PNA/PI at all incubation times (compared to 50 and 100% SP; $p \leq 0.05$). With the CB stain, the highest percentages of sperm without acrosomes were also observed in 0% SP samples, but only at 3 h of incubation ($p \leq 0.05$). In porcine, the addition of 10% SP to *in vitro* fertilization media prevents sperm from penetrating the oocyte and it is thought that this inhibition is caused by the decapacitating effect of SP on pig sperm (Suzuki et al., 2002). This was also reported by Heise et al. (2011) in a study in equine semen, where these authors observed that epididymal sperm exposed to SP maintained their fertilizing capacity better than epididymal sperm that had never come into contact with SP. Similarly, in alpaca epididymal sperm incubated with 0% SP, lower percentages of acrosome-intact sperm were observed when compared to those incubated with 10 and 100% SP (Kershaw-Young and Maxwell, 2011). These studies, together with our own, where the samples treated with the highest percentages of SP showed the lowest percentages of sperm with reacted acrosomes, reinforce the idea that factors present in SP can reversibly inhibit the process of capacitation and that in the absence of them, spontaneous capacitation can occur, followed naturally by the acrosome reaction.

The significant decrease in sperm motility, membrane integrity and function and the increase in dead sperm with reacted acrosomes in the samples incubated for 3 h with 100% SP could also be due to the depletion of nutrients, the accumulation of waste and/or reactive oxygen species (ROS), as has been reported in other species (Morton et al., 2010). On the other hand, the samples with 0, 10 and 50% SP, having a higher proportion of HT, could thus have greater access to nutrients and a better survival of the spermatozoa over time aided by the buffer in the medium.

The second objective of the study was to correlate the techniques routinely used to evaluate llama sperm viability and acrosome status (CFDA/PI and CB) with a modern fluorescent technique (FITC-PNA/PI). A high positive correlation was found between both techniques for sperm viability. In human sperm, a similar correlation was obtained when viability was evaluated with Hoescht 33258/FITC-PSA (*Pisum sativum* agglutinin) and yellowish eosin (Ozaki et al., 2002). Similarly, Way et al. (1995) compared different techniques (Fast green FCF/eosin B, eosin B-aniline blue and propidium iodide/*Pisum sativum* agglutinin) in bull ejaculates to determine the percentage of live spermatozoa, and although they did not use correlation, they did not observe statistical differences in sperm viability between techniques.

When acrosomal status was analyzed, higher percentages of sperm with reacted acrosomes were found in the FITC-PNA/PI stain compared to the CB. This could be due to the different mechanisms between the techniques. PNA is a lectin that selectively binds the outer acrosomal membrane, thus identifying sperm with intact acrosomes and also those with reacting acrosomes. These sperm show an intense green fluorescence in the acrosome cap as the bound fluorochrome (FITC) enters the cap through the points of fusion between the plasma membrane and the outer acrosome membrane. This category of sperm shows two staining patterns: sperm with spotted fluorescence in the acrosome region and sperm with a band of fluorescence in the equatorial segment. Both patterns express the interaction of the fluorochrome with the remnants of the outer acrosome membrane that remain attached after the acrosome reaction. Perhaps, the differences observed between techniques could be the CB binding to remnants of the outer acrosome membrane, thus categorizing those sperm that are reacting or reacted as having an acrosome present. This could be the reason behind the moderate correlation we observed. As mentioned before, samples incubated with 100% SP showed a significant decrease in the percentages of live sperm over time with both viability techniques. With the FITC-PNA/PI this decrease seemed to be at the expense of an increase in dead, acrosome-reacted sperm. Although these significant changes in acrosome status were not detected with the CB stain, a slight decrease in percentage of sperm with intact acrosomes was observed in this assay. A comparison of different techniques to determine acrosome status was also performed in human semen by Ozaki et al., (2002). A high positive correlation ($r = 0.92$, $p < 0.001$) was found for the percentages of acrosome reacted sperm when using two fluorescent stains (FITC-PSA and FITC-Concanavalin A). This was in contrast with our study, were only a moderate correlation was observed, but this could be due to the fact that they used lectins in both techniques (hence with a similar mechanism of action for the evaluation of sperm acrosomes).

5. Conclusions

This study shows that the motility pattern of llama sperm is influenced by SP and by the proportion of SP present with the cells. In addition, it was established that a certain percentage of SP in the media is necessary to exert a decapacitating role, to avoid spontaneous acrosome reactions. On the other hand, the use of SP as the only medium for incubating spermatozoa is not advisable as this is unable to maintain sperm motility, viability, membrane function and acrosome integrity for 3 h.

A positive correlation was established between the FITC-PNA/PI and CFDA/PI stains for evaluation of sperm viability and a moderate correlation was observed between the FITC-PNA/PI and CB assays used for evaluation of acrosome status. These results could help to shorten the time it takes to evaluate llama sperm and reduce costs in sperm laboratories by using only one technique to jointly evaluate llama sperm viability and acrosome status.

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