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Evaluation of the acrosomal status in *Lama glama* sperm incubated with acrosome reaction inducers

M.I. Carretero[a,d,*], F.G. Fumuso[a,d], D.M. Neild[a], S.M. Giuliano[b], P. Cetica[c,d], M.H. Miragaya[a]

[a] *Cátedra de Teriogenología, Instituto de Investigación y Tecnología en Reproducción Animal, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina*
[b] *Cátedra de Física Biológica, Instituto de Investigación y Tecnología en Reproducción Animal, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina*
[c] *Cátedra de Química Biológica, Instituto de Investigación y Tecnología en Reproducción Animal, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Argentina*
[d] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina*

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ABSTRACT

The objectives of this study were to evaluate the effect of different acrosome reaction (AR) inducers on viability and acrosomal status in llama spermatozoa, by using the FITC-PNA/PI technique and evaluate if there is a positive correlation between the FITC-PNA/PI and the Coomassie blue (CB) staining techniques. After incubating twenty ejaculates in 0.1% collagenase the centrifuged pellets were resuspended in TALP–BSA medium. An aliquot was sonicated to remove the acrosomal content (positive control). The rest of the sample was incubated for 3 h at 38 °C with 5% CO_2 and 100% humidity.
Treatments: Three aliquots were further incubated 1 h with one of the following AR inducers: calcium ionophore, ionomycin or progesterone.
Controls: One without inducers and the other, incubated with dimethyl sulfoxide (vehicle of the inducing agents). Acrosomes were evaluated at time 0 and after 4 h incubation. Calcium ionophore was the most potent agent for inducing the AR (67.2 ± 14.4% live + dead AR sperm) ($P < 0.05$). These samples showed no motility and viability was very low (0–30%). Both ionomycin and progesterone presented significantly higher ($P < 0.05$) percentages of total AR sperm than the controls, but had similar percentages of dead reacted sperm to the controls. A positive correlation was observed between the intact acrosome FITC-PNA/PI pattern (live + dead sperm) and the acrosome-present CB pattern ($r = 0.64$; $P = 0.000$) in all the evaluated samples.
Conclusions: the FITC-PNA/PI technique simultaneously evaluates viability and acrosomal status in llama spermatozoa and calcium ionophore could be used as a control of AR.

1. Introduction

The acrosome reaction (AR) is an irreversible process during which the sperm plasma membrane fuses with the external acrosome membrane, resulting in the releasing of the acrosomal content and the exposition of the inner acrosome membrane (Kopf and Gerton, 1991). During

* Corresponding author at: Chorroarín 280, Ciudad Autónoma de Buenos Aires (CABA), CP: C1427CWO, Argentina. Tel.: +54 011 4524 8425; fax: +54 011 4524 8425.
E-mail address: ignaciacarretero@fvet.uba.ar (M.I. Carretero).

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fertilization, spermatozoa carry out the acrosome reaction, an event that is required for sperm to penetrate the zona pellucida and subsequently fuse with the oocyte's membrane. After undergoing the acrosome reaction, sperm survival is limited if they do not fertilize an oocyte. Visualization of sperm acrosomal status would be beneficial because one of the possible causes of male infertility is an absence or a shortage of spermatozoa with intact acrosomes at ejaculation (Blach et al., 1988; Zhang et al., 1990). In addition, this evaluation can be used to indicate the quality of an ejaculate and even to evaluate the effects that different *in vitro* techniques have on spermatozoa. This becomes especially relevant when taking into account that after cooling and freeze-thawing sperm from various species show changes similar to those of capacitation, an effect which has been termed "cryocapacitation" (Watson, 1995; Neild et al., 2003; Silva and Gadella, 2006). Besides, evaluation of a spermatozoa's ability to carry out the acrosome reaction is a useful tool to detect defects in its fertilizing capacity. Several techniques have been proposed to differentiate acrosome-intact from acrosome-reacted spermatozoa, including cytochemical staining (Talbot and Chacon, 1981; Brum et al., 2006), indirect immunofluorescence using monoclonal antibodies (Sanchez et al., 1991), labeling with fluoresceinated lectins (Cross et al., 1986; Aitken et al., 1993; Cheng et al., 1996; Kershaw-Young and Maxwell, 2011) and phase-contrast microscopy to examine partial head decondensation (Silvestroni et al., 2004). Currently, the use of lectins that bind external acrosome membrane glycoconjugates or the acrosomal matrix have been among the most employed, both with fluorescence microscopy (Mortimer et al., 1987; Vázquez et al., 1993; Ou, 1994; Cross and Waston, 1994; Kershaw-Young et al., 2013) and with flow cytometry (Tao et al., 1993; Rathi et al., 2001; Cheuquemán et al., 2013). The FITC-PNA stain (Fluorescein isothiocyanate – *Arachis hypogaea* agglutinin) has been used to evaluate acrosomal status with fluorescence microscopy in alpaca spermatozoa (Kershaw-Young and Maxwell, 2011; Morton et al., 2012). Nevertheless, these studies did not include fluorochromes that allow differentiation of the live acrosome reacted sperm population from the dead one. Recently, Kershaw-Young et al. (2013) used FITC-PNA together with another fluorochrome, Propidium iodide (PI), to identify live reacted alpaca spermatozoa in samples previously fixed with 0.1% neutral buffered formalin. Cheuquemán et al. (2013) evaluated the acrosomal status of alpaca sperm using flow cytometry. These authors used FITC-PSA (Fluorescein isothiocyanate – *Pisum sativum* agglutinin) with PI to identify live reacted spermatozoa. However, many authors prefer to use PNA because it presents less unspecific union sites with sperm when compared to PSA (Graham, 2001). In addition, PNA is the chosen lectin when evaluating sperm that are diluted in extenders containing egg yolk because PSA also presents affinity for non-specific sites in egg yolk (Thomas et al., 1997). The presence or absence of the acrosomal cap has been evaluated in llama sperm using the Coomassie blue stain (Giuliano et al., 2012; Fumuso et al., 2014). This simple technique is inexpensive and can be evaluated using light microscopy, but it does not permit evaluation of sperm viability, it only assesses the presence or absence of the acrosomal cap.

The acrosome reaction has been induced *in vitro* in various species using both pharmacological and physiological inducers. Among the pharmacological inducers, one of the most used has been calcium ionophore (A23187), not only in laboratory animals such as the guinea pig (Yanagimachi, 1975; Green, 1978) but also in bovine (Byrd, 1981; Beorlegui et al., 1997; Pereira et al., 2000), caprine (Pereira et al., 2000), equine (Cheng et al., 1996; Spizziri et al., 2010) and humans (Liu and Gordon Baker, 1998; Cardona-Maya et al., 2006). Among the physiological inducers of the AR, incubation with follicular fluid has been the most used in different species (golden hámster: Yanagimachi, 1969; mouse: Iwamatsu and Chang, 1969; equine: Cheng et al., 1998). It has been reported that the progesterone present in the follicular fluid would be responsible for inducing the AR (equine: Cheng et al., 1998; human: Schuffner et al., 2002). Various authors have added this hormone to capacitated spermatozoa to induce the AR in a physiological way (equine: Cheng et al., 1998; caprine: Somanath et al., 2000; human: Schuffner et al., 2002; porcine: Wu et al., 2006). To date, there are no studies that evaluate acrosomal status in llama sperm induced to acrosome react *in vitro*, nor any reports that have simultaneously evaluated the acrosome reaction and viability in llama sperm.

The objectives of this study were: (1) to evaluate the effect of different acrosome reaction (AR) inducers on viability and acrosomal status in llama spermatozoa, by using the FITC-PNA/PI technique and (2) evaluate if there is a positive correlation between the FITC-PNA/PI and the Coomassie blue staining techniques.

2. Materials and methods

2.1. Reagents

Collagenase, dimethyl sulfoxide (DMSO), calcium ionophore A23187, ionomycin, FITC-PNA, Propidium iodide and the reagents for the TALP–BSA medium were purchased from Sigma Chemicals (Sigma Aldrich, Buenos Aires, Argentina). Type I collagenase (*Clostridium peptidase A* from *Clostridium histolyticum*) and Lectin from *Arachis hypogaea* (peanut) were used. TALP medium (Parrish et al., 1986) was supplemented with 6 mg ml^{-1} of Bovine Serum Albumin (TALP–BSA). Progesterone was purchased from Spectrum Chemical MFG-CORP (New Brunswick, NJ, USA). The reagents for the Coomassie blue stain were purchased from Tecnolab, S.A. (Buenos Aires, Argentina).

Ionomycin, calcium ionophore and progesterone were solubilized in dimethyl sulfoxide in the following concentrations: 1000 μM of calcium ionophore, 100 μM of ionomycin and 1500 μg/ml of progesterone.

2.2. Animals and location

The study was carried out at the Institute of Research and Technology for Animal Reproduction, 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, 5 male *Lama glama* ranging between 6 and 10 years of age and weighing 132.72 ± 18.22 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. To minimize heat stress, all males were shorn during the month of November.

2.3. Semen collection

Semen collections were carried out using electroejaculation (EE) under general anesthesia with 0.2 mg/kg of xilazine IV (Xilazina®, Vetec, Argentina) and 1.5 mg/kg of ketamine IV (Ketamina®, Clínica Equina, PRO-SER, Argentina), 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, and eligible males were randomly chosen for EE. 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 2014/16).

2.4. Raw semen evaluation

The following seminal characteristics were evaluated in raw semen: ejaculate volume, sperm motility (oscillatory, progressive and total motility), concentration, membrane function, membrane integrity and DNA condensation. Sperm motility was evaluated, using a phase contrast microscope (100×) and a warm stage (37 °C). Concentrations were calculated using a Neubauer hemocytometer (dilution 1/100). The hypoosmotic swelling (HOS) test was used for assessing membrane function, and the fluorochromes 6-Carboxyfluorescein Diacetate (CFDA) and Propidium Iodide (PI) were used for assessing membrane integrity (viability). These techniques were carried out according to Giuliano et al. (2008). Briefly, for the HOS test semen was incubated (37 °C) in a hypoosmotic solution containing fructose and sodium citrate (50 mOsm). After incubation, a minimum of 200 spermatozoa was evaluated using a phase contrast microscope. For assessing membrane integrity, samples of semen were incubated (37 °C) with CFDA and PI in an isotonic saline solution. A minimum of 200 spermatozoa was evaluated per sample using an epifluorescence microscope with a rhodamine and standard fluorescein filter set. The Toluidine blue (TB) stain was carried out according to Carretero et al. (2009) to evaluate the degree of chromatin condensation. Briefly, each sample was smeared on clean, non-greasy 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 (1000×) evaluating a minimum of 200 spermatozoa per smear. Sperm 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).

2.5. Semen processing

A total of 20 ejaculates were collected from 5 male llamas ($n = 5$, $r = 4$). Each whole ejaculate was diluted 4:1 in 0.1% collagenase in TALP–BSA medium (Parrish et al., 1986), incubated 4 min at 37 °C with the objective of decreasing thread formation and facilitating manipulation of the samples (Giuliano et al., 2010). Afterwards, the ejaculates were centrifuged for 8 min at 800 g and the pellets were resuspended at a concentration of 20×10^6 sperm/ml in TALP medium with 6 mg ml^{-1} of BSA.

2.5.1. Sonication

As a positive control, an aliquot of 500 μl of semen was resuspended and submitted to sonication with the objective of removing the acrosomal content (Yu et al., 2006). Briefly, the sample was submitted to 3 pulses, of 30 s each; at maximum intensity using a Vibracell Sonicator model 600 W (Sonics & Materials Inc., Newton, CT, USA).

2.5.2. Inducing the acrosome reaction

The sample was incubated for 3 h at 38 °C with 5% CO_2 and 100% humidity and was then subdivided into 5 aliquots to induce the acrosome reaction (AR). Three aliquots were incubated using the following inducers: 5 μM calcium ionophore (A23187) or 10 μM ionomycin or 30 μg/ml progesterone. The last two aliquots were used as controls: one with spermatozoa incubated in TALP–BSA (6 mg ml^{-1}) without any inducer and the other, incubated with DMSO (vehicle of the inducing agents). All samples (with and without inducers) were incubated 1 hour at 38 °C with 5% CO_2 and 100% humidity. The concentrations of AR inducers used in this study were based on those published in other species (De Jonge et al., 1989; Cheng et al., 1996; Villaverde et al., 2009).

2.6. Acrosomal status evaluation

2.6.1. FITC-PNA/PI technique

FITC-PNA is a lectin that selectively binds the outer acrosomal membrane. This technique was modified from Cheng et al. (1996). Briefly, samples were incubated 10 min at 37 °C with a FITC-PNA solution (final concentration: 20 μg/ml) and were then incubated 10 min at 37 °C with a solution of Propidium iodide (PI) (final concentration: 10 μg/ml). 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 Mycrosystems Co., Wetzlar, Germany).

2.6.2. Coomassie blue stain (CB)

This stain permits detection of the presence or absence of the acrosomal cap. The technique was carried out according to Giuliano et al. (2012). 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

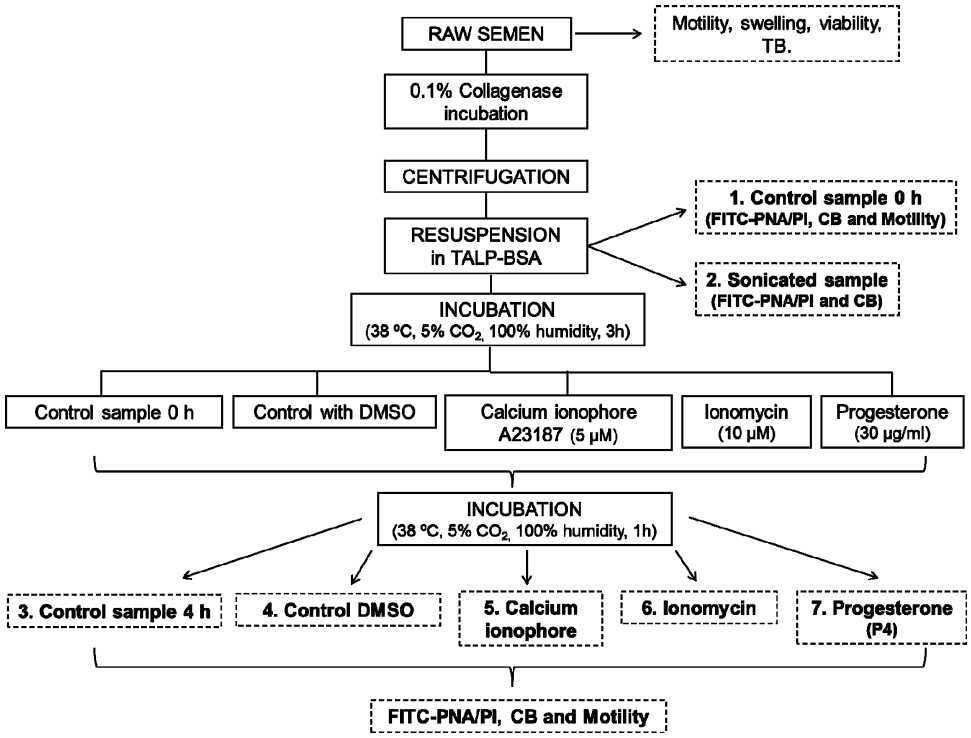

Fig. 1. Diagram showing the experimental design used to evaluate routine seminal characteristics and acrosomal status in llama sperm ($n = 5$, $r = 4$). The dotted-line boxes indicate each time and sample in which evaluations were carried out. Swelling: percentage of spermatozoa showing functional membranes evaluated with the hypoosmotic (HOS) test. Viability: percentage of spermatozoa with intact membranes evaluated with the fluorochromes 6-Carboxyfluorescein Diacetate and Propidium Iodide. TB: Toluidine blue stain for evaluating chromatin condensation. FITC-PNA/PI: Fluorescein isothiocyanate – *Arachis hypogaea* agglutinin/Propidium iodide for simultaneous evaluation of viability and acrosomal status. CB: Coomassie blue stain for evaluating present or absent of the acrosome cap. DMSO: dimethyl sulfoxide (vehicle of the acrosome reaction inducers used as a control).

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: violet/blue acrosome cap (acrosome-present) and lack of color in the acrosomal region (acrosome-absent).

2.7. Experimental design

The acrosome status was evaluated at time 0 (sample 0 h and sonicated samples) and after each of the controls and treatments (controls: sample 4 h and DMSO; treatments: calcium ionophore, ionomycin and progesterone).

The experimental design can be seen in Fig. 1.

2.8. Statistical analysis

Statistical analysis was conducted using the R 2.2.1. Program. Descriptive statistics was carried out for routine seminal characteristics. A factorial design (5 levels) was carried out to compare the different patterns of FITC-PNA/PI and CB techniques (5 levels: control sample 4 h, control DMSO, calcium ionophore, ionomycin and progesterone), using the male as a blocking factor. In addition, sample 0 h was compared to the different samples (control sample 4 h, control DMSO, calcium ionophore, ionomycin and progesterone) using a paired T test. A Kruskal–Wallis's test was used to evaluate sperm motility and a Pearson's test was used to correlate FITC-PNA/PI with CB stain.

3. Results

3.1. Raw semen evaluation

Routine seminal characteristics (semen volume, sperm motility, concentration and membrane function and integrity) and sperm chromatin condensation in raw llama semen were within the normal range for these species. The results are summarized in Table 1.

3.2. Sperm evaluations

3.2.1. Motility

No significant differences ($P > 0.05$) were observed for total motility between the sample evaluated at time 0 and the controls incubated for 4 h (sample 4 h and DMSO; Fig. 2). Although the percentages of total motility observed in the samples at time 0 h and the controls at 4 h were higher than the percentages observed in the samples incubated with the AR inducers, these differences were only significant between the samples induced with either ionomycin or progesterone and the control sample at 4 h ($P < 0.05$).

Table 1
Routine seminal characteristics: volume, motility, concentration, membrane function (positive swelling), membrane integrity (viability) and normal chromatin condensation (Toluidine blue negative) observed in raw llama semen ($n = 5$, $r = 4$).

	Volume (ml)	Oscillatory motility (%)	Concentration (10^6 sperm/ml)	Sperm swelling (%)	Sperm viability (%)	Toluidine blue negative sperm (%)
Mean	2.1	18.4	75.3	29.7	55.4	86.4
SD	±1.2	±11.5	±58.3	±10.9	±13.1	±6.7

The values are expressed as means ± standard deviation (SD).
Sperm swelling: percentage of spermatozoa showing functional membranes evaluated with the hypoosmotic (HOS) test.
Sperm viability: percentage of spermatozoa with intact membranes evaluated with the fluorochromes 6-Carboxyfluorescein Diacetate and Propidium Iodide.
Toluidine blue negative sperm: percentage of spermatozoa showing normal chromatin condensation with the Toluidine blue stain.

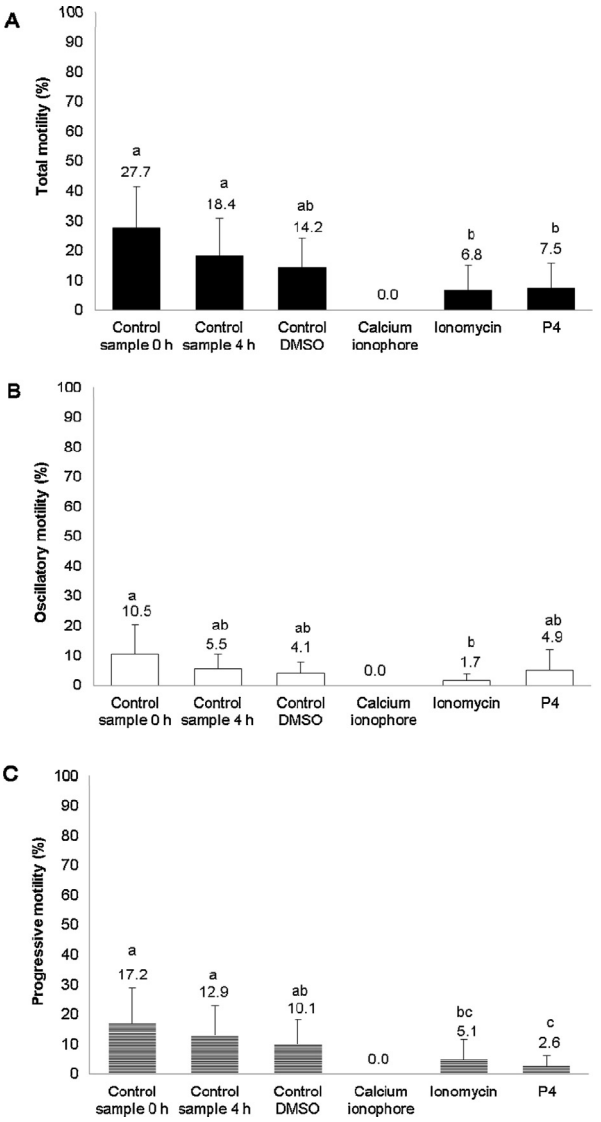

Fig. 2. Llama sperm motility observed in samples incubated without inducers of the acrosome reaction (control sample 0 h, control sample 4 h and control DMSO) and in samples incubated in the presence of different AR inducers (calcium ionophore, ionomycin and P4). (A) Total motility, (B) oscillatory motility and (C) progressive motility. [a,b,c] Different letters between different treatments indicate significant differences ($P < 0.05$). The results obtained with calcium ionophore were not included in the statistical analysis because all values were 0 (all sperm were non-motile). P4: progesterone. DMSO: dimethyl sulfoxide.

No motile sperm were observed in the samples incubated with the calcium ionophore (Fig. 2).

3.2.2. Acrosomal status and sperm viability

The FITC-PNA/PI technique was effective for establishing acrosomal status and viability simultaneously, presenting six different staining patterns (Fig. 3): (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 acrosomes: 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; (vi) dead, acrosome reacted spermatozoa: red fluorescent head with spotted or half-moon green fluorescence in the acrosome cap or green fluorescent equatorial segment. All spermatozoa submitted to sonication showed red fluorescent heads and no tails, indicating they were dead sperm. They also showed an absence of acrosome caps (green fluorescent equatorial segments or spotted green heads or heads without traces of the acrosome cap) (Fig. 4).

The percentage of total (live + dead) acrosome intact sperm in time 0 h samples was significantly greater ($P < 0.05$) than the percentage of any of the samples incubated 4 h. The percentage of total (live + dead) acrosome reacted sperm was significantly greater ($P < 0.05$) in the samples incubated with AR inducers with regard to the samples without inducers (Controls: sample 4 h and DMSO). The samples incubated with calcium ionophore showed the highest percentages of AR of all ($P < 0.05$) (Table 2).

With regard to viability, all AR inducers significantly decreased the percentage of live acrosome intact spermatozoa ($P < 0.05$); nevertheless, this decrease was more notorious in the samples incubated with calcium ionophore (Fig. 5A). The percentage of live acrosome reacted spermatozoa was significantly greater ($P < 0.05$) in the samples incubated with ionomycin and with P4 with respect to the samples evaluated at time 0 h. The percentage of live acrosome reacted sperm was greater in the presence of ionomycin and P4 with regard to the controls incubated 4 h (sample 4 h and DMSO) although no significant differences were observed.

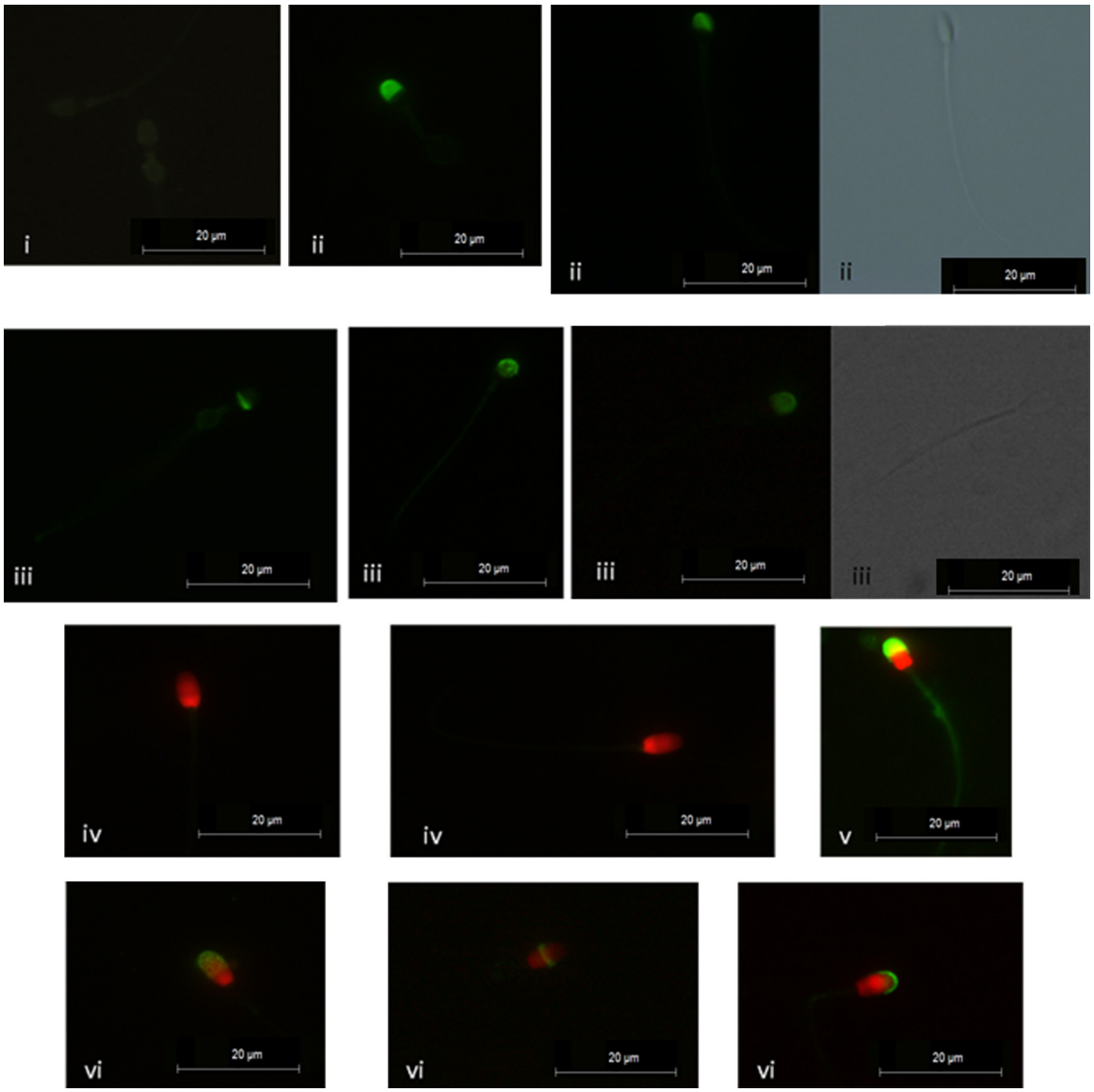

Fig. 3. Patterns of llama sperm observed with the FITC-PNA/PI technique: (i) live spermatozoa with intact acrosomes, (ii) live, acrosome reacting spermatozoa, (iii) live, acrosome reacted spermatozoa, (iv) dead spermatozoa with intact acrosomes, (v) dead, acrosome reacting spermatozoa and (vi) dead, acrosome reacted spermatozoa. The two top right images show sperm observed using bright field microscopy and the rest of the images show sperm evaluated by epifluorescence microscopy. Magnification 1000 × 0.05.

Table 2
Percentages of total (live + dead) llama sperm with intact, reacting and reacted acrosomes, evaluated using the FITC-PNA/PI technique.

	Intact acrosome (%)	Reacting acrosome (%)	Reacted acrosome (%)
Control sample 0 h	64.4 ± 13.6 [a]	5.2 ± 4.7 [ab]	30.4 ± 12.9 [a]
Control sample 4 h	49.3 ± 15.2 [b]	5.5 ± 5.2 [ab]	45.2 ± 15.8 [b]
Control DMSO	50.0 ± 12.4 [b]	4.4 ± 4.4 [b]	45.6 ± 11.9 [b]
Calcium ionophore	22.7 ± 11.3 [c]	10.1 ± 9.6 [a]	67.2 ± 14.4 [c]
Ionomycin	37.2 ± 13.7 [d]	8.3 ± 7.5 [ab]	54.5 ± 14.5 [d]
P4	37.0 ± 16.0 [d]	9.5 ± 6.8 [ab]	53.5 ± 16.2 [d]

The values are expressed as mean percentages ± SD ($n = 5$, $r = 4$).
[a,b,c,d] Different superscripts within a column indicate significant differences ($P < 0.05$). DMSO: dimethyl sulfoxide.
P4: progesterone.

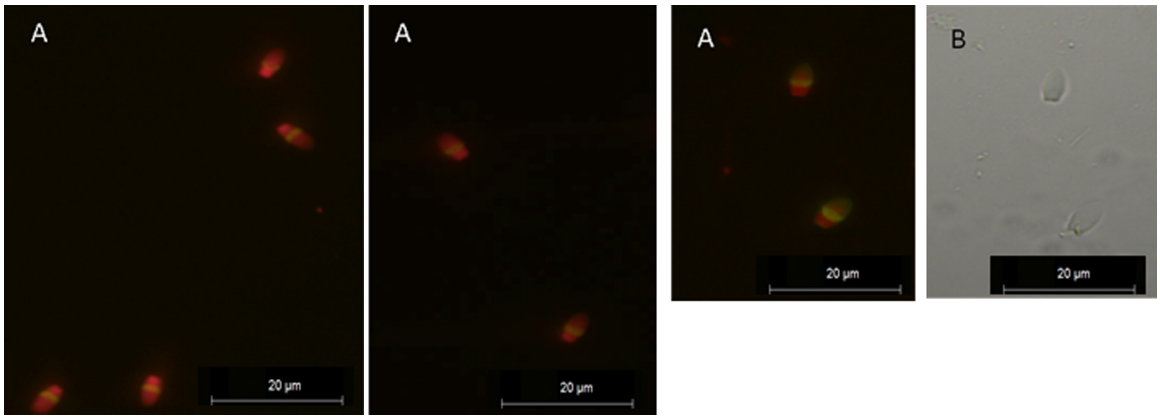

Fig. 4. Llama sperm submitted to sonication observed with the FITC-PNA/PI technique using epifluorescence microscopy (A) and bright field microscopy (B). Magnification 1000 × 0.05.

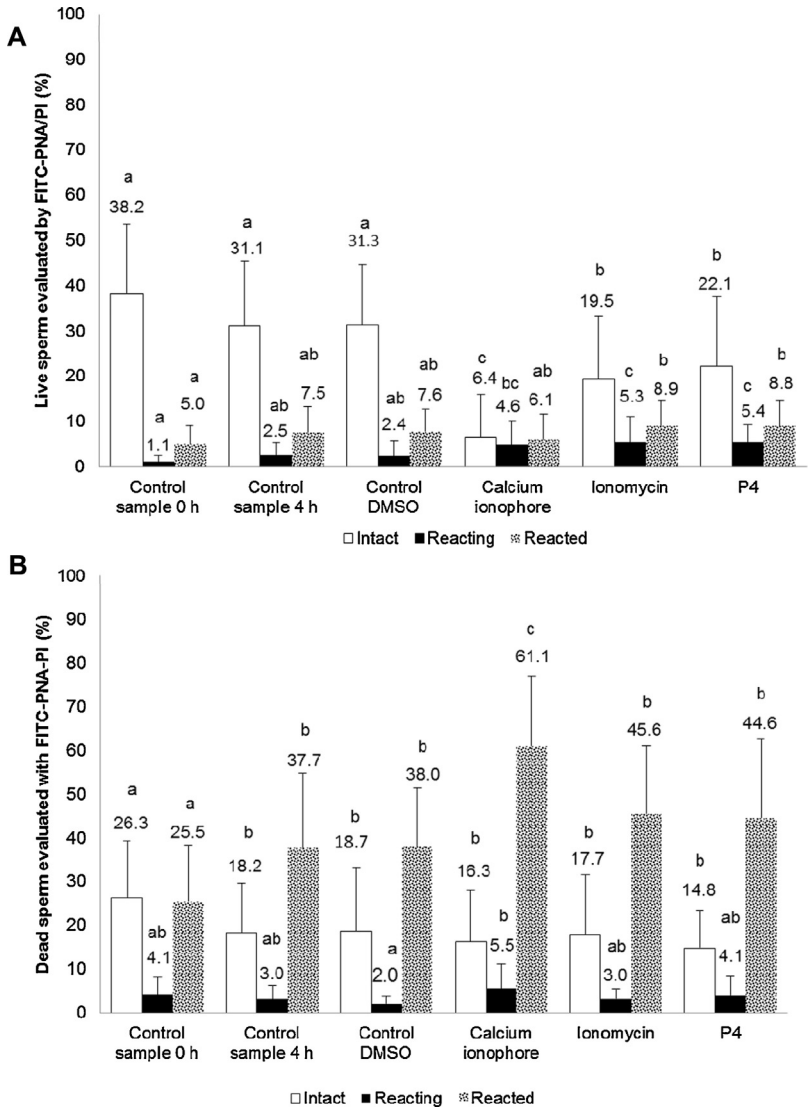

Fig. 5. Llama sperm evaluated with FITC-PNA/PI. (A) live sperm and (B) dead sperm. The different patterns of acrosome status are represented: intact, reacting and reacted. [a,b,c] Different letters indicate significant differences between the samples evaluated (control sample 0 h, control sample 4 h, control DMSO, calcium ionophore, ionomycin and progesterone) within each acrosomal pattern ($P < 0.05$). DMSO: dimethyl sulfoxide. P4: progesterone.

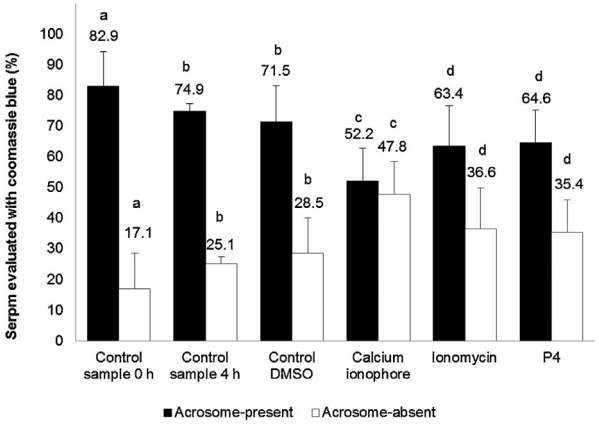

Fig. 6. Llama sperm evaluated with Coomassie blue (CB). The acrosome-present and acrosome-absent patterns are shown for the different samples evaluated. [a,b,c,d] Different letters indicate significant differences between the CB patterns for each of the evaluated samples (control sample 0 h, control sample 4 h, control DMSO, calcium ionophore, ionomycin and progesterone). DMSO: dimethyl sulfoxide. P4: progesterone.

With regard to dead sperm, calcium ionophore showed significantly higher ($P < 0.05$) percentages of dead acrosome reacted spermatozoa than the rest of the samples (Fig. 5B).

Two acrosome patterns were observed in the sperm submitted to sonication: 1-FITC-PNA unstained sperm, due to complete removal of acrosomal content ($35.1 \pm 26.2\%$) and 2-sperm with a remnant acrosome that showed staining in the equatorial segment ($64.9 \pm 26.2\%$). All spermatozoa showed positive staining with PI.

Regarding the Coomassie blue stain, the percentage of sperm with acrosome-present decreased significantly ($P < 0.05$) after incubating the samples 4 h (Fig. 6). This decrease was more prominent in the samples incubated with AR inducers, and even more so with calcium ionophore ($P < 0.05$). Smears of the sonicated samples stained with CB presented a $96.7 \pm 2.4\%$ (mean $\pm$ SD) of detached heads without acrosomes.

A positive correlation was observed between the intact acrosome FITC-PNA/PI pattern (live + dead sperm) and the acrosome-present CB pattern ($r = 0.64$; $P = 0.000$) in all the evaluated samples (Fig. 7).

4. Discussion

To the best of our knowledge this is the first report on simultaneous viability and acrosomal status evaluation of llama sperm incubated with and without acrosome reaction inducers.

When evaluating the action of the different acrosome reaction inducers with FITC-PNA/PI all presented significantly higher percentages of total reacted sperm (live + dead) than the control samples (sample 0 h, sample 4 h and DMSO). This demonstrates that in llama spermatozoa, calcium ionophore, ionomycin and progesterone can all induce the acrosome reaction (AR). When analyzing the behavior of the different AR inducers in our study, it was evident that calcium ionophore A23187, at the concentration and length of incubation we used (5 μM, 60 min), was the inducer that presented the highest significant percentages of both total (live + dead) and dead sperm with reacted acrosomes, showing total values of 50 to 80% acrosome reacted sperm ($67.2 \pm 14.4\%$). However, sperm showed no total motility whatsoever (0% in all the samples that were processed) and viability was very low (0–30%). Similarly, in other species very low to no motility was observed after incubation with the ionophore A23187 (human: Tesarik, 1985; Byrd and Wolf, 1986; equine: Cheng et al., 1996).

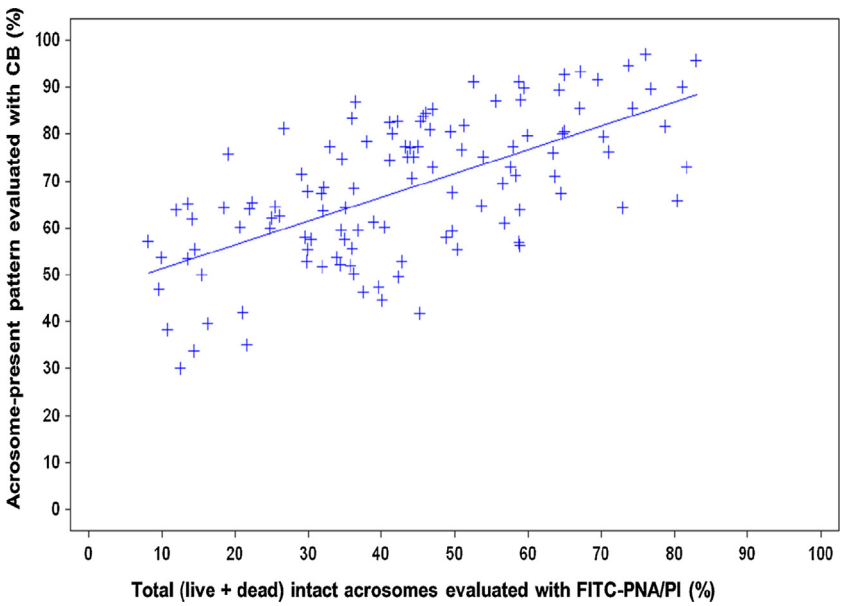

Fig. 7. Correlation between the percentage of total sperm (live + dead) with intact acrosomes evaluated with FITC-PNA/PI stain and the acrosome-present pattern of Coomassie blue (CB) stain in all the evaluated samples (control sample 0 h, control sample 4 h, control DMSO, calcium ionophore, ionomycin and progesterone) ($r = 0.64$; $P = 0.000$).

With regard to the acrosome reaction, the values we observed with calcium ionophore (50–80%) were similar to those observed in cats (73.3%; Long et al., 1996) and in horses (62–89.5%; Cheng et al., 1996) both reports using similar concentrations to those of our study (4 μM and 5 μM, respectively) but with different incubation times (30 min in cats and 2 h in horses). Conversely, lower percentages of reacted cells (40–57%) were observed after inducing human sperm with calcium ionophore (Tesarik, 1985; Byrd and Wolf, 1986; De Jonge et al., 1989). In our study, the high percentage of acrosome reacted sperm observed in the samples incubated with calcium ionophore would seem to indicate that the concentration and length of incubation used (5 μM during 60 min) could be appropriate as a control of the AR for the FITC-PNA/PI and CB techniques. Nonetheless, it will be necessary to carry out more experiments to find a more adequate combination of concentration and/or length of incubation to use this inducer in assisted reproductive techniques (ART) in this species.

Ionomycin (10 μM) and progesterone (30 μg/ml) showed a similar behavior after one hour of incubation. Both presented significantly higher percentages of total (live + dead) acrosome reacted sperm to those of the controls (sample 4 h and DMSO). However, they proved to be similar to the controls in the percentage of dead reacted sperm, indicating that although they are less effective to induce AR than calcium ionophore at the concentrations and incubation times evaluated in this study, they have less deleterious effects on sperm viability. The percentage of total sperm (live + dead) with reacted acrosomes in the samples incubated with ionomycin (54.5%) was similar to that observed in human sperm (50.0%) induced with the same concentration used in this study (10 μM) but incubated for only 15 min (De Jonge et al., 1989); and was lower than the percentage in cat semen (75.3%) incubated with 8 μM ionomycin during 30 min (Villaverde et al., 2009). With regard to progesterone, we observed an average 53.5% of total sperm (live + dead) with reacted acrosomes which was similar to that observed in cat semen (54.4%) incubated with a lower concentration of progesterone and time of incubation (20 μg/ml during 30 min) (Villaverde et al., 2009) and was higher than the 44.6% observed by McPartlin et al. (2008) in equine sperm incubated with 3.2 μM (approximately 1 μg/ml) during 30 min. These results could be indicating that llama sperm would be less sensitive to react to these inducers (progesterone and ionomycin) than human, cat and equine sperm because using a higher concentration and longer incubation time the percentages of total sperm (live + dead) with reacted acrosomes are similar or lower than those observed in these other species.

When evaluating the control samples at time 0 h, the percentage of total (live + dead) acrosome reacted sperm observed in our study (30.4%) was greater than that reported for alpaca epididymal (23.4%) and ejaculated sperm (21.1%), also evaluated with FITC-PNA at time 0 h (Kershaw-Young and Maxwell, 2011 and Morton et al., 2012 respectively) and to that reported by Cheuquemán et al. (2013) in alpaca ejaculated sperm (12.1%) evaluated using FITC-PSA/PI and flow cytometry. These differences could be due to the different origin of sperm (epididymal *vs.* ejaculated) or to the fact that in the Morton and Cheuquemán studies the samples were not treated with enzymes, only diluting the seminal plasma and thus perhaps the lower results could be attributed to the seminal plasma interfering with the stain. This hypothesis would be supported by a more recent study by Kershaw-Young et al. (2013) in alpaca sperm where they obtained percentages from 37 to 64.3% of total acrosome reacted sperm evaluated with FITC-PNA/PI when they incubated the semen samples with different enzymes prior to the evaluation.

When evaluating the control samples at time 4 h, the percentage of total acrosome reacted sperm in our study was lower (45.2%) than that observed by Kershaw-Young and Maxwell (2011) in alpaca epididymal sperm after 3 h of incubation (64.4%). Among the differences between these studies, in addition to working with different species (llama *vs.* alpaca) and with sperm of different origins (epididymal *vs.* ejaculate) is the different media in which sperm were incubated (H-TALP in our study and PBS in Kershaw-Young's study). With regard to the incubation media, it is interesting to note that in our study we obtained a lower percentage of total (live + dead) reacted sperm using higher concentrations of BSA (6 mg ml^{-1}) and longer incubation times (4 h) than those used with alpaca epididymal sperm (1 mg ml^{-1} BSA and 3 h of incubation) (Kershaw-Young and Maxwell, 2011). This could be indicating that TALP medium is possibly better than PBS for incubating SAC spermatozoa for long periods of time because it would seem to produce a lower percentage of spontaneous acrosome reactions.

In most of the reported studies using the FITC-PNA/PI technique, the smears are fixed and permeabilised prior to evaluation under a microscope (Aitken et al., 1993; Cross and Waston, 1994; Ou, 1994; Fazeli et al., 1997; Morton et al., 2012). This methodology does not allow one to distinguish the true acrosome reaction (live reacted sperm) from what has been termed the false acrosome reaction (dead reacted sperm) (Christensen et al., 1994). In our study we adapted the technique in such a way as to be able to evaluate viability and the acrosome reaction simultaneously. In addition, the semen samples were treated in suspension, which would permit the use of flow cytometry to evaluate the acrosome reaction with this stain. The patterns we observed with the FITC-PNA/PI technique in our study in non-permeabilised llama sperm coincide with those reported by Fazeli et al. (1997) in non-permeabilised pig sperm bound to oocyte hemizonas.

In this study, the Coomassie blue (CB) stain was also used to evaluate the presence or absence of sperm acrosomes. This technique is simple and can be evaluated with light microscopy allowing its use in the field. When the effect of the AR inducers was evaluated with the CB stain, similar results were obtained to those observed with FITC-PNA/PI. That is to say, the percentage of sperm without acrosomes was greater in the samples incubated with the inducers, with the calcium ionophore showing the highest values of all. These results reinforce the use of CB under field conditions. Although the CB stain does not evaluate viability, the positive correlation observed between the total percentage of acrosome intact sperm (live + dead)

evaluated with FITC-PNA/PI and the acrosome-present sperm observed with CB, serves to validate this simple stain.

It is interesting to highlight that the sonication technique used in other species to remove the acrosome content was also effective in llama sperm. In this case, acrosome removal is considered to have been successful when no fluorescence is observed or only the equatorial region is fluorescent, because when the acrosome content is removed, the lectin can no longer bind or only does so to the remains of the acrosomal membrane. Likewise, sonicated sperm stained with CB did not present an acrosome, which also reinforces the possibility of using this technique to evaluate acrosomal status.

5. Conclusions

It was possible to evaluate the effect of different acrosome reaction (AR) inducers on viability and acrosomal status in llama spermatozoa, by using the FITC-PNA/PI technique. Calcium ionophore induced a high percentage of AR in llama sperm (showing that 5 μM can be used as a control of AR) but negatively affects motility and viability, whereas ionomycin (10 μM) and progesterone (30 μg/ml) had less adverse effects but also induced less AR. The correlation between the FITC-PNA/PI and CB patterns reinforce the use of this simple stain to evaluate the presence or absence of acrosomes in llama sperm. The results of this study would indicate that different concentrations and incubation times should be assayed before using these AR inducers in ART.

Conflict of interest

The authors declare there is no conflict of interest, actual or perceived, that could be perceived as prejudicing the impartiality of the research reported.

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