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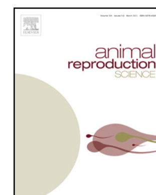

Evaluation of the acrosomal status in *Lama glama* sperm incubated with acrosome reaction inducers

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.
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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

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