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Evaluation of DNA fragmentation in llama (*Lama glama*) sperm using the sperm chromatin dispersion test

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ABSTRACT

The integrity of sperm chromatin is now viewed as an important factor in male fertility and in early embryonic development. The objectives of this study were: (1) adapt the simple and inexpensive sperm chromatin dispersion (SCD) test to evaluate DNA fragmentation in llama sperm and establish the halo patterns observed in this species, (2) determine an effective and reliable positive control for this technique and (3) evaluate correlation between the SCD test and the toluidine blue (TB) stain. To adapt the SCD test, three different mercaptoethanol (ME) concentrations were assayed (2.5%, 5% and 10% ME). To determine an effective positive control, three treatments (incubation at 100 °C for 30 min, incubation with 0.3 M NaOH for 30 min at room temperature and exposure to UV light for 2 h) were assayed. The concentration selected to use in the SCD test was 5% ME, because it produced the largest halo while still conserving the structure of the core. Four DNA dispersion patterns were clearly observed: (I) nuclei with large DNA dispersion halos; (II) nuclei with medium halos; (III) nuclei with very small halos and (IV) nuclei with no halo. All treatments used as positive controls were effective in producing DNA fragmentation. A high correlation ($r = 0.84$, $P = 0.03$) was observed between spermatozoa without halos and TB positive cells. To conclude, SCD patterns in llama sperm have been established as well as a repeatable positive control for the assay. The SCD test and TB stain are simple and inexpensive techniques that can be used to evaluate DNA damage in llama sperm.

1. Introduction

Standard semen characteristics such as ejaculate volume, sperm concentration, plasma membrane function and integrity, sperm motility and sperm morphology are used to evaluate seminal quality. In addition, the integrity of sperm chromatin is now viewed as an important factor in male fertility and in early embryonic development (Zini et al., 2001). However, studies on DNA integrity are not simple and, therefore, the addition of these tests to routine sperm evaluation is still not widespread. When assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF) or intracytoplasmatic sperm injection (ICSI) are used, the evaluation of chromatin integrity is even more important. The presence of defects in the genetic material, such

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as abnormalities in chromatin condensation, DNA integrity or the presence of chromosome abnormalities such as aneuploidies, are closely associated to infertility (Aravindan et al., 1997; Tsarev et al., 2009). In addition, Sharma et al. (2004) observed a correlation between DNA damage and ART outcome in human semen.

Cortés-Gutiérrez et al. (2007) classified the techniques that evaluate DNA fragmentation into two groups. The first includes the methodologies used to mark double and simple strand breaks, for example, the use of enzymatic processes to incorporate marked nucleotides *in situ* such as terminal dUTP nick-end labeling (TUNEL) or *in situ* nick translation (ISNT). Owing to the fact that strand breaks increase DNA susceptibility to denaturation, the second strategy includes the techniques that measure the ability of chromatin to denaturalize after treatment. In this group are the sperm chromatin structure assay (SCSA), the DNA breakage detection-fluorescence *in situ* hybridization (DBD-FISH), single-cell-gel-electrophoresis (SCGE) or comet assay and the sperm chromatin dispersion (SCD) test. However, some DNA fragmentation techniques require expensive equipment for optimal analysis, are labor intensive, or require the use of enzymes whose activity and accessibility to DNA breaks may be irregular (Fernández et al., 2005). With the SCD test, spermatozoa are immersed in an agarose matrix on a slide, treated with an acid solution to denature DNA that contains breaks, and then treated with a lysing buffer to remove membranes and proteins. Removal of nuclear proteins results in nucleotides with a central core (residual nucleus) and a peripheral halo of dispersed DNA loops. Sperm nuclei with elevated DNA fragmentation produce very small or no halos of DNA dispersion, whereas sperm with low levels of DNA fragmentation release their DNA loops forming large halos. These results have been confirmed by DBD-FISH in human sperm (Fernández et al., 2003). Thus, DNA fragmentation can be accurately determined using the SCD test, a simple, rapid, accurate, highly reproducible, and inexpensive technique (Fernández et al., 2005). Spermatozoa may be visualized using fluorescent or bright-field microscopy to assess the relative size of the halos. Visual differentiation of categories is very clear and evident besides which, the flagellum are easily visualized thus making it possible to easily distinguish between spermatozoa and other kinds of cells.

Another alteration of the DNA is chromatin decondensation, which can be evaluated using the toluidine blue (TB) stain, a nuclear dye used to evaluate the degree of sperm chromatin condensation by detecting the rupture or absence of disulfide bonds. TB has been used to evaluate DNA condensation in our laboratory in horse, llama, guanaco and alpaca sperm (Sardoy et al., 2008; Carretero et al., 2009, 2010a,b); however DNA fragmentation has not yet been evaluated in South American Camelids.

The objectives of this study were: (1) to adapt the simple and inexpensive SCD test to evaluate DNA fragmentation in llama sperm and establish the halo patterns observed in this species, (2) to determine an effective and reliable positive control for this technique and (3) to evaluate correlation between the SCD test and the TB stain.

2. Materials and methods

2.1. Animals and location

The study was conducted 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, nine male *Lama glama* ranging between 6 and 10 years of age and weighing 144.75 ± 16.28 kg (mean ± SD) were used. Animals were maintained on pasture in pens and supplemented with alfalfa; they also had free access to fresh water throughout the study. All males were shorn during the month of November.

2.2. Semen collection

Semen collections were conducted using electroejaculation (EE) under general anesthesia according to the technique described by Director et al. (2007). All procedures were approved by the Committee for the Use and Care of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2010/24).

2.3. Semen evaluation

The following semen characteristics were evaluated: ejaculate volume, sperm concentration, motility, membrane function and integrity and sperm morphology. Sperm counts were performed using a Neubauer hemocytometer. Sperm motility (percentage of total motile sperm) was evaluated in raw semen using a warm stage (37 °C) and a phase contrast microscope (100×). 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 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 evaluating 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. Sperm morphology was evaluated in a drop of raw semen (between a slide and a coverslip) using phase contrast microscopy (1000×) with 200 sperm per sample evaluated. The same person conducted all evaluations.

2.4. SCD test

2.4.1. Positive controls

In a short assay (one ejaculate from each of five males), three different treatments were used to produce DNA damage: (1) incubation at 100 °C for 30 min, (2) incubation with 0.3 M NaOH for 30 min at room temperature and (3) exposure to UV light (36 W) for 2 h. To corroborate their effectiveness, slides were prepared for each treatment with