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Characterization of functional variables in epididymal alpaca (*Vicugna pacos*) sperm using imaging flow cytometry

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ABSTRACT

Epididymal alpaca sperm represent an alternative model for the study of alpaca semen. The objective of this study was to characterize the normal values of some functional variables in epididymal alpaca sperm using imaging flow cytometry. Alpaca testicles ($n = 150$) were processed and sperm were recovered from the cauda epididymides. Only 76 samples with acceptable motility and sperm count were considered for assessment by imaging flow cytometry. Acrosome integrity and integrity/viability were assessed by FITC-PSA/PI and FITC-PNA/PI. Mitochondrial membrane potential (MMP) was assessed by MitoTracker CMXRos and MitoTracker Deep Red FM. Lipid peroxidation was evaluated using BODIPY 581/591 C11. Results show that the mean values for acrosome-intact sperm were 95.03 ± 6.39% and 93.34 ± 7.96%, using FITC-PSA and FITC-PNA, respectively. The mean values for acrosome-intact viable sperm were 60.58 ± 12.12% with FITC-PSA/PI and 58.81 ± 12.94% with FITC-PNA/PI. Greater MMP was detected in 65.03 ± 15.92% and 59.52 ± 19.19%, using MitoTracker CMXRos and MitoTracker Deep Red FM, respectively. Lipid peroxidation was 0.84 ± 0.95%. Evaluation of acrosome-intact and acrosome-intact viable sperm with FITC-PSA/PI compared with. FITC-PNA/PI or MMP with MitoTracker CMXRos compared with MitoTracker Deep Red FM were correlated ($P < 0.05$). The MMP using MitoTracker CMXRos was the only variable correlated ($P < 0.05$) with sperm motility ($r = 0.3979$). This report provides a basis for future research related to alpaca semen using the epididymal sperm model.

1. Introduction

Alpaca (*Vicugna pacos*) and llamas (*Lama glama*) are South American camelids of considerable economic importance in Peru, Bolivia, Chile and Argentina. In Peru, alpaca are a source of wool fiber and meat for many families living in the Andean highlands. Advances in genetic improvement in South American camelids are limited by the lack of an adequate sperm cryopreservation protocol that ensures success after artificial insemination with frozen semen. Sperm motility and viability have been the traditional variables used for evaluating sperm quality in different studies (Santiani et al., 2005, 2013; Morton et al., 2007, 2010; Banda et al., 2010). Currently, however, new variables have been incorporated to assess sperm function in alpacas, such as DNA fragmentation (Santiani et al., 2012), acrosome integrity, mitochondrial membrane potential

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(Cheuqueman et al., 2013) and lipid peroxidation of plasma membrane (Evangelista et al., 2014).

The assessment of acrosome integrity is important because acrosome enzymes are essential for penetrating the egg investments in order to achieve fertilization (Edy, 2006). For the evaluation of acrosome integrity, Pisum sativum (PSA) and Arachis hypogaea (PNA) lectins conjugated with fluorescein isothiocyanate (FITC-PNA and FITC-PSA) can be used. The PNA lectin binds to β-galactose terminals located within the outer acrosomal membrane (Silva and Gadella, 2006), while PSA lectin binds to glycoproteins found in the acrosomal matrix (Celeghini et al., 2010; Silva and Gadella, 2006). In sperm with reacted or damaged acrosomes, fluorochromes are specific for the acrosomal membrane, binding to receptors, and green fluorescence is observed. These lectins have been used for the evaluation of acrosome integrity in humans (Risopatrón et al., 2001) and rams (Celeghini et al., 2010). In alpacas, Cheuqueman et al. (2013) evaluated the viability and acrosome integrity in ejaculated sperm collected by artificial vagina with FITC-PSA and PI, while Ugarelli et al. (2015) evaluated different concentrations of FITC-PSA and FITC-PNA to assess acrosome integrity in frozen/thawed sperm obtained by post-coital sperm washing.

Mitochondrial membrane potential (MMP) is another variable that has been evaluated in humans (Amaral and Ramalho-Santos, 2010), bulls (Hallap et al., 2005) and alpacas (Cheuqueman et al., 2013). The flagella movement of sperm results from the presence of energy derived from ATP which is produced by the mitochondrial organelles located in the middle part of the sperm (Hallap et al., 2005). Any failure in mitochondrial membrane potential would affect sperm functionality directly. Therefore, measuring mitochondrial membrane potential could be a more objective and useful alternative for measurement of sperm quality. MitoTracker probes are cell-permeant mitochondrion-selective dyes that passively diffuse across the plasma membrane and accumulate in active mitochondria (Gregory, 2002). These probes have not been used in alpaca sperm.

Furthermore, the study of lipid peroxidation has also been described in alpacas and related to oxidative damage during cryopreservation (Evangelista et al., 2014) but using the thiobarbituric acid method. Sperm cells are particularly sensitive to oxidative stress due to the low content of antioxidant enzymes, large proportion of polyunsaturated fats in the plasma membrane and presence of NADPH membrane-bound oxidase (Guthrie and Welch, 2012). Moreover, alpaca sperm are highly sensitive to lipid peroxidation due to the greater proportion of abnormalities that result from this condition in this species as compared to many other species (Santiani et al., 2012). The BODIPY 581/591 C11 probe has been used to study lipid peroxidation in stallion (Neild et al., 2005) and buffalo (Kadirve et al., 2014) sperm.

In recent years, due to the difficulty of obtaining semen samples from alpaca males, many researchers have used epididymal sperm in conducting research (Morton et al., 2007, 2010; Banda et al., 2010; Vásquez et al., 2012). These samples are characterized by an absence of viscosity because the sperm has not been exposed to seminal plasma. For this reason, some characteristics of sperm function, such us viability, acrosome integrity and MMP may result in different values as compared with those reported in semen samples from alpacas (Cheuqueman et al., 2013). Even so, other variables such as acrosome integrity assessment using FITC-PNA, assessing MMP with MitoTracker or determining lipid peroxidation using BODIPY 581/591 C11 have not been reported for alpaca sperm. In addition, assessment by flow cytometry has the advantage of involving an automated process that can evaluate more than 10,000 cells objectively in a few seconds, compared to traditional microscopy, with which the technology can only be used to subjectively evaluate no more than 200 cells (Muratori et al., 2008). Additionally, imaging flow cytometry combines the use of standard flow cytometry with digital microscopy, providing a rapid and accurate analysis of cell populations (Basiji et al., 2008). Therefore, the objective of the present study was to assess normal values of viability and acrosome integrity (FITC-PSA/PI and FITC-PNA/PI), MMP (MitoTracker CMXRos and MitoTracker Deep Red FM) and lipid peroxidation (BODIPY) in epididymal alpaca sperm using imaging flow cytometry.

2. Materials and methods

2.1. Reagents

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich.

2.2. Samples and sperm recovery

Testes ($n = 115$) with attached epididymides were obtained from alpacas slaughtered around noon during the spring of 2015. Samples were washed and placed in plastic bags with saline solution (0.9% NaCl). The bags were placed on top of a cold pack in a foam cool box and transported to the laboratory and arrived at this site approximately 20 h post collection. Samples were processed after being delivered to the laboratory. For recovery of epididymal sperm, the testes and epididymides were removed from the bags and washed in fresh saline solution. Parietal vaginal tunic was removed, and the weight and length of testicles were recorded. Cauda epididymides, including about 1 cm of the proximal ductus deferens were separated and transferred to 35 mm plastic dishes. Tris-based medium (1 mL; 2.71 g Tris, 1.4 g citric acid, 1 g fructose in 100 mL) was added to each sample and samples were subsequently minced with a scalpel, allowing for sperm motility to occur. Suspensions of cells and medium were placed in 1.5 mL plastic tubes.

Sperm motility was assessed under a coverslip (18 mm × 18 mm) on a warm glass slide using light microscopy (400× magnification), and sperm concentration was assessed using a hemocytometer. Only samples with motility ≥20% and a sperm concentration $\geq 30 \times 10^6$ cells/mL from testicles with a weight of ≥10 g and length of ≥3 cm were processed. Suitable samples were centrifuged at 800 $g \times 5$ min. Pellets were re-suspended in 300 μL PBS and aliquoted in groups of 100 μL. Half of the suitable samples ($n = 38$) were used for evaluation of acro-