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Assessment of Sperm Function Parameters and DNA Fragmentation in Ejaculated Alpaca Sperm (*Lama Pacos*) by Flow Cytometry

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Contents

Flow cytometry has been shown to be an accurate and highly reproducible tool for the analysis of sperm function. The main objective of this study was to assess sperm function parameters in ejaculated alpaca sperm by flow cytometry. Semen samples were collected from six alpaca males and processed for flow cytometric analysis of sperm viability and plasma membrane integrity using SYBR-14/PI staining; acrosomal membrane integrity using FITC-conjugated *Pisum Sativum Agglutinin*/PI labelling; mitochondrial membrane potential ($\Delta\psi$m) by staining with JC-1 and DNA Fragmentation Index (DFI) by TUNEL. The results indicate that the mean value for sperm viability was 57 ± 8 %. Spermatozoa with intact acrosome membrane was 87.9 ± 5%, and viable sperm with intact acrosomal membrane was 46.8 ± 9%, high mitochondrial membrane potential ($\Delta\psi$m) was detected in 66.32 ± 9.51% of spermatozoa and mean DFI value was 0.91 ± 0.9%. The DFI was inversely correlated with high $\Delta\psi$m ($p = 0.04$; $r = -0.41$) and with plasma membrane integrity ($p = 0.01$; $r = -0.47$). To our knowledge, this is the first report of the assessment on the same sample of several parameters of sperm function in ejaculated alpaca sperm by flow cytometry.

Introduction

The alpaca industry in our country is still under development but considered highly attractive in economic terms, and it has great potential for long-term commercial development. Despite the commercial potential of these camelids, their introduction in the market place has been limited due to their low fertility rate (50–60%) (Fernandez-Baca 1993). The atypical reproductive physiology of these species limits extrapolation of the results obtained using reproductive technologies in other domestic animals (Reyna 2005). Outside their natural habitat, alpaca animals are considered a non-seasonal breeder and an induced ovulating species (Sumar 1999). The South American camelid ejaculate is characterized by having a small volume, lower sperm concentration and greater viscosity than that found in other species, making it difficult to evaluate the macroscopic and microscopic parameters of the ejaculate and to establish a relationship between normal semen characteristics and fertility thresholds (Giuliano et al. 2008). The sperm parameters that are usually evaluated in these species include sperm motility, acrosome integrity, membrane integrity, sperm viability and sperm morphology (Bravo et al. 1997; Buendía et al. 2002; Morton et al. 2010a,b). Information generated to date has been useful in establishing general guidelines for breeding with regard to semen parameters (Tibary and Vaughan 2006).

Flow cytometry has been shown to be an accurate and reproducible means for the analysis of sperm function, in addition to the high throughput that this technique can provide in terms of the analysis of thousands of cells within seconds (Martínez-Pastor et al. 2010; Cheuquemán et al. 2012). Therefore, characterization of sperm function by flow cytometry could be of paramount importance in establishing sperm function parameters cut-offs (Ax et al. 2000) and in improving fertility rates in camelids (Bravo et al. 1997; Buendía et al. 2002). Currently, there is limited information concerning the assessment of sperm function in South American camelid sperm.

The main objective of this study was to assess sperm function parameters in ejaculated alpaca spermatozoa by flow cytometry, including plasma membrane integrity, mitochondrial membrane potential, acrosomal membrane integrity and DNA fragmentation, in order to provide reliable data that can be used for the standardization of normal alpaca sperm function. To our knowledge, this is the first report of assessment in the same semen sample of important parameters associated with the sperm function in ejaculated alpaca sperm by flow cytometry.

Materials and Methods

This study was approved by the Institutional Review Board Committee of Universidad de La Frontera, Chile. A flow sheet of the experimental design of this study is shown in Fig. 1. Unless otherwise indicated, all chemicals used were purchased from Sigma (St Louis, MO, USA). All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA).

Animals and location

Six alpaca males of proven fertility, ranging between 6 and 10 years of age and weighing 75.5 ± 3.5 kg (mean ± SD), were used in this study and their semen collected during the months of January to March, which is well within the breeding season of the alpaca males. Animals were maintained in the Llama del Sur Ranch, in the City of Temuco, Chile (38°S, 72°W, at sea level). Alpacas were kept in natural pastures with free access to fresh water and housed in outdoor pens at night. The health status of the alpaca males was periodically monitored by a veterinarian. Alpaca males were trained for semen collection before this study was initiated and

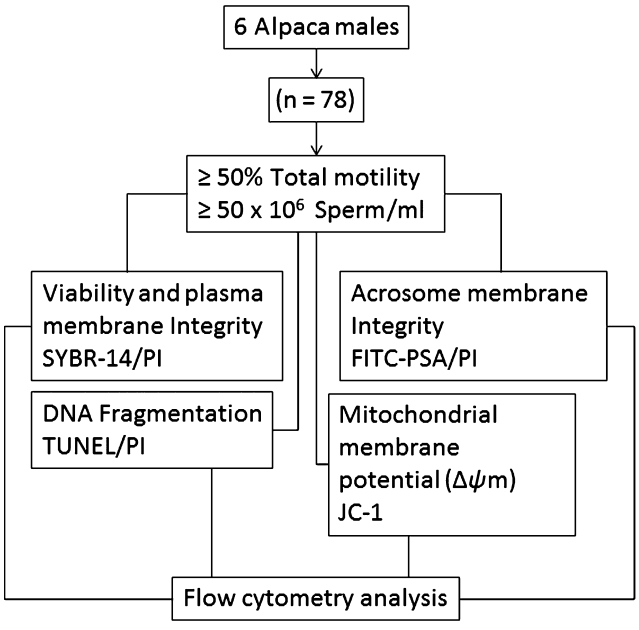

Fig. 1. Diagram of the experimental design of the study

were routinely used as semen donors. Flow cytometric analysis were performed at the Centre of Reproductive Biotechnology (CEBIOR) laboratories, Faculty of Medicine, University of La Frontera, Temuco, Chile.

Semen collection and evaluation

Thirteen ejaculates from each of the six alpaca males (n = 78) were utilized. Ejaculates were collected once a week from each of the six alpaca males included in this study using an artificial vagina, according to the technique described by Von Baer and Hellemann (1998). In each experimental trial, aliquots of semen samples collected each week from the six alpaca males were mixed to minimize sample-to-sample and male-to-male variability, as well as to increase the volume for processed samples by different techniques of flow cytometry. The semen samples were collected between the months of January and March 2011 and used for the assessment of sperm function by flow cytometry at CEBIOR laboratories.

Immediately after semen collection from the alpaca male with the artificial vagina, a 10-μl aliquot of each ejaculate was placed on a slide mounted with a cover slip and sperm concentration and per cent sperm motility assessed. Sperm motility was measured by phase-contrast microscopy using a light microscope (Carl Zeiss, Jena, Germany) with heated stage at 37°C and a magnification of 400 × ***g***. Non-progressive as well as progressive sperm were considered as motile. Sperm concentration was determined using a Neubauer counting chamber. Only samples containing ≥ 50% total motility and ≥ 50 × 10^6 sperm/ml were used in our study, as previously described (Kershaw-Young and Maxwell 2011).

In each experimental trial, the semen sample was diluted with a lactose-based extender (Morton et al. 2007) for their transport (approximately 15 min.) to the CEBIOR laboratories for flow cytometric analysis of sperm function. Subsequently, the semen sample was centrifuged at 720 × ***g*** for 5 min, the supernatant was discarded, and the pellet was washed twice by centrifugation at 720 × ***g*** for 3 min in PBS medium, and the sperm pellet was resuspended in PBS medium to obtain a final concentration of 2 × 10^6 cells/ml. Subsequently, aliquots for the different sperm evaluation techniques were prepared from this sperm suspension.

Forward-Scatter-Height (FSC-H) was plotted against Side-Scatter-height (SSC-H) (Fig. 2a) and autofluorescence control (Fig. 2b) were used to define the specific settings used for flow cytometric analysis of sperm function parameters in ejaculated alpaca sperm.

Flow cytometric analysis of sperm function parameters

Flow cytometric analysis was performed on a BD FACS Canto II™ Flow Cytometer (BD Biosciences, San José, CA, USA) and a BD FACSDIVA™ software (updated for version 6.0) was used. The collection optics configuration included two detector arrays, consisting in photomultiplier tubes (PMTs) arranged in one octagon and one trigon. The octagon contained five PMTs detecting light from the 488 nm (blue) laser. One PMT in the octagon collects side-scatter (SSC) signals. The trigon contains two PMTs detecting light from the 633 nm (red) laser. For each sample, 10 000 events were captured.

The following sperm function parameters were evaluated:

Viability and plasma membrane integrity

Viable spermatozoa with intact plasma membranes were detected using SYBR-14 /PI (LIVE /DEAD®Sperm Viability kit; Molecular Probes cat no L-7011, Eugene, OR, USA) according to the manufacturer's instructions. As a positive control for SYBR-14 (Fig. 3a), a sperm swim-up was performed to obtain spermatozoa with intact plasma membrane. Aliquots of 2 μl of SYBR-14 (100 nM final concentration) were added to 400 μl of the sperm suspension in PBS (2 × 10^6 cells/ml). As a positive control for propidium iodide (PI) (Fig. 3b), spermatozoa were incubated in a 4% formaldehyde solution for 30 min at room temperature and centrifuged at 300 × ***g*** for 5 min. The supernatant was discarded, and the pellet resuspended in a final volume of 400 μl of PBS (final volume) and a 2-μl aliquot of PI (stock solution of 2.4 mM) added. Both positive controls

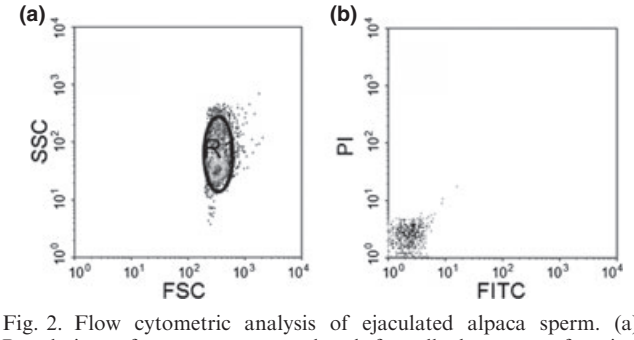

Fig. 2. Flow cytometric analysis of ejaculated alpaca sperm. (a) Population of spermatozoa analyzed for all the sperm function parameters evaluated. (b) Autoflorescence control of alpaca sperm

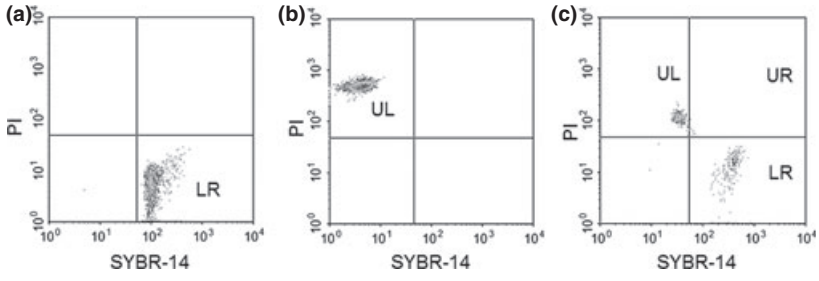

Fig. 3. Sperm viability and plasma membrane integrity analysis. (a) Positive control for SYBR-14(LR). (b) Positive control for PI (UL). (c) Analysis of pooled semen samples with SYBR-14/PI stain; viable spermatozoa (LR), dead spermatozoa (UL), and moribund spermatozoa (UR)

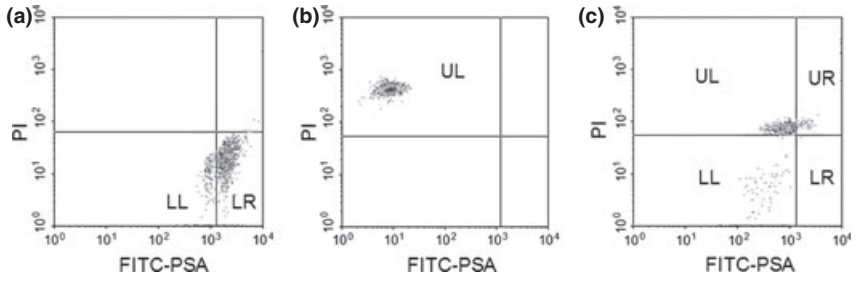

Fig. 4. Acrosomal membrane integrity analysis. (a) Positive control for FITC-PSA; intact acrosome membrane (LL), damaged acrosomal membrane (LR). (b) Positive control for PI (UL). (c) Analysis of pooled semen samples with FITC-PSA/PI stain; viable spermatozoa with intact acrosome membrane (LL), viable spermatozoa with damaged acrosomal membrane (LR), dead spermatozoa with intact acrosomal membrane (UL), and dead spermatozoa with damaged acrosomal membrane (UR)

were incubated for 8 min at 37°C in the dark and immediately analysed by flow cytometry. Each test was run in triplicate.

For each experiment, a volume of 2 μl of SYBR-14 (100 nM final concentration) and 2 μl of PI (stock solution 2.4 mM) was added to 400 μl sperm suspension in PBS (2×10^6 cells/ml) and incubated for 8 min at 37°C in the dark and immediately analysed by flow cytometry.

Spermatozoa were classified as viable with an intact plasma membrane (PI-negative/SYBR-14-positive), dead (PI-positive/SYBR-14-negative), or moribund (PI-positive/SYBR-14-positive). (See Fig. 3c). Each test was run in triplicate.

Acrosomal membrane integrity

Acrosomal membrane integrity was assessed by fluorescence staining with FITC-conjugated *Pisum SativumAgglutinin* (FITC-PSA)/PI according to the manufacturer's instructions (kit FITC-PSA/PI; St Louis, MO, USA) (Peña et al. 1999). As positive control for FITC-PSA (Fig. 4a), a 4 μl of the calcium ionosphere solution (stock 1 μM) was added to 400 μl of sperm suspension in PBS (2×10^6 cells/ml) and incubated for 40 min at 37°C. As a positive control for PI, spermatozoa were incubated in a 4% formaldehyde solution for 30 min at room temperature and centrifuged at $300 \times \boldsymbol{g}$ for 5 min. The supernatant was discarded and the pellet resuspended in a final volume of 400 μl of PBS and 2 μl aliquot of PI (stock solution 2.4 mM) added (Fig. 4b). Both positive controls were incubated for 8 min at 37°C in the dark and immediately analysed by flow cytometry. For each experiment, 20 μl of FITC-PSA (0.1% stock) was added to a volume of 400 μl of the sperm suspension in PBS (2×10^6 cells/ml) followed by incubation for 8 min at 37°C in the dark. After centrifuging at $300 \times \boldsymbol{g}$ for 5 min, the supernatant was discarded and the pellet resuspended in 400 μl of PBS and immediately analysed by flow cytometry. The spermatozoa were classified as viable with an intact acrosomal membrane (PI-negative/FITC-PSA-negative) or viable with a damaged acrosomal membrane (PI-negative-/FITC-PSA-positive), dead with an intact acrosomal membrane (PI-positive/FITC-PSA-negative) or dead with a damaged acrosomal membrane (FITC-PSA-positive/PI-positive) (Fig. 4c). Each test was run in triplicate.

Mitochondrial membrane potential ($\Delta\psi m$)

Mitochondrial membrane potential ($\Delta\psi$m) of spermatozoa was assessed using 5, 5′, 6 6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolcarbocyanine iodide known as JC-1. This test was performed using the Mitochondrial Permeability Detection kit AK-116 (MIT-E-ψTM; BIOMOL International LP, Plymouth Meeting, PA, USA) following the manufacturer's instructions. As a positive control for high $\Delta\psi$m, a sperm swim-up was performed. Aliquots of 2 μl of JC-1 were added to 400 μl of the sperm suspension (2×10^6 cells/ml) selected by swim-up (Fig. 5a). As a control for low $\Delta\psi$m, 2×10^6 spermatozoa/ml were incubated in 400 μl of carbonyl cyanide 3-chlorophenylhydrazone solution (CCCP) (final concentration of 100 μM) for 30 min at 37°C (Fig. 5b). Spermatozoa were washed and resuspended in PBS to a final volume of 400 μl and a 2 μl aliquot of JC-1 added. A volume of 400 μl of the sperm suspension in PBS (2×10^6 cells/ml) was centrifuged at $300 \times \boldsymbol{g}$ for 5 min, and the supernatant discarded. The resulting pellet was resuspended in PBS and a 2 μl aliquot of JC-1 added (the stock solution was prepared with 1 vial of JC-1 and 125 μl of DMSO, according manufacturer's instructions). All

samples, including controls, were incubated for 8 min at 37°C in the dark. The cell suspensions were then centrifuged at 300 × *g* for 5 min, the supernatant discarded and the sperm pellet resuspended in 400 µl of PBS and immediately analysed by flow cytometry (Fig. 5c). Each test was run in triplicate.

Sperm DNA fragmentation

Sperm DNA fragmentation was evaluated by terminal deoxinucleotidil transferase uracil nick end labelling (TUNEL). This test was performed using the TUNEL protocol according to the manufacturer's instructions (In Situ Cell Death Detection Kit with Fluorescein, Roche®, Mannheim, BW, Germany). The sperm suspension in PBS (2×10^6 cell/ml) was fixed in 4% of formaldehyde for 15 min at 4°C. The sperm suspensions were then washed twice in PBS and permeabilized in 0.5% Triton X-100 and 0.1% of citrate solution for 30 min at room temperature. A positive control was used by treating the sperm samples with DNase I (Promega Co., Madison, WI, USA) and incubated for 10 min at 37°C (Fig. 6a). All samples were incubated with a 5-µl aliquot of the enzymatic solution (deoxinucleotidil terminal transferase enzyme) and with 45 µl of the labelling solution (2′-deoxyuridine 5′-triphosphate-dUTP + fluorescein isothiocyanate- conjugated-FITC) for 60 min in a humid chamber at 37°C in the dark. The negative control was incubated containing only 50 µl of labelling solution (thus excluding the enzymatic solution). After washing, a positive control for PI (Fig. 6b) and experimental samples were stained with 2 µl aliquots of PI (2.4 mM stock solution) and resuspended in 400 µl of PBS and immediately analysed by flow cytometry (Fig. 6c).Each test was run in triplicate. The percentage of spermatozoa with DNA fragmentation was expressed as the DNA Fragmentation Index (DFI).

Statistical analysis

Data were analysed using GraphPad Prism ® software, version 5.0 (GraphPad Software, San Diego, CA, USA). For the different functional parameters evaluated by flow cytometry, the data are represented as the mean percentage ± standard deviation (SD) recorded for the 13 experiments performed. Spearman's correlation for non-parametric data was used to detect correlations among the sperm variables examined. The level of significance was set at a p-value < 0.05.

Results

The use of flow cytometry allows evaluation in the same sperm sample of the most important parameters associated with the function of the different membranes present in the sperm: plasma membrane, acrosomal and mitochondrial membranes.

Evaluation of sperm membranes

Flow cytometric analysis of sperm viability in alpaca sperm showed that the percentage of viable spermatozoa with intact plasma membrane was 57 ± 8%. Total intact acrosome were 87.9 ± 5%, percentage of viable spermatozoa with intact acrosomal membrane was 46.8 ± 9%, dead with intact acrosome membrane were 42.24 ± 12%, viable with damaged acrosomal membrane were 10.2 ± 5% and dead with a damaged acrosome membrane were 0.7 ± 0.6%. The percentage of sperm with high $\Delta\psi$m was 66.32 ± 9.51%.

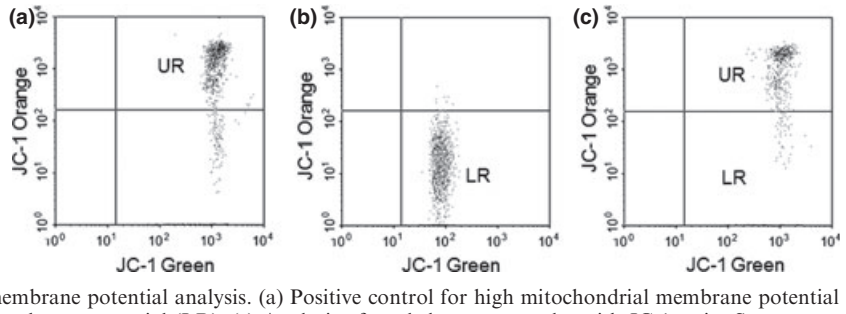

Fig. 5. Mitochondrial membrane potential analysis. (a) Positive control for high mitochondrial membrane potential (UR). (b) Positive control for low mitochondrial membrane potential (LR). (c) Analysis of pooled semen samples with JC-1 stain; Spermatozoa with high mitochondrial membrane potential (UR), spermatozoa with low mitochondrial membrane potential (LR)

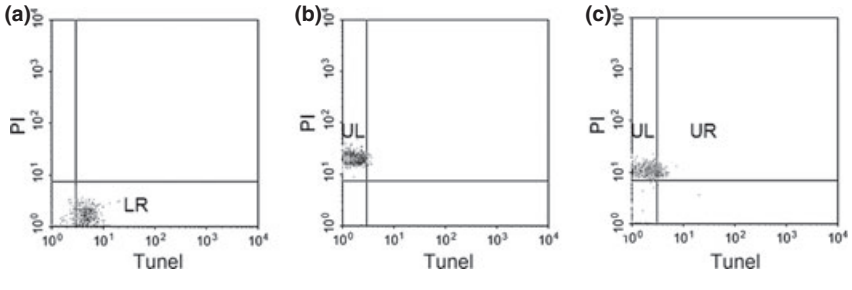

Fig. 6. DNA fragmentation assay. (a) Positive control for TUNEL; damaged DNA (LR). (b) Positive control for PI; fixed spermatozoa PI+ (UL). (c) Analysis of pooled semen samples by TUNEL/PI assay; Spermatozoa without DNA fragmentation (UL), spermatozoa with damaged DNA (UR)

Determination of DNA fragmentation

The mean of DNA fragmentation value obtained in ejaculated sperm by TUNEL/flow cytometry was 0.91 ± 0.9%.

Correlations between the studied sperm parameters

Correlation analysis of all the sperm function parameters evaluated indicated that sperm DNA fragmentation was inversely correlated with high $\Delta\psi m$ ($p = 0.04$; $r = -0.41$) and plasma membrane integrity ($p = 0.01$; $r = -0.47$). No significant correlation was found between the other sperm function parameters evaluated (Table 1).

Discussion

In recent years, flow cytometry has become a powerful tool for the evaluation of sperm function and has been increasingly used for this purpose and also in veterinary science research (Martínez-Pastor et al. 2010).

South American camelids are species with high economic potential as they provide fibre and meat for High-Andean people (Santiani et al. 2005). However, the fertility rates of South American camelids are relatively low and there is a pressing need to optimize pregnancy rates in these species. For this reason, the characterization of sperm function in alpaca sperm becomes of paramount importance in obtaining the fertility thresholds required to optimize pregnancy outcome in these species and also in the selection of breeding animals.

Plasma and acrosomal membranes integrity, mitochondrial function and DNA integrity are key parameters in the evaluation of sperm function (Cheuquemán et al. 2012). In addition, sperm DNA integrity is a *sine qua non* condition for normal embryo and foetal development (Zini et al. 2001; Carretero et al. 2012). This is precisely the aim of this study; characterize these sperm function parameters in ejaculated alpaca sperm through the different tests by flow cytometry to achieve a better understanding of the functionality of the alpaca spermatozoa.

Flow cytometric analysis in the same semen sample allowed assessment of plasma and acrosomal membrane integrity, mitochondrial membrane potential and DNA integrity in ejaculated alpaca sperm. The use of sample pooling in our study allowed us to obtain sperm function values that are representative of alpaca sperm and that can be used to obtain cut-off fertility values and, at the same time, minimize sample-to-sample and male-to-male variability (Giuliano et al. 2008).

Table 1. Correlations between the sperm parameters evaluated

Functional parameters	p-value	*r*	Correlation
Plasma membrane integrity – Acrosomal membrane integrity	0.05	0.38	ns
Acrosomal membrane integrity – DNA fragmentation	0.75	−0.06	ns
Acrosomal membrane integrity – Mitochondrial membrane potential	0.25	0.23	ns
DNA fragmentation – Mitochondrial membrane potential	0.04	−0.41	Inverse correlation[a]
DNA fragmentation – Plasma membrane integrity	0.01	−0.47	Inverse correlation[a]
Mitochondrial membrane potential – Plasma membrane integrity	0.81	−0.05	ns

[a]Statistically significant.

The sperm viability and plasma membrane integrity values obtained in the present study were similar than those obtained in alpaca sperm assessed with Hancock's stain (57 ± 8 % vs 65%, respectively) (Bravo et al. 1997), by epifluorescence microscopy with CFDA/PI dyes (57 ± 8 % vs 44 ± 14%, respectively) (Carretero et al. 2012) and by flow cytometry with Syto-16/PI (57 ± 8 % vs 47%, respectively) (Kershaw-Young and Maxwell 2011). Furthermore, our results are lower than obtained using the trypan blue standard technique with different extenders (81.58 ± 2.8% vs 84.6 ± 3.5, 87.6 ± 3.2, 82.0 ± 4.0, 80.6 ± 5.8 and 79.6 ± 7.5%) reported by other authors in Dromedary camel (Wani et al. 2008). The lower sperm viability and plasma membrane integrity values reported in most camelids studies against the high viability described in other species that are near to 80% (Cheuquemán et al. 2012) may be also related to the high semen viscosity found in alpaca semen which make it difficult to handle during laboratory procedures (Tibary and Vaughan 2006), which could impact the results of our analysis.

The total acrosomal membrane integrity is according with other authors for ejaculated alpaca sperm using the Giemsa staining (87.9 ± 5% vs 86–88%) (Morton et al. 2010a); FITC-PNA in epididymal sperm (87.9 ± 5% vs 89.3%) (Morton et al. 2010b) are lower than those reported by dual Trypan blue and Giemsa staining in Dromedary camels (87.9 ± 5% vs 95%, respectively) (Wani et al. 2008) but are higher than those reported by Kershaw-Young and Maxwell (2011) (87.9 ± 5% vs 60.1 ± 1.05%) using isothiocyanate-conjugated lectin from *Arachis hypogaea* and fluorescence microscopy. Differences between our viability and plasma and acrosomal membranes integrity values and those reported by other authors can be explained to a great extent by the fact that the analysis of sperm function parameters in our study was performed using an extensively validated flow cytometry protocol. Most of these studies were performed using either phase-contrast or fluorescence microscopy where only a relatively low number of cells and in a subjective way are evaluated. In sharp contrast, flow cytometric analysis allows for the objective, unbiased analysis of thousands of sperm cells within seconds in a highly reproducible manner, capturing multiple sperm function parameters, and thus providing more reliable information concerning sperm function assessment (Martínez-Pastor et al. 2010). In addition, it also allows working with small-size samples, increasing the sensitivity of the analysis and still allowing for the measurement of multiple fluorochromes (Gillan et al. 2005).

To our knowledge, this is the first study that describes the analysis of mitochondrial function in ejaculated alpaca sperm by flow cytometry using the JC-1 stain. The mean percentage of mitochondrial membrane potential obtained in our study is close to that obtained

in canine (66.32 ± 9.51% vs 72% and 80.9 ± 17%) (Volpe et al. 2009; Cheuquemán et al. 2012) ram (66.32 ± 9.51% vs 68.82 ± 8.26) (Câmara et al. 2011) and bull sperm (66.32 ± 9.51% vs 76.8 ± 1.4%) (Celeghini et al. 2008).

It is worth noting that the mean DFI value obtained for ejaculated alpaca sperm in our study was less than 1% (0.9%). This value is lower than that reported by Kershaw-Young et al., in ejaculated alpaca spermatozoa (near 5%) (Kershaw-Young and Maxwell 2011), in ejaculated human spermatozoa from sperm bank donors (15.9%and 7%) (De Iuliis et al. 2009; Aitken et al. 2010), in ejaculated equine spermatozoa (12.48%) (Gutiérrez-Cepeda et al. 2012) and in ejaculated chicken sperm (6.2%) (Gliozzi et al. 2011). This low DFI value observed in alpaca ejaculated sperm could also be related to the protective effect of seminal plasma in these species (Tibary and Vaughan 2006). On other hand, the differences observed between different mammalian species maybe also related to the degree of artificial selection in each domestic animal (Carretero et al. 2012). The other important conclusion that can be drawn from the low DFI value obtained in our study is that sperm function was well preserved during transport of the samples to our laboratories in the extender lending more credibility to the data obtained in this study.

The inverse correlation between the DFI value, sperm viability and plasma membrane integrity is in good agreement with the results reported by other authors in human ($r = -0.4788$ vs $r = -0.65$) (Zribi et al. 2011) and bull sperm ($r = -0.4788$ vs $r = -076$) (Kasimanickam et al. 2006). Although this correlation is usually found, it cannot always be established because both parameters may behave in an independent manner (Fernández et al. 2009; Gosálvez et al. 2009; Gosálvez et al. 2011).

The inverse correlation found in our study between the DFI value and the high mitochondrial membrane potential is also in good agreement with the reports of other authors showing that sperm DNA fragmentation and loss of the mitochondrial function in bull (Bollwein et al. 2008) and human sperm (Donnelly et al. 2000; Marchetti et al. 2002) may be correlated. More specifically, a low mitochondrial membrane potential has been correlated with high DFI values in ejaculated human spermatozoa (La Vignera et al. 2012).

In summary, flow cytometry is a powerful tool for the reliable analysis and characterization of sperm function parameters in ejaculated alpaca sperm. Application of this technology in camelid reproduction could be of significance importance in (i) establishing the fertility cut-off values for alpaca sperm; (ii) improving pregnancy rates in alpaca females; and (iii) the selection of breeding males.

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Conflict of interest

None of the authors have any conflicts of interest to declare.

Author contributions

All authors contributed to the reviewing of the scientific literature and writing of the text of the manuscript. The work was coordinated by Professor Risopatrón.

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