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مقایسه سه روش رنگ آمیزی مختلف برای توصیف مورفومتریک اسپرم آلپاکا (*Vicugna pacos*) با استفاده از سیستم ISAS® CASA-Morph

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چکیده. مورفومتری اسپرم بخشی از آنالیز مایع منی مبتنی بر فناوری CASA است و نقش مهمی در پیش‌بینی باروری مردان نشان داده است. این آنالیز برپایه استفاده از تکنیک های مختلف رنگ‌آمیزی است، اگرچه نشان داده شده است که رنگ‌آمیزی می تواند تغییرات چشمگیری در مورفومتری سلول در گونه‌های مختلف ایجاد کند. هدف از این مطالعه، بررسی تفاوت‌های موجود در مورفومتری اسپرم درآلپاکا بود که با استفاده از تکنیک‌های مختلف رنگ‌آمیزی انجام شد. یک انزال ازهریک از پنج آلپاکای بالغ مورد استفاده قرار گرفت. نمونه‌ها با استفاده از روش جراحی مجرای دفران جمع‌آوری شدند. سه روش رنگ‌آمیزی (یعنی Hemacolor، Harri's Henatoxylin و Diff-Quik) مورد بررسی قرار گرفت. تجزیه و تحلیل مورفومتریک با استفاده از سیستم ISAS® v1 CASA-Morph، با بزرگنمایی x۱۰۰ و میکروسکوپ زمینه روشن و یک دوربین فیلمبرداری دیجیتالی با وضوح نهایی ۰/۰۸ میکرومتر بر پیکسل انجام شد. تقریباً تعداد ۲۰۰ سلول به صورت تصادفی از هر نمونه انتخاب شد وانتخاب تکنیک رنگ‌آمیزی با توجه به اهمیت پارامترهای اندازه سر اسپرم وبخش میانی انتخاب شد. بین تکنیک‌های به کار رفته تقریباً کلیه پارامترها در سطوح متفاوت تفاوت‌هایی را از خود نشان دادند. به طور کلی، سلول‌هایی که سر بزرگ داشتند با تکنیک Harri's Henatoxylin و آن‌هایی که دارای سر کوچک بودند با Diff-Quik رنگ آمیزی شدند. پارامترهای تفکیک کننده برای تمایز بهتر بین حیوانات، عرض سر، سطح و درصد آکروزوم بود. در نتیجه، مانند سایر گونه‌ها، نتایج مورفومتری اسپرم آلپاکا نیز به تکنیک رنگ‌آمیزی به کاررفته، حساس بود. این دلالت بر لزوم ذکر واضح و دقیق تکنیک رنگ‌آمیزی استفاده شده در هر مورد برای انجام مقایسه بین مطالعات مختلف روی گونه‌های مشابه دارد. هیچ یک از تکنیک‌های به کار گرفته شده برتر از سایرین نبود. علاوه بر این، روشی که برای به دست آوردن نمونه‌ها استفاده شد، سودمندی و سادگی آن را برای نمونه‌برداری‌های مکرر نشان داد.

واژه‌های کلیدی. آنالیز منی، دیف کوئیک، ریخت‌شناسی اسپرم، هما کالر، هماتوکسیلین هری

Comparison of three different staining methods for the morphometric characterization of Alpaca (*Vicugna pacos*) sperm, using ISAS® CASA-Morph system

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Abstract. Sperm morphometry is a part of the semen analysis based on CASA technology and has shown a big role in the

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prediction of male fertility. This analysis implies the use of stain techniques, although it has been shown that staining can make dramatic changes in the cell morphometry in different species. The aim of the present work was to evaluate the differences in sperm morphometry in Alpaca, introduced by the use of different stain techniques. Single ejaculates from five adult alpacas were used. Samples were recovered by deferent duct deviation surgery technique. Three stain techniques (i.e. Hemacolor, Harri's Henatoxylin and Diff-Quik) were evaluated. Morphometric analysis was done using ISAS® v1 CASA-Morph system, at 100x bright field objective magnification and a digital video camera with a final resolution of 0.08 µm/pixel. Almost 200 randomly selected cells were automatically analysed per sample and stain technique, considering several sperm-head and midpiece parameters regarding size and shape. Almost all of the parameters showed different levels of difference among the employed techniques. In general, the largest cell heads were the ones stained with Harri's Hematoxylin and the smallest ones were the ones stained with Diff-Quik. The discriminant parameters used to differentiate between animals better were the head width, area and acrosome percentage. In conclusion, like in other species, alpaca sperm morphometry results were found to be sensitive to the applied stain technique. This implies the necessity of referring clearly the stain technique used in each case to perform comparisons between different works on the same species. None of the employed techniques was superior to the others. In addition, the method used to obtain the samples showed its usefulness and simplicity for repeated samplings.

Keywords. Diff-Quik, Hemacolor, Harri's Hematoxylin, semen analysis, sperm morphometry

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INTRODUCTION

Semen analysis is the way to determine the potential fertility of a male. Traditionally, sperm concentration, motility and morphology are the basic characteristics included in a spermiogram for all mammalian species, among which sperm morphology is most relevant with the genetics of the male (Landry *et al*., 2003; Sun, Ko & Martin, 2006; Kosova *et al*., 2014; Ray *et al*., 2017).

The early development of Computer Assisted Semen Analysis (CASA-Morph) technology allowed passing from the subjective morphology to the metric semi-automatic or full-automatic analysis of the spermatozoa in a sample (Casey *et al*., 1997; Barroso *et al*., 1999; Soler *et al*., 2005b; Yániz *et al*., 2015; Maroto-Morales *et al*., 2016).

Nevertheless, it was demonstrated that both fixatives (Sancho *et al*., 1998; Cooper, 2012) and staining techniques (Gago *et al*., 1998; Soler *et al*., 2005a; Maree *et al*., 2010; Banaszewska *et al*., 2015) could introduce dramatic changes in head sperm morphometry, which is species-specific. This implies the necessity to analyse the effect of different staining techniques on sperm morphometry prior to developing a scientific research, unless it is impossible to compare the results obtained from the application of diverse techniques in different species (Soler *et al*., 2015).

The sperm characterization in camelids is not so much developed as in other mammalian species (Valverde *et al*., 2016; Yániz *et al*., 2016: Soler *et al*., 2017), although some works were developed on sperm characterization in camelids (Bravo *et al*., 1997; Bravo *et al*., 1999; Soler *et al*., 2014b, 2014c; Carretero *et al*., 2015; Laruta-Limachi *et al*., 2016; Carretero *et al*., 2017; Panahi *et al*., 2017; Meza *et al*., 2018). The aim of the present work was to evaluate the effect of three different staining techniques on alpaca sperm morphometry analysed by CASA-Morph system.

MATERIALS AND METHODS

Broodstock conditions

This study was conducted in the Investigation Center in South American Camelids (CICAS – La Raya), belonging to the Agrarian Sciences Faculty of the National University of San Antonio Abad del Cusco (UNSAAC) localized in Cusco, Peru.

The natural life area of this investigation is subalpine wet subtropical wilderness (Holdridge LR 1982). Bio-temperature range from 0 and 6ºC with high currency frost, rainfall is in the range of 500-1000 mm and the most precipitation occurs during the months of December to March. The maximum and minimum temperatures are -7ºC and 15 ºC, respectively, with an average of 7.49ºC.

The UNSAAC has not established a Bioethics Committee (which is being defined), but the Dirección de Investigación certifies that the protocols of the study were developed following the norm indicated on https://ec.europa.eu/environment/chemicals/lab_animals//legislation_en.htm.

The study was conducted from October 2015 to March 2016 and on five Huacaya-breed male alpacas randomly selected, with a weight average of 56 Kg and an age average of 5 years. Animals were maintained to the same conditions of handling and feeding.

Surgical technique

Alpacas males were submitted to a deferent duct deviation surgery to collect the spermatozoa by friction of the epididymal tail and ductus deferens (Meza *et al*., 2018). In brief, before starting surgery, males were weighed to calculate the dose of anaesthetic agent, and were then tranquilized by promazil (Montana, Lima, Perú) at a dose of 1 ml/10 kg by deep intramuscular injection. After thirty minutes, a local lidocaine (1 mL per sensation point, Genfar, Bogotá, Colombia) was applied subcutaneously. After disinfecting the operation zone, small lateral cuts were made in the scrotal bag to

locate the ductus deferens. Then, the ductus was moved to the inner thigh zone, where a 2-3 cm subcutaneous cut was made.

During the post-operative treatment, bioflan (Myralis pharma, Sao Paulo, Brazil) at 12 to 12 hours (dose of 1 ml / 25 kg) and biomizona (Biomont, Lima Perú, dose of 1 ml / 10 kg), both for 3 days, were administered to animals. Scars were disinfected regularly to prevent possible complications.

Semen collection

Alpaca males were placed in a quiet place on lateral position and the semen collection area was disinfected. To stimulate the semen output, friction of the epididymal tail and ductus deferens was realized. Spermatozoids were conducted to the output of the fistula and collected into a 37°C pre-heated syringe. Semen was immediately placed in a graduated tube with 0.4 ml tris-based dilution medium at 37º. After collecting the semen, Vaseline was applied on the recovering area to prevent the fistula from drying. Collections were done close to the laboratory in order to facilitate the handling and transportation of samples.

Sperm staining

Hemacolor (Merck, Darmstadt, Germany), Diff-Quik (Baxter, Düdingen, Switzerland) and Harris' Hematoxylin (Merck) were used to stain the smears from each sperm sample. First, two staining methods were applied, following kit recommendations. Hematoxylin staining was performed by a 30 min immersion of samples into stain and subsequent washing with tap water. All samples were air dried and permanently mounted on a slide with Eukitt mounting medium (O. Kindler GMBH & CO., Freiburg, Germany).

Sperm morphometric analysis

Morphometric analysis of sperm samples was performed using the ISAS® v1 (Proiser® R+D SL, Paterna, Spain) CASA-Morph system. Slides were examined and cells were captured, using a phase-contrast microscope (UOP-Proiser) equipped with a 100x bright field objective (AN 0.17) and a digital video camera (Proiser 782M). The array size of the video frame grabber was 768x576x8 bits and 256 grey levels. Resolution of images was 0.08 μm per pixel in both the horizontal and vertical axes. Two hundred sperm cells per sample, presenting no overlapping with other cells or with background particles, were randomly captured. Several sperm-head parameters of size (length, width, area and perimeter), shape (ellipticity, rugosity, elongation and regularity), midpiece area, width and insertion distance as well as angle between head and midpiece axis were measured (Soler *et al*., 2005; Fig. 1).

Sperm Statistical analysis

For multiple comparisons between staining methods and animal sperm samples, normality distributions and variance homogeneity were checked by the tests of Kolmogorov–Smirnov and Levene, respectively. For samples that were normally distributed, one-way ANOVA was performed, followed by a Bonferrini a posteriori test. For non-normally distributed populations, the Kruskal–Wallis one-way ANOVA on ranks was performed. Moreover, considering only 250 randomly selected cells from those correctly analysed after each stain technique, Pearson's correlation analyses between morphometric parameters were also done. All statistical calculations were performed using the SPSS v 23.0 (IBM SPSS Inc., Chicago, USA).

RESULTS

Head length, perimeter, acrosome and rugosity were different for each of the three stain techniques, the highest values being those of Harris' Hematoxylin and the lowest being those of Diff-Quik. Also, the area of the midpiece was different for the three techniques, and in this case the highest value corresponded to Diff-Quik and the lowest to Hematoxilin. Values of the head area were not different for Hematoxylin and Hemacolor but higher than that corresponding to Diff-Quik. Ellipticity, Elongation and the distance of the midpiece insertion point were significantly higher for Hematoxylin and similar for the other two techniques. The midpiece width was similar for Hemacolor and Diff-Quik, and lower for Hematoxylin. Finally, the head width and the midpiece insertion angle showed no changes among stain techniques (Table 1, Fig. 2).

Regarding the differences among animals, most of the parameters showed differences for the different staining methods, however, Hemacolor was found to be more appropriate for discriminating purposes. In this case, head area, acrosome percentage and ellipticity were the best parameters for differentiating animals (Table 2).

DISCUSSION

Subjective evaluation of sperm morphology has been considered a key factor in the prediction of male fertility in a variety of species (Yániz *et al*., 2015, 2016). Only few studies have been performed with the aim of establishing standard morphological criteria to estimate the South American camelids sperm quality (Buendía *et al*., 2002; Schwalm *et al*., 2007; Morton *et al*., 2010; Ordoñez *et al*., 2012).

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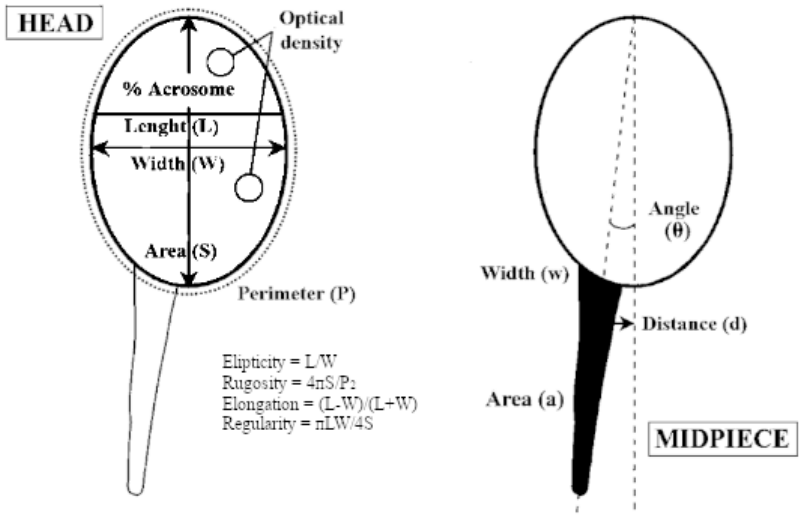

Fig. 1. Morphometric parameters examined in this study.

Table 1. Shape and size morphometric values of alpaca spermatozoa associated with each staining technique.

	Diff-Quick		**Hemacolor**		**Hematoxylin**	
	Mean	SD	Mean	SD	Mean	SD
N	5625		6954		5646	
Lenght (µm)	5.8[a]	0.01	5.8[a]	0.01	6.0[b]	0.01
Width (µm)	3.2[a]	0.00	3.2[a]	0.00	3.2[a]	0.00
Area (µm^2)	15.1[a]	0.02	15.4[b]	0.02	15.6[c]	0.02
Perimeter (µm)	17.0[a]	0.02	17.0[a]	0.02	17.9[b]	0.02
Elipticity	1.8[a]	0.00	1.8[a]	0.00	1.9[b]	0.00
Rugosity	0.7[b]	0.00	0.6[a]	0.00	0.7[b]	0.00
Elongation	0.3[a]	0.00	0.3[a]	0.00	0.3[a]	0.00
Regularity	1.0[a]	0.00	1.0[a]	0.00	1.0[a]	0.00
Widt-Mp	1.2[a]	0.01	1.2[a]	0.01	1.1[b]	0.01
Area-Mp	3.2[a]	0.02	2.9[b]	0.02	2.6[c]	0.02
d.h.-Mp	0.2[a]	0.0	0.2[a]	0.00	0.2[a]	0.00
a.h.-Mp	8.9[a]	0.21	8.9[a]	0.21	8.6[a]	0.21

Values with different letters in the same row are significantly different ($P < 0.05$).

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The average value of normal and abnormal spermatozoa had been reported previously in alpaca submitted to the same surgery and collection methodology and was not calculated here again. The ejaculated spermatozoa had a greater proportion of normal spermatozoa, showing a percentage of 74.98% of normal forms and 25.02% of abnormalities (Quintano, 2001), and 75.87% of normal spermatozoa and 24.13% of abnormal spermatozoa (Cárdenas, 2002). Nevertheless, the classical subjective analysis approach has two main limitations, the high variability due to technicians (Eusteche & Auger, 2003) and the staining procedures (Soler & Cooper, 2016).

Regarding the sperm preparation, it is well known that the spermatozoa structure can be modified by fixation (Hardy, 1899), either air dried (Yeung *et al*., 1997; Soler *et al*., 2000) or using chemical fixatives (Sancho *et al*., 1998). This limitation implies that any conclusion about the real meaning of sperm morphology must be relative when analysed under these technical conditions (Soler & Cooper, 2016).

The problem related to the technician variability was partially solved by the introduction of CASA-Morph systems, which analyse the sample in a quantitative way changing the classical morphological to the computerized morphometry approach (Yániz *et al*., 2015; Soler *et al*., 2016). CASA technology not only dramatically reduces the Coefficient of the Variance in the final results, increasing their reliability but also offers a big amount of quantitative morphometric data for use in a variety of studies (for revision look the special issue published by the Asian Journal of Andrology in 2016, Vol 18, issue 6).

In human beings, it was observed that spermatozoa, which was classified as normal following the criteria defined by WHO (WHO, 2010), showed statistically significant differences among individuals when morphometric analysis was done. This indicates the concept of what is "normal" must be revisited even in a species as the human being, in which an enormous effort in the definition and standardization of sperm morphology was performed for a long time (Bellastela *et al*., 2010).

Nevertheless, the use of CASA-Morph systems does not solve the problem of artefacts production during the staining procedure. This made it necessary to define a good protocol definition and a correct evaluation of its impact in the final morphometric results, as it was done in the present work. Depending on the species, the optimal stain for the sperm morphometric analysis using CASA-Morph systems were Diff-Quik (for goat, Hidalgo *et al*., 2006; for red deer, Soler *et al*., 2003), Harris' Hematoxyline (boar, García-Herreros *et al*., 2006; Cynomolgus monkey, Gago *et al*., 2000; stallion, Hidalgo *et al*., 2005) and Hemacolor (Soler *et al*., 2003).

The recent introduction of Trumorph® was proposed as a new approach that prevents the staining

Table 2. Shape and size morphometric values of alpaca spermatozoa stained with Hemacolor for different animals.

Animal	1	2	4	5	6
Length-h (μm)	5.8 ± 0.03^{ab}	5.9 ± 0.03^{a}	5.7 ± 0.03^{b}	6.1 ± 0.03^{c}	5.8 ± 0.03^{b}
Width-h (μm)	3.5 ± 0.02^{a}	2.9 ± 0.02^{b}	3.3 ± 0.02^{c}	3.0 ± 0.02^{b}	3.1 ± 0.02^{d}
Area-h (μm²)	16.4 ± 0.1^{a}	14.4 ± 0.01^{b}	15.9 ± 0.01^{c}	15.0 ± 0.01^{d}	14.7 ± 0.01^{bd}
Perimeter-h (μm)	17.0 ± 0.08^{a}	16.6 ± 0.08^{b}	16.9 ± 0.08^{ab}	18.5 ± 0.08^{c}	16.7 ± 0.09^{ab}
Acrosome (%)	56.6 ± 0.3^{ab}	55.9 ± 0.3^{b}	57.7 ± 0.3^{ac}	58.9 ± 0.3^{c}	54.3 ± 0.3^{d}
Ellipticity	1.7 ± 0.01^{a}	2.0 ± 0.01^{b}	1.8 ± 0.01^{c}	2.1 ± 0.01^{b}	1.9 ± 0.01^{d}
Rugosity	0.7 ± 0.004^{a}	0.6 ± 0.004^{b}	0.7 ± 0.004^{a}	0.6 ± 0.004^{b}	0.7 ± 0.004^{a}
Elongation	0.2 ± 0.003^{a}	0.3 ± 0.003^{b}	0.3 ± 0.003^{b}	0.3 ± 0.033^{b}	0.3 ± 0.003^{b}
Regularity	1.0 ± 0.002^{a}	0.9 ± 0.002^{b}	0.9 ± 0.002^{b}	0.9 ± 0.002^{b}	0.9 ± 0.002^{b}
Widt-Mp (μm)	1.1 ± 0.03^{a}	1.1 ± 0.03^{a}	1.2 ± 0.03^{b}	1.2 ± 0.03^{b}	1.3 ± 0.02^{c}
Area-Mp (μm2)	2.7 ± 0.04^{a}	2.7 ± 0.04^{a}	3.3 ± 0.04^{b}	2.8 ± 0.04^{a}	3.3 ± 0.04^{b}
d.h.Mp (μm)	0.2 ± 0.01^{a}	0.1 ± 0.02^{b}	0.2 ± 0.01^{a}	0.2 ± 0.01^{a}	0.2 ± 0.01^{a}
a.h.Mp (°)	9.9 ± 0.4^{ac}	9.6 ± 0.4^{ac}	6.4 ± 0.4^{b}	10.3 ± 0.4^{a}	8.2 ± 0.5^{c}

Values with different letters in the same row are significantly different ($P < 0.05$).

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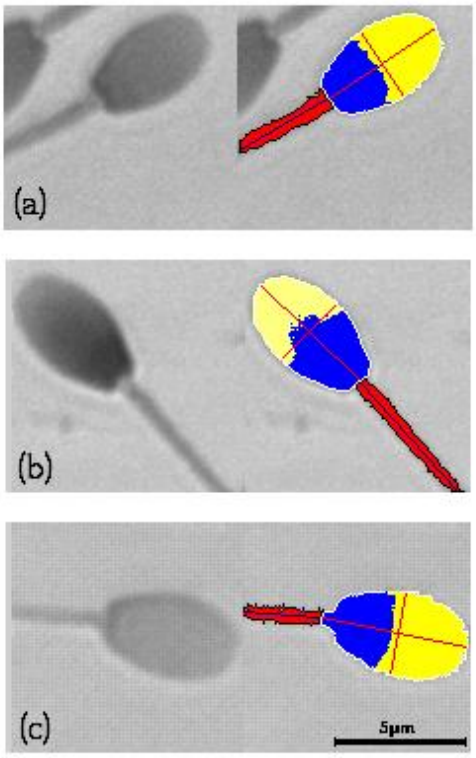

Fig. 2. Microscopic images of alpaca spermatozoa and its digitized mask stained with **a**. Diff-Quik, **b.** Hemacolor, **c.** Hrmatoxylin

process, allowing the analysis of sperm morphology in live cells (Soler *et al*., 2014a, 2015). The images obtained by this method showed big differences as compared with classical methods, which introduce different levels of artefact production. When the use of CASA-Morph technology is available for morphometric analysis, it will be needed to revise the classical concepts related to sperm morphology and morphometry (Soler *et al*., 2016).

Finally, it is necessary to compare the results obtained under similar technical conditions, e.g., using artificial vagina or using the new method proposed here. In general, the cells analysed here were shorter and thinner than those observed after artificial vagina sampling, using the Hemacolor staining (Buendía *et al*., 2002). This can be due to the fact that two different CASA-Morph systems were used or most probably because, as it was shown in different species, there are differences among spermatozoa from the epididymis directly and post-ejaculation (Yeung *et al*., 1997; Pérez-Sánchez *et al*., 1998; Gago *et al*., 1999, 2000; Soler *et al*., 2000). Therefore, it is necessary to consider collecting methods and/or the origin of the sample before performing any morphometric data comparison.

It can be concluded that, as in other species, alpaca sperm morphometry is sensitive to the stain technique used, making it necessary to refer to the employed technique when comparison between different works is done. In any case, it is not possible to conclude that any of the techniques used here can be considered better than others. However, based on previous works, we decided to use Hemacolor as a reference. Finally, the method to obtain the samples used here showed to be useful and simple for repeated samplings.

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