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Azteca Breed Horse Epididymal Sperm Evaluation: A Comparison of Head, Corpus and Cauda Sperm Quality

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Abstract

The main objective of this study was to determine differences in sperm recovered from the three segments of the epididymis, *i.e.*, head, corpus and cauda, using routine measurements of sperm quality and DNA fragmentation assessment. There was a larger sperm concentration ($P < 0.05$) in the head (3.34×10^6 sperm/mL) compared with the corpus and cauda. The percentage of normal morphology was higher in the cauda but had lower motility ($P < 0.05$). The DNA fragmentation index was higher for sperm obtained from the head and body of the epididymis ($P < 0.01$) compared with the cauda. The DNA fragmentation index values were 12% higher ($P < 0.01$) using Wright stain compared with DAPI stain in sperm from the head and corpus of the epididymis, but similar in the cauda. Spermatobioscopy parameters match the maturation changes that occur in the epididymal tract with higher quality sperm being from the cauda, but with a lower concentration compared to sperm found in the head.

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Keywords

Epididymal, Horse, Sperm, DAPI, Wright Stain, DNA Fragmentation

1. Introduction

The collection of epididymal sperm can be used to preserve the genetic material of animals, including humans that present an inability to ejaculate naturally because of prostration due to health problems, lack of erection, obstructive azoospermia [1] [2], and emergency or elective castrations [3]. Epididymal sperm collection has also been useful in animals that die suddenly or in the case of Equidae because of their susceptibility to gastrointestinal problems that may end with humane euthanasia and death [4].

Epididymal sperm can be useful for fertilization, either *in vivo* or *in vitro*, as it has been shown to have a high enough quality to be used in assisted reproduction procedures. Epididymal sperm viability at room temperature for the 24 h following an orchiectomy is important for freezing protocols [1] [5] [6]. The sperm concentrations of the samples collected from the epididymides can be similar to those of ejaculates obtained with an artificial vagina, but the quality is reduced when epididymal sperm are subjected to multiple freezing and thawing steps [7]. Pregnancy rates with epididymal sperm have varied from 8% to 92% with frozen-thawed epididymal semen and 9% to 55% with fresh epididymal semen [8]. The differences in pregnancy rates using frozen-thawed semen can be due to the following: stallion semen quality, the type of diluent utilized, and the use or removal of the seminal plasma, as well as other factors [9].

There is a lack of information in previous studies with regard to epididymal sperm quality in terms of the state of sperm DNA integrity within the epididymides. It is in the epididymides where the major morphological changes in sperm occur [10], such as chromatin condensation, due mainly to the replacement of histones by protamines in the testes [11] [12]. However, the lack of seminal plasma makes the epididymal sperm less motile than would be considered ideal for direct insemination techniques and the sperm can also be easily damaged by oxidizing agentsp [13]-[15]. Garcia-Macias *et al*. [16] noted that there was a greater possibility of finding sperm with damaged DNA in epididymal samples compared with samples obtained by artificial vagina. Given the aforementioned issue, it would be beneficial to identify the potential differences in sperm obtained from the different sections of the epididymis and to determine the presence of altered DNA. The primary objective of this study was to determine the epididymal sperm DNA fragmentation index for Azteca breed stallions, while also comparing DAPI (4'-6-diamidino Obtained 2-phenylindole) and Wright stain protocols.

2. Materials and Methods

2.1. Epididymal Sperm Retrieval

Elective castrations involving Azteca breed horses (n = 46) of 1 - 2 years of age, were performed to obtain each individual's testes and epididymides. The collected testes and epididymides were immersed in saline solution (NaCl 0.9%), penicillin-G (100 UI/mL) and streptomycin sulfate (100 mg/mL), kept at a temperature between 4 and 8˚C, and transported to the laboratory (**Figure 1**), a trip of approximately 30 min. Epididymal sperm was obtained using the technique described by Muradas *et al*. [17] and the tubules of the body and head were minced when necessary. More specifically, the head, body and cauda were separated with Nylon No.1 and each segment was washed with 40 mL of Dulbecco's Phosphate Buffered Saline (DPBS) at a temperature of 37˚C ± 2˚C (Dulbecco's Phosphate Buffered Saline D8662 [2], performing 3 washes for each testicle and epididymis obtained. All of the animals used in this research were treated in accordance with the Federation of Animal Science Societies (FASS) guide for use of farm animals in research and teaching [18].

2.2. Sperm Evaluation

On average 37 ± 2 mL of DPBS containing semen was obtained from each epididymal segment. The number of sperm based on the concentration was calculated using a Neubauer Marienfield® cell counting chamber. For sperm quality evaluations, 25.4 × 76.2 mm glass microscope slides (Corning®) and 22 × 22 mm #1.5 coverslips (Corning®) were used. Progressive motility was evaluated using an optical light microscope (200 ×) (Eclipse

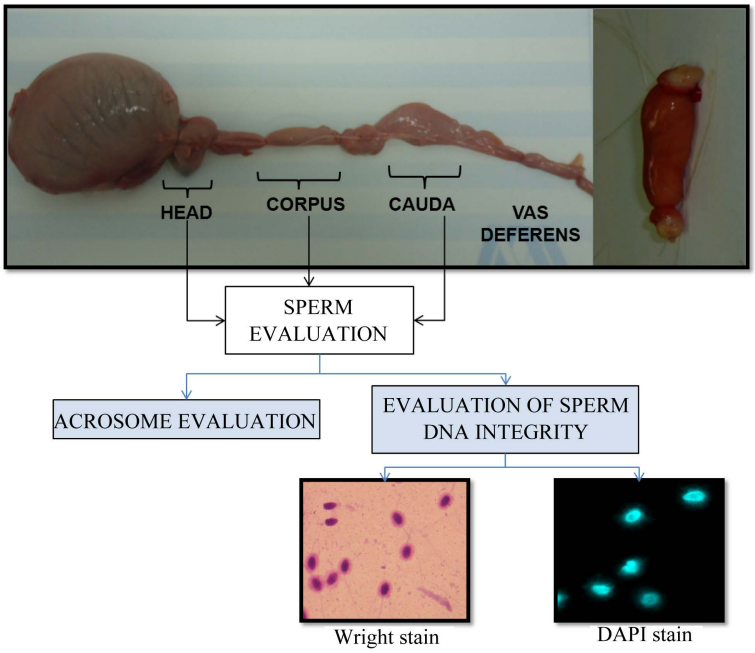

Figure 1. Azteca breed horse testicle and epididymide showing sperm DNA fragmentation analysis using Wright stain and DAPI stain.

Ni-U, Nikon®, Japan) with 10 µL of sample, sperm diluted 1:1 with DPBS. The percentage of dead sperm was determined with smears stained with eosin-nigrosin and with Diff Quick (Kubus®) to determine the percentage of abnormal morphologies, based on [6] [13], using a 400× optical light microscope. A total of 300 cells were counted per sample (**Figure 1**).

2.3. Acrosome Evaluation

A total of 1.5 mL of DPBS containing collected semen was centrifuged at 50 *g* × 5 min to obtain a sperm pellet from which 10 µL were removed and diluted with 1000 µL of fixing solution (2% paraformaldehyde, 2% formaldehyde buffered) and stored at 4˚C until analysis [19] [20]. For the acrosome structure assessment, five washes of the sample with DPBS were performed. Afterwards, the sample was incubated for 30 min (while protected from light) with 10 uL of lectin *Pisum sativum* (PSA) (FITC-PSA, Sigma, St Louis, MO, USA) at a 1:10,000 dilution, plus an antifade solution (Vectashield, Burlingame, USA) in a 1:1 ratio and evaluated using a fluorescence microscope (FV10i, OYMPUS) with a 1000X magnification, similar to the procedure in Farlin *et al*. [21].

2.4. Evaluation of Sperm DNA Integrity

The Halomax kit for horses (Halotech SL, Madrid, Spain) was used for assessing DNA fragmentation. The Sperm-Halomax® methodology is based on the sperm chromatin dispersion test (SCDt) [22]-[24]. Details of this technique have previously been reported for human and other mammalian species [23] [25]-[27].

In the present study, a sample of 15×10^6 sperm/mL was diluted in agarose to give a final concentration of 1×10^6 sperm/mL, placed in racks, lysed, and stained with DAPI (SIGMA, 4'-6-diamidino Obtained 2-phenylindole) and Wright stain (Accustain, Sigma) to obtain two slides for each epididymal segment sample. Kjelland *et al*. [28] provides a more detailed sperm sample preparation protocol using the Halomax kit (**Figure 1**). The DNA fragmentation analyses were conducted using a fluorescence microscope (FV10i, OYMPUS) using a magnification of 1000X with the DAPI stain, but without filters for the WRIGHT stain. Three hundred sperm per slide were counted to determine the sperm DNA fragmentation index which is the proportion of sperm with damaged DNA. For DNA fragmentation analysis using the Halomax kit for horses, sperm with fragmented DNA are identified by the formation of a halo around the head of the sperm produced by chromatin dispersion in agarose,

signaling the single-strand and/or double-strand breaks in the DNA. The DNA fragments tend to move away from the sperm head with larger pieces of chromatin typically moving less distance compared to smaller pieces and thereby forming a "halo" around the sperm head. When the diameter of the halo exceeds twice the sperm head's radius then the sperm is considered fragmented [5] [22].

2.5. Statistical Analysis

The results of the sperm evaluation were analyzed as a completely randomized design where the model included the site of semen collection (head, corpus and cauda). The fragmentation index values of sperm assessed using DAPI and Wright stain were compared using a Student's *t*-test. The statistical software SPSS v. 16.0 (SPSS Inc. 2007) was used to determine the significance of the results [29] [30]. Statistical differences were determined to exist at $P < 0.05$ (*i.e.*, Type I error set at $\alpha = 0.05$).

3. Results

The highest total concentration of sperm ($P < 0.05$) was found in the head of the epididymis. The percentage of normal morphology was higher in the cauda epididymis, but cauda samples also had a lower motility ($P < 0.05$) compared to the samples from the head and corpus segments. The highest percentage of damaged acrosomes was obtained in sperm from the head of the epididymis while the lowest percentage came from the cauda ($P < 0.05$). The DNA fragmentation indices were higher in the head and corpus ($P < 0.01$) compared to the cauda (**Table 1**). Notably, the values obtained regarding the DNA fragmentation index were 12% higher ($P < 0.01$) using Wright stain in sperm from the head and corpus (**Table 2**) compared to those stained with DAPI. **Figure 2** and **Figure 3** show epididymal sperm stained with DAPI and Wright stain.

4. Discussion

Sperm maturation occurs in the tract of the epididymis, with the sperm acquiring important functions essential

Table 1. Spermatobioscopy parameters and sperm DNA fragmentation index (DNA FI) in the head, corpus, and cauda of Azteca breed horse epididymides.

	Epididymal Segment				
Spermatobioscopy Parameters	Head	Corpus	Cauda	SEM	*P*-Value
Total Concentration (×10^6)	3.34^a	1.39^b	1.34^b	0.089	<0.0001
Motility (%)	69.30^a	67.02^a	54.91^b	0.873	<0.0001
Normal Morphology (%)	74.37^b	75.26^b	80.78^a	0.637	<0.0001
Mortality (%)	25.04^a	17.17^b	18.21^b	0.479	<0.0001
Acrosomes Damaged (%)	23.76^a	20.61^b	16.09^c	0.472	<0.0001
DNA FI DAPI	20.96^a	20.40^a	16.47^b	0.559	<0.0001
DNA FI Wright	23.48^a	23.22^a	16.72^b	0.672	<0.0001

Different letters indicate statistically different values, $P < 0.0001$.

Table 2. Comparison of staining methods to assess the DNA fragmentation index (DNA FI) of epididymal sperm obtained from Azteca breed horses.

	Stain		
Epididymal Segment	DAPI (DNA FI)	WRIGHT (DNA FI)	*P*-Value (Student's *t*-Test)
Head	20.91 (0.66)	23.42 (0.69)	0.009
Body	20.35 (0.52)	23.16 (0.82)	0.005
Cauda	16.42 (0.50)	16.66 (0.49)	0.729

Standard error in parentheses.

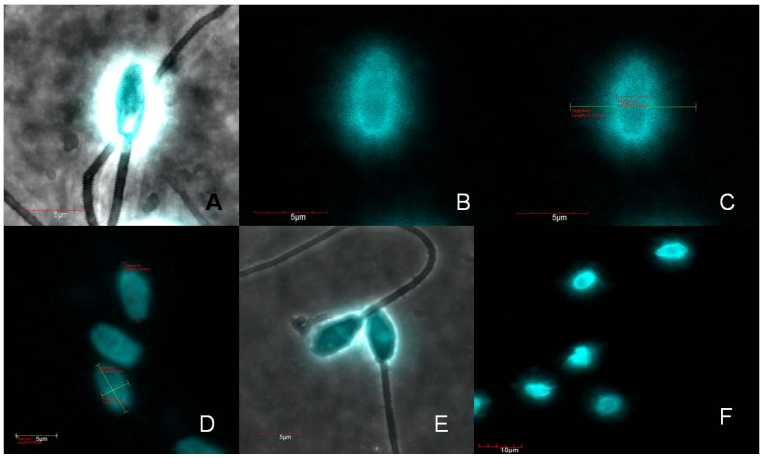

Figure 2. Azteca breed horse sperm staining with DAPI. (A) visualization of a sperm using phase contrast; (B) and (C) sperm exhibiting DNA fragmentation (fluorescence microscopy); (C) and (D) measurements of halos (fluorescence microscopy); (E) sperm without fragmentation (combination of phase contrast and fluorescence microscopy); (F) technical application of the sperm chromatin dispersion test.

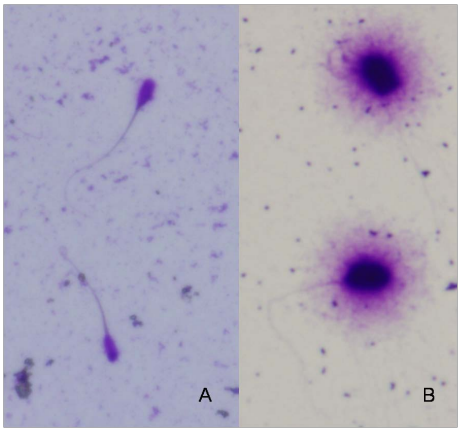

Figure 3. Azteca breed horse sperm stained with Wright stain. (A) sperm with-out fragmentation; (B) fragmented sperm.

for fertilization such as motility, differentiation of the midpiece and acrosome [11] [12] and changes and modifications to the chromatin of the nucleus [5] [13] [31]. The differences detected in the sperm collected in different segments of the epididymis reflect the biochemical changes that occur during sperm passage [32] [33]. When sperm reach the cauda they come in contact with various substances including immobiline which greatly reduces their metabolism to minimize the energy demand and minimize the occurrence of residues; primarily because the epithelium has not been developed for absorbing wastes, which are higher in the segment of the head and somewhat less in the corpus of the epididymis [33] [34].

The greater concentration of sperm was obtained from the head of the epididymis, and was higher than that reported by others, e.g., James *et al*. [35] and Guimarães *et al*. [3]. In other studies the unit of measure is given as the total sperm/mL [2] [36] but they do not report the total number of mLs used for conducting the evaluation; as such, it was reported as total concentration in the present study. The differences reported for epididymal sperm concentrations in these other studies can be attributed to the differences in the age of animals and/or environmental temperatures utilized. The age of the animals and the individual effect should be considered as important variables [37] [38].

The decrease in sperm motility in the cauda segment of the epididymis has been reported by Cary *et al*. [39] and Braun *et al*. [1]. The aforementioned authors compared the motility of ejaculates obtained with an artificial

vagina and epididymal sperm from orchiectomy on the same animals and found that cryopreserved sperm with seminal plasma produced better rates of progressive motility. Their results have been verified by several authors [3] and attributed to inhibitory factors present in the seminal plasma, which is contrary to a study by Monteiro *et al*. [40] who found that frozen/thawed motility values of sperm obtained from the cauda epididymis were higher than those obtained with artificial vagina ejaculates. Monteiro *et al*. [40] also reported that sperm obtained from the epididymis are more resistant to thermal shock and freezing, which is attributed to the different phospholipid distribution in apoptotic sperm demonstrated in the evaluation of caspase activation.

Muradas *et al*. [17] obtained a value of 45% live sperm with intact acrosomes recovered from the epididymis at 0 h of analysis (lower than that found in ejaculates, *i.e*., 65% at 0 h of analysis) and only 17% live sperm with intact acrosomes after 24 h, indicating that these sperm can be used in reproductive clinics. The differentiation of the acrosome during spermatogenesis occurs in the tract of the epididymis, with mature sperm resulting in the end [11] [41]; this includes an intact acrosome [42] [43]. The results of the present study are in agreement with the aforementioned results, since the lowest percentage of damaged acrosomes were obtained from the cauda region of the epididymis.

The increase in dead sperm in the head of the epididymis may be due to the biochemical regionalization of the epididymis. Fouchécourt *et al*. [34] identified the secretion of proteins during epididymal sperm transit, with the head segment secreting the most (73%), followed by the corpus (20.5%), and finally the cauda (6.5%). In the head of the epididymis, 44 different proteins were detected of which 22 were specific for this region, e.g., PGDS, GPX, and with the most abundant being the clusterines [44]-[46].

It is important to note that the horses used in this study were prepubertal, which likely affected the sperm DNA fragmentation index found in the present study. Monteiro *et al*. [40] reported a sperm DNA damage of 6.7% in cryopreserved washed ejaculates and 5.7% from epididymal sperm from adult horses. The aforementioned values are at least 10% below those found in the present study. López-Fernández *et al*. [22] performed sperm DNA fragmentation analysis using frozen-thawed straws of semen from stallions of various breeds and found that the average values at the time of thawing ranged between 10.3% and 25%, although in that study the Azteca breed was not included.

It is necessary to consider that the physiology of each segment of the epididymis may affect the extent to which sperm DNA becomes fragmented [14] [47], with associated influence on the quality of the embryo and its implantation [8] [48]. For instance, pregnancy rates in horses with the use of epididymal sperm have ranged from 8% - 92% [49] and this variation could be associated with sperm DNA fragmentation.

Statistically significant differences were found among the spermatobioscopy variables in different epididymal segments, meaning that there may be a higher risk of lower embryo production using sperm from one segment of the epididymis versus the other. The sperm tail must be analyzed and sperm separated by screening tests in which the largest number of immature or DNA fragmented sperm are excluded from being used in assisted reproductive techniques. Additionally, a full flushing of the epididymis, in terms of dissection of segments, is unnecessary as one would end up combining a high concentration of immature sperm with mature sperm, likely resulting in a less than ideal reproductive outcome. The epididymal sperm selection method must be conducted using such a segment selected method, most commonly the cauda, even though one sacrifices obtaining a greater volume of sperm. More advanced studies involving cultured sperm maturation should be evaluated for the head and body segments of the epididymis and the testis for Equine reproductive assisted techniques [42] [50] [51].

In a comparison of the DNA fragmentation index values using two different stains, Wright stain showed higher sperm DNA fragmentation index values than that evaluated with DAPI stain, with differences of around 12% ($P < 0.05$). Wright stain and the HALOMAX® kit have been used in several studies using bright field microscopy [25] [52], since it contains Methylene Blue which has an affinity for proteins with a characteristic acidic property, binding to them via its positive charge thereby marking them a blue color. Given that the Wright Stain only requires bright field microscopy the sperm DNA fragmentation analysis can be much more economical than using a fluorochrome, if it would require the purchase of a microscope with fluorescence capabilities. DAPI is widely used in various techniques to detect DNA damage by its affinity to join to the nitrogenous bases adenine and thymine [25] [53] [54], although its use has declined in DNA studies as it also binds to RNA, considered as having low specificity relative to other intercalating, commercially available fluorochromes.

The two dyes, DAPI and Wright stain, have an affinity for DNA fragments and we believe that the results of the present study are not mutually exclusive, despite being statistically different (**Table 2**). The implementation of one or the other will be dependent upon the possibility of acquiring fluorescence microscopy capabilities.

Studies will be needed to establish the correction for error with regard to the method of analysis of the sample, which must be conducted by individuals with other fluorochromes and different equipment characteristics found in each laboratory.

5. Conclusion

Some differences can be found in the characteristics of semen collected from the epididymal headcauda, and corpus; but less DNA fragmentation and less acrosome damage is observed in sperm from the cauda segment. Sperm DNA fragmentation analyses using Wright's stain result in higher DNA fragmentation index values compared with analyses made with DAPI.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

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