Acta Scientiarum
http://periodicos.uem.br/ojs
ISSN on-line: 1807-8672
Doi: 10.4025/actascianimsci.v45i1.58593

Histomorphological comparisons in testicles between Barb horse, donkey and mule

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ABSTRACT. The mule is a sterile hybrid domestic animal that results from the breeding of a male donkey with a female horse, understanding the reproductive biology of these species is very critical. The goal of this paper was to perform a comparative and more accurate histomorphometric of the testicles in Barb horse, donkeys and mules. Microscopic examinations and histological description were carried on genital tract of horses, donkeys and mules healthy and mature; this study was conducted during April-May 2018. The histological and the morphological results shows a similarity between the two equine species and the infertile hybrid for the testicles, the epididymis and the vas deferens. However, the difference was presented on the morphometric data; vas deferens was more voluminous in the horse and donkey than a mule. Moreover, the differences were significantly higher for the surface of the seminiferous tubules and for the epididymis. The lumen of the seminiferous tubules in mule was significantly higher than in the horse and donkey. Absence of gametes in the epididymal cavity and lower number of gametes in the mule. Furthermore, we have noted the presence of spermatozoa in one mule 16.67%. Therefore, the mule could complete development of spermatogenesis.

Keywords: equine; fertility; histological; morphometric; spermatogenesis.

Received on April 8, 2021.
Accepted on October 5 2021.

Introduction

The equine family is represented in Algeria by two species: Equus asinus (donkey) and Equus caballus (horse). The appearance of equine animals in Algeria dates back to prehistoric era of the fourth millennium (Houssou, Bouzebda-Afri, Bouzebda, Benidir, & Boujakji, 2021). The male mule (Equus mulus mulus, 63 chromosomes) is a sterile domestic animal caused by the breeding of a male (Equus asinus, 62 chromosomes) to a mare (Equus caballus, 64 chromosomes) (McLean, Varnum, Ali, Heleski, & Navas González, 2019).

Many studies have been reported from different countries on the physiological, histological and reproductive pathway of horses. In addition, the histology of the male reproductive system varies generally among mammal species (Danmaigoro, Onu, Sonfada, Umaru, & Oyelowo, 2014). The testis is the important dual glandular organ of the male reproductive system, with exocrine and endocrine components (Houssou et al., 2021). Its exocrine function is to produce male sex cells; meanwhile, its endocrine function is mainly focused on producing male sex hormone (Moustafa, Sayed, Zayed, & Abd El-Hafeez, 2015). Testis biometric parameters are very important for the development of assisted reproductive protocols in different species (Houssou et al., 2021). The Sertoli cells are the first cell type known to differentiate in the gonad (Svingen & Koopman, 2013). There is strong genetic evidence that the pre-Sertoli cell expresses the testis-determining factor (Sry) (Svingen & Koopman, 2013).The general organization of spermatogenesis is essentially the same in all mammals, largely regulated by many genes (Li et al., 2019). It is well known that production of fertile spermatozoa is a consequence of normal mitosis and meiosis of germ cell (Tripathi et al., 2015). Nevertheless, there are some specific traits related to the types and number of morphological traits of germ cells that exist in the different stages of spermatogenesis between different species (Neves, ChiarinI-Garacia, & Fança, 2002). The main criterion for stage identification lies in the morphological properties of spermatids, especially their nucleus and acrosomal systems (Neves et al., 2002).

Equine hybrids, composed of a fertile mule showed a mosaic of genes sequences a suggesting that other mechanisms than chromosomal numbers contribute to hybrid sterility in equids (Steiner & Ryder, 2013). Aiming mainly to perform studies related to germ cells transplantation from fertile equid species (donors) to mules. The structural results obtained for mule, horse and donkeys indicated that both Leydig and Sertoli cells were functionally normal in mule, strongly suggesting that mule seminiferous tubules are able to sustain complete development of spermatogenesis (Costa et al., 2012; Neves, Costa, & França, 2014).

This study used traditional histological analysis to characterize the structure of the testes and to analyze spermatogenesis in mules compared to horse and donkey.

Material and methods

Animals

The testicles of a mules (n=6), Barb horses (n=3) and donkeys (n=3), all mature >7 years and in good health, were obtained from a local slaughterhouse, during the equine breeding season, which is from February to June in the northern hemisphere. In our case, we cleaned the male genitals and we removed the scrotal and adipose tissue, we weighed testicles without the epididymis, we measured the testicular using a hand caliber. We made two types of cuts, transverse and longitudinal. Due to the large size of the testicles, we divide into four numbered parts, which were kept in 10% formaldehyde solution, processed to paraffin, sectioned at 4 µm thickness, the epididymal tissue were also sectioned with 4 µm using an automatic microtome. In the laboratory, tissue samples were prepared using standard histological methods described in Vilar et al. (2017).

Staining technique

We used three types of coloring. Hematoxylin Eosin (HE), Hematoxylin dyes in particular the nuclei in blue / purple, eosin colors the cytoplasm in pink and the other basic cellular elements in pink / red. Masson's trichrome, highlights the elements of connective tissue, in particular the collagen in green, the nucleus in black and the cytoplasm in red. Congo red, this coloration marks in red the deposits of amylose, red fibers and polynuclear red eosinophils.

Morphometry and histology of testicle

Histological sections (300 slides) were observed under a light microscope at different magnifications (40x, 100x, 400x and 1000x). To draw a tissue image comparing the sizes of the seminiferous tubes and the epididymis of these species. Pictures are captured by a digital camera (HIROCAM, MA88-500, BME lab and Science, St. Paul, USA) connected to a light microscope (Optika B 235, Italy) via TS View software (Microscopes America, Cumming, GA, USA). This image analysis system was calibrated using a graduated micrometric slide. In order to obtain the measurements of the diameters and perimeter in µm and the surfaces in µm2. The diameter and the perimeter of the seminiferous tubules and epididymis were measured from 50 areas, under 400x magnification. The contours of epididymis were observed from 20 measurements under 1000x magnification, using analysis and image processing software "Axio Vision 4.6.3.0" developed by the company Carl Zeiss.

Results

The results of the study are exposed in Figure 1 to 11, under 100x, 400x and 1000x magnifications as well as in Table 1. The results are presented as mean ± standard error.

Morphometry and histology of testicle

The observations of histological sections in the testicle of the two equine species and the infertile hybrid studied show that: in the horse and the donkey, the seminiferous tubes are voluminous, separating from each other by an interstitial space; they were formed by a seminiferous epithelium with a reduced lumen. However, in the mule, the seminiferous tubules were of medium size, possessing a large lumen (Figure 1).

The surface of the seminiferous tube in the horse approximates that of the donkey. The variation of the area of the seminiferous tube and the area of the lumen of the seminiferous in these species ($p < 0.001$) studied were significant (Figure 2).

Table 1. Biometric and morphometric data in horse, donkeys and mule.

Parameters	Horse (n=3)	Donkey (n=3)	Mule (n=6)
Age (years)	14±0.81	14±0.81	10±1.29
Testes weight (g)	305±8.5	254±11.9	173±12.5
Testicular length (cm)	10±0.97	9.5±0.66	8±0.96
Testicular height (cm)	7.5±0.20	6.5±0.40	4.5±0.64
Testicular width (cm)	6.5±0.73	5±0.81	4.4±051
Total length of epididymis (cm)	22.5±2.27	14.5±1.77	14±1.32
The surface of seminiferous tubules (µm²)	26170.3 ± 795.4	25081.3±720.7	21101.7 ± 923.5
The lumen of seminiferous tubules (µm²)	706.2 ± 76.3	4304.9 ± 186.8	9299.7 ± 332.8
Seminiferous tubule diameter (µm)	191.2±3.18	174.7±4.21	164.4±4.82
The area of the epididymal ducts (µm²)	284653.5 ± 17239.5	580351± 46799.1	778399.8 ± 48897.9
The lumen of the epididymal ducts (µm²)	1 50276.2 ± 11779	442600± 40789.1	543995.4 ± 38115
The length of the epithelial (µm)	89.3±2.4	59.4 ±1.3	76 .06±1.7
The length of basal nuclei(µm)	43.9± 2.2	27.7±1.2	26.5±1.1,2
The surface of the ductus deferens (µm²)	2849994 ±253854.1	1623709±57433.2	2487166±118281.2
The lumen of ductus deferens (µm²)	934359.7±109061.3	543831.4±41885.4	702782.2±157388.1

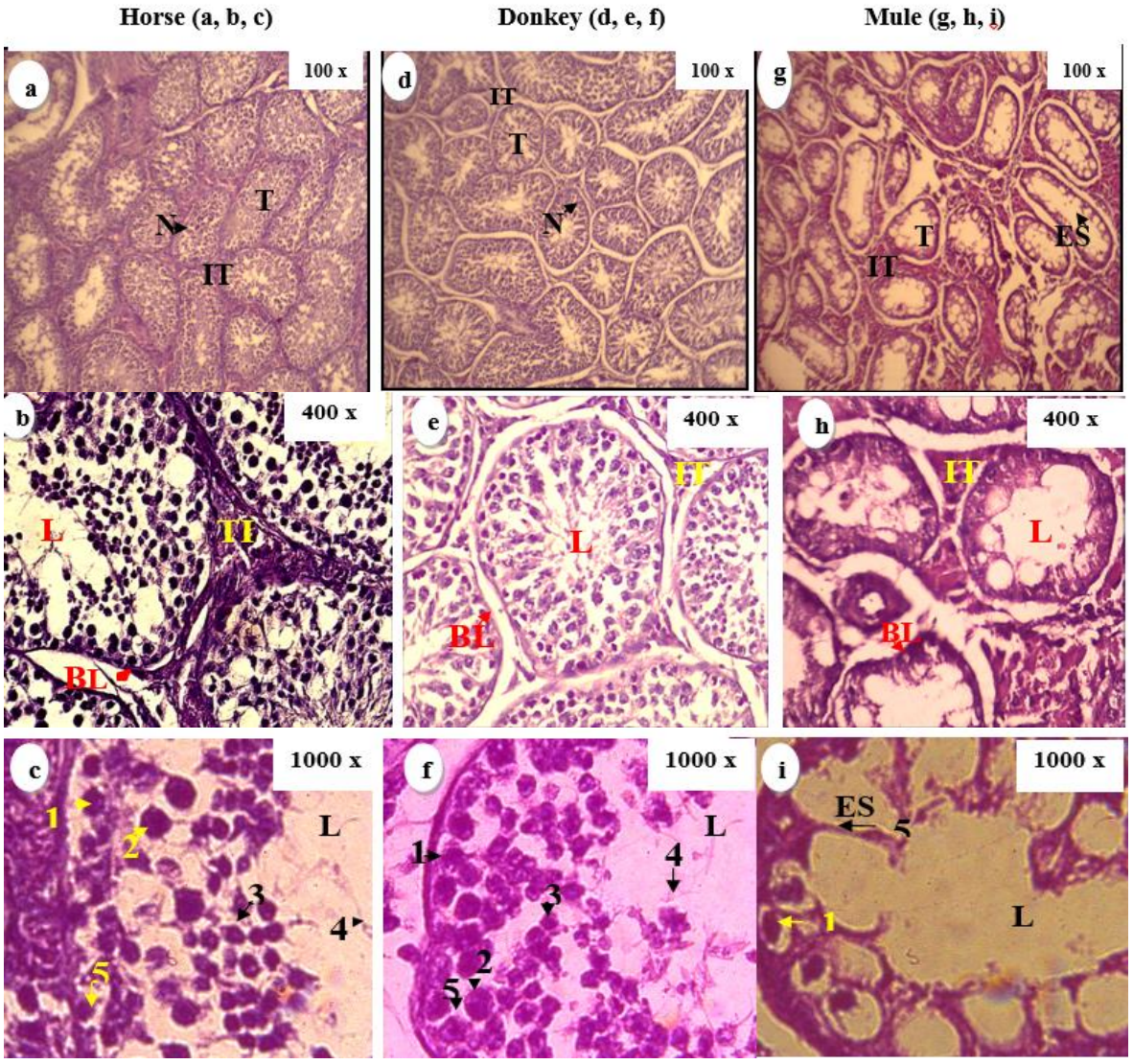

Figure 1. Structural appearance of the seminiferous tube in the three animals studied stained with eosin hematoxylin, under 10x, 40× and 400x magnifications. Scal bar: 100 µm. (100 x 400 x, 1000 x, 400 x), a: Horse. b: Donkey. c: Mule. T: Tube séminifère. TI: Interstitiel Tissue. N: Nucleus. Es: Empty space. L:Lumen. BL: Basic layer. 1: Spermatogonia; 2: Spermatocyte I; 3: Spermatid; 4: Sperm; 5: Sertoli cell.

The observations of the histological sections under 1000 x magnification, show that in the horse as in the donkey spermatogenesis is normal, with the presence of spermatogonia, spermatocyte I, spermatids and spermatozoa, while in the mule we have noted the presence of some spermatogonia (Figure 3).

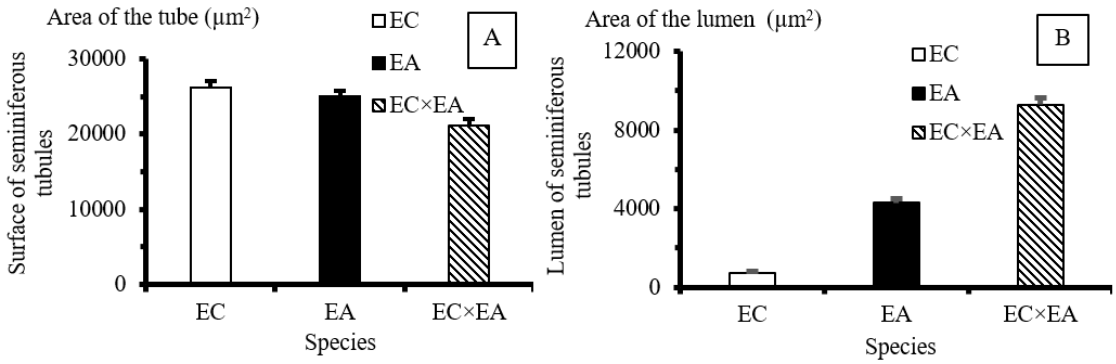

Figure 2. Variation of the area of the seminiferous tube (A) and the area of the lumen of the seminiferous tube (B) in horses, donkeys and mules. EC: Horse. EA: Donkey. EC×EA: Mule.

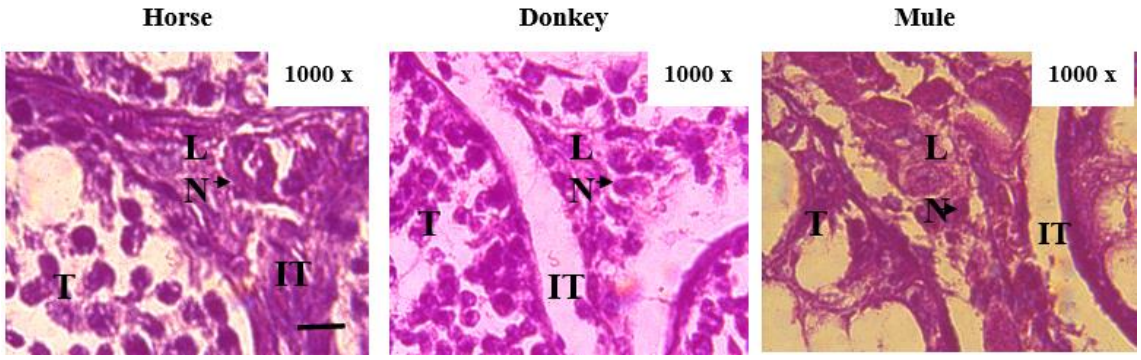

Figure 3. Structural aspect of the Leydig cell in the three animals studied stained with Congo red and observed under a light microscope at1 000×, Scal bar: 10 µm. T: Seminiferous tube; TI: Interstitial Tissue; L: Leydig cell; N: Leydig cell nucleus.

Morphometry and histology of epididymis

Figure 4 showed that in the horse and in the donkey the epididymal duct was fuller of spermatozoa. In the mule, the lumen of the epididymal duct was poor in spermatozoa.

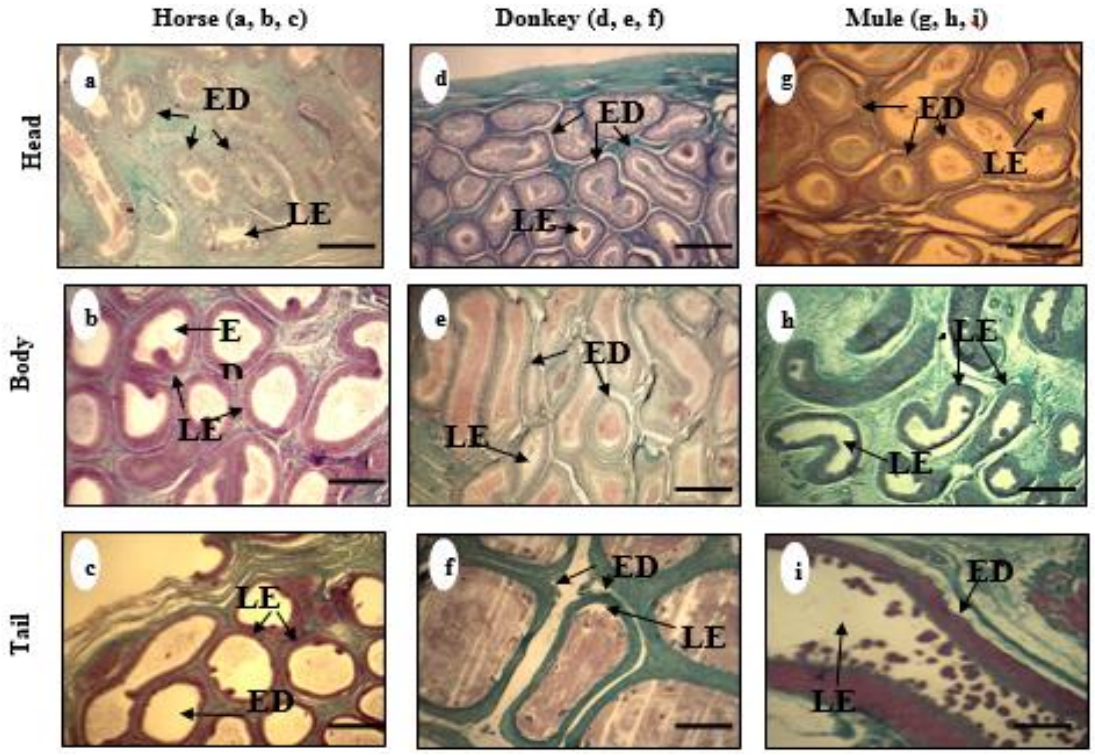

Figure 4. Structural appearance of the epididymis in the three animals studied stained with Masson's trichrome (TM) observed under 400× magnification; ED: Epididymal duct; LE: Lumen of the epididymal duct; SPZ: spermatozoa. Scal bar: 200 µm.

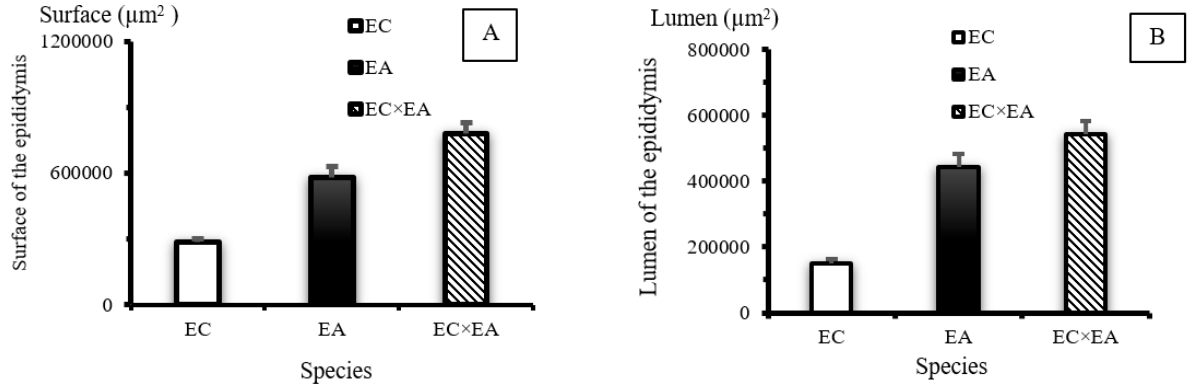

Figure 5. Epididymis morphometric, the surface area of the epididymal duct (A) and that of the lumen of this duct (B). EC: Horse. EA: Donkey. EC×EA: Mule hybrid.

Figure 6 illustrated that in the horse the lumen of the epididymis channels was surrounded by a pseudostratified cylindrical epithelium. The height of the epithelium length of the stereocils were developed in the body compared to the other segments, the light of the three segments was full of sperm. In the donkey, the height of the epithelium varied according to the epididymal segment. In the mule, the height of the epithelium was greater in the head and body regions and lower in the tail.

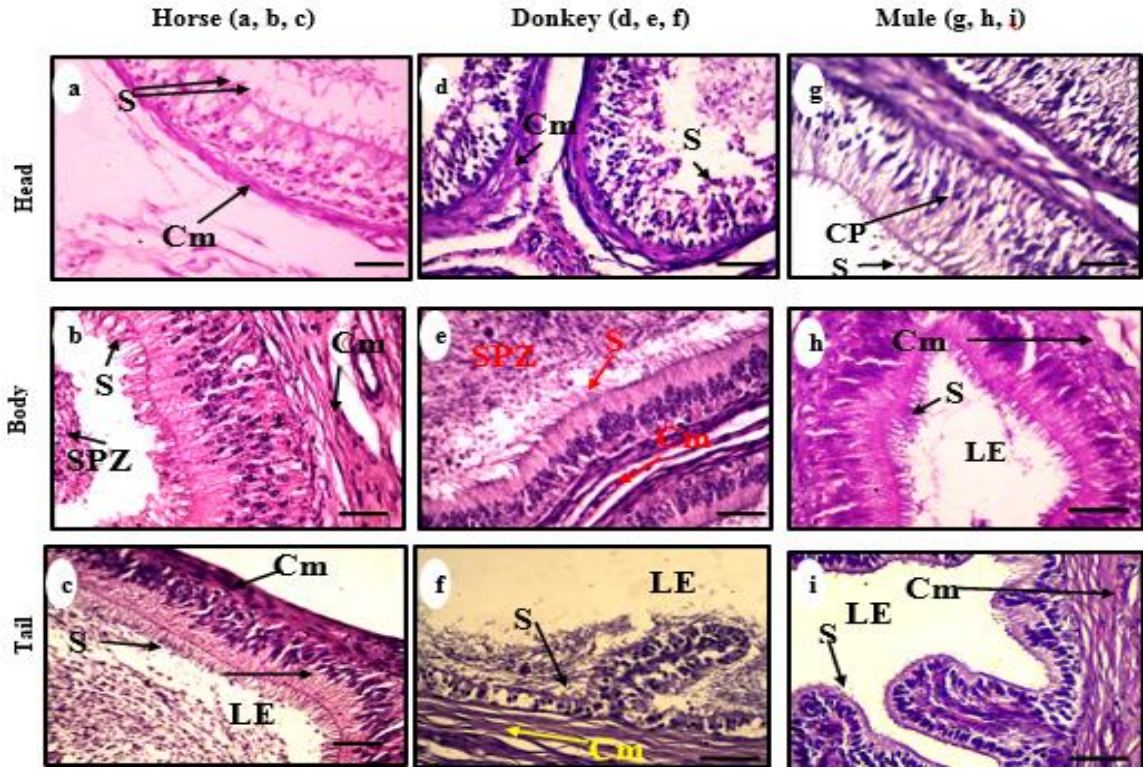

Figure 6. Structural appearance of the epididymis in the three animals stained with Hematoxylin Eosin and observed under 400× magnification. Cm: Circular layer of smooth muscle cell; S: Stereocilia; SPZ: spermatozoa; LE: lumen of the epididymal duct. Scal bar: 50 µm.

Morphometric results reinforce and confirm histological results (Figure 7).

The statistical study showed that there was a significantly highly difference between the horse and the donkey ($p < 0.001$) for the length of the epithelial cells and for the length of the basal nucleus. Significantly highly difference between horse and mule, ($p < 0.001$) for epithelial cell length and for basal nucleus length. A Significantly highly difference between donkey and mule ($p < 0.001$) for epithelial cell length and ($p > 0.05$) for basal nucleus length.

Morphometry and histology of Vas Deferens

A significantly difference between the horse and the donkey ($p < 0.001$) for the surface of the duct ($p < 0.05$) for the lumen of this duct. There is a significantly difference between donkey and mule ($p < 0.001$)

for surface of the duct, and no difference ($p < 0.05$) for the lumen of this duct. No significantly differences were reported between horse and mule for the surface and for the lumen of this duct ($p > 0.05$) (Figure 8).

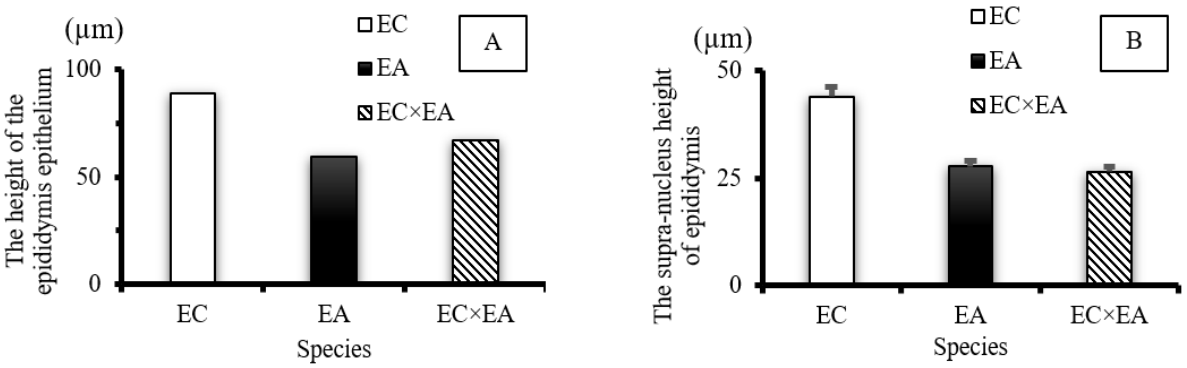

Figure 7. The height of the epididymis epithelium (A), the supra-nucleus height of epididymis (B). EC: Horse. EA: Donkey. EC×EA: Mule.

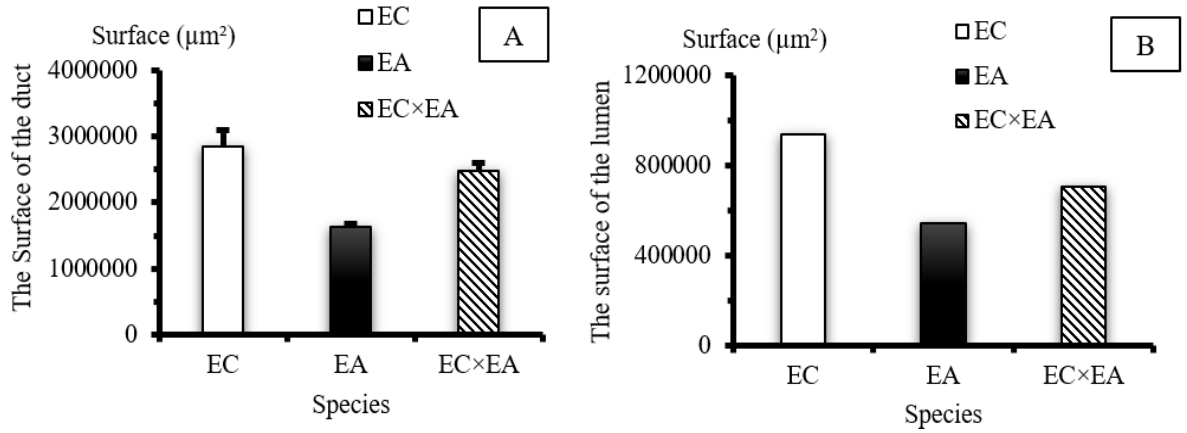

Figure 8. Morphometric of the vas deferens in the two species and the hybrid. (A): the surface of the canal, (B): the surface of the lumen. EC: Horse EA: Donkey, EC × EA: Mule (hybrid).

The Figure 9 showed that the length of invaginations in the lumen of the vas deferens varied according to the region and according to the species.

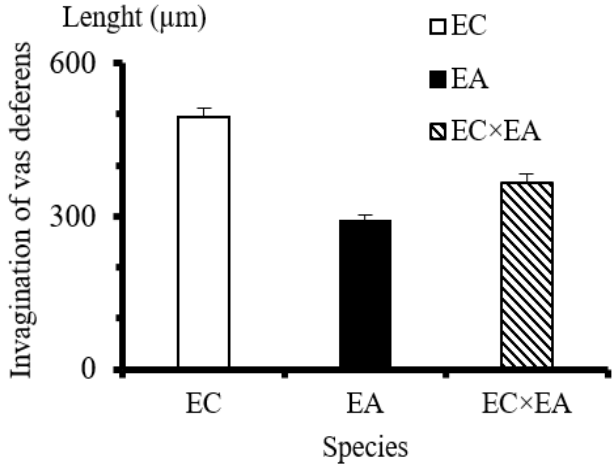

Figure 9. Morphometric of the length of vas deferens invagination in the two species and the hybrid. EC: Horse. EA: Donkey. EC × EA: Mule (hybrid).

Under 400x magnification, for the horse as for the donkey, the lumen is full of spermatozoa; in the mule we have the total absence of spermatozoa in the deferens duct in five cases or 83.33% (Figure 10).

Under 1000x magnification we have noted the presence of a spermatozoa in the deferens duct of one hybrid species (mule) or 16.67% (Figure 11).

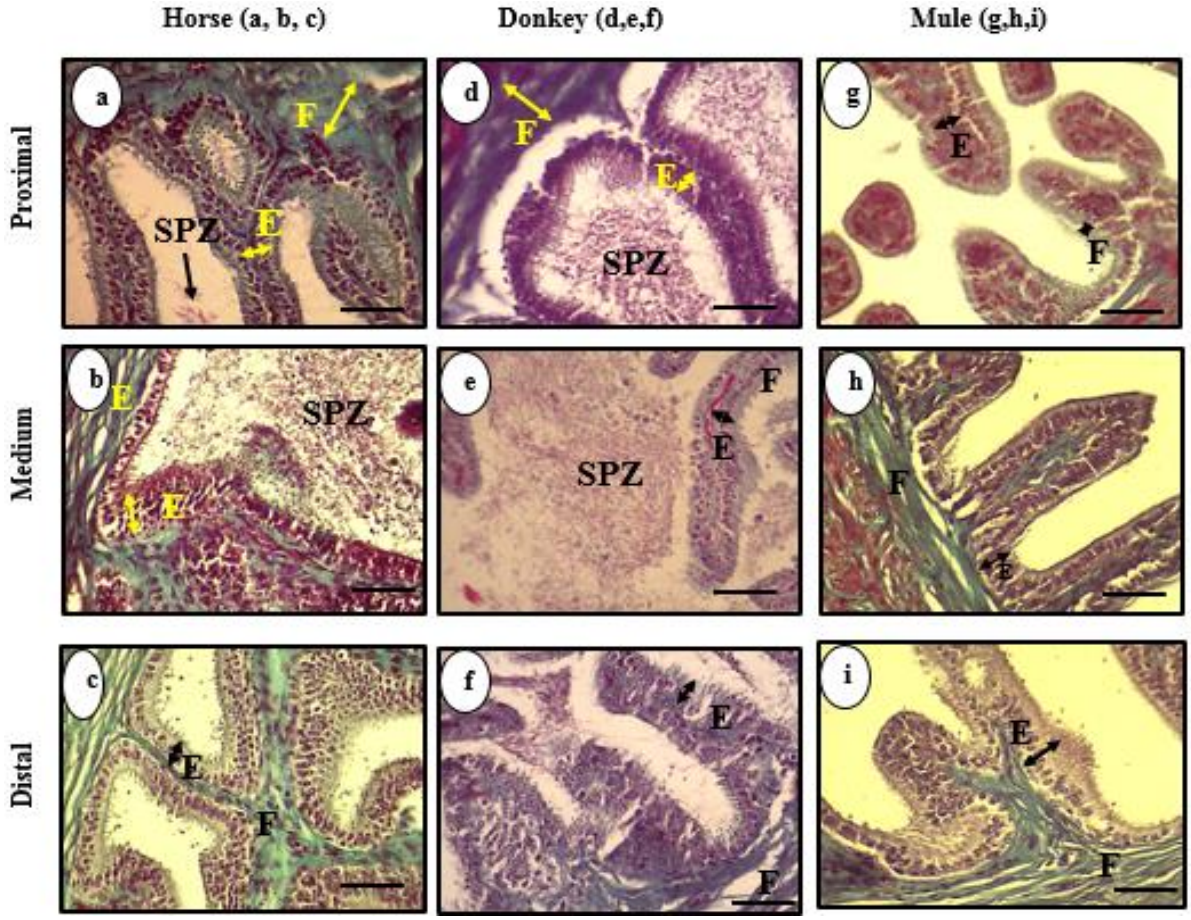

Figure 10. Structural aspect of the vas deferens in the three animals studied, stained with Masson's trichrome (TM) and observed under 400x magnification. Scal bar: 50 µm. SPZ: Spermatozoa. F: collagen fiber E: Epithelium.

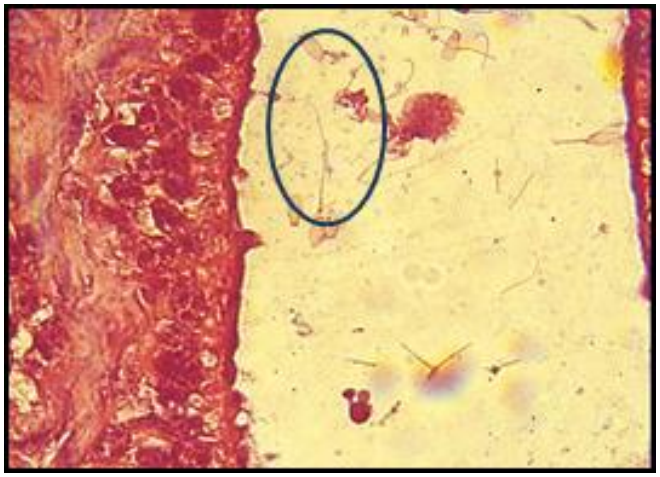

Figure 11. Presence of spermatozoa the vas deferens of the mule, stained by Congo red under 1000× magnification. Scal bar: 10 µm.

Discussion

The testicles gonad structure is conserved among vertebrates. These gonads are compound of several lobes, within each lobe of the testis there are convoluted seminiferous tubules. These are lined by a stratified epithelium of spermatogenic cells, Sertoli cells and Leydig cells. Thus, quantitative data can be used to investigate testicle function. A few works have reported upon the spermatogenesis process and testis morphometry in horses, donkeys and their hybrids (Han et al., 2016). This is the first comparative study of testicular structure and spermatogenesis in two equine species and their sterile hybrid. Likewise, an analysis was carried out on the stage of sperm block that is the cause of infertility, which could serve as a baseline data in further study of this animal.

Morphometry of testicle, epididymis and vas deferens

The results of this study highlight some differences in testicular morphology between the mule and the horse and the donkey two parental species. The testicle of the hybrid were smaller than those of the two parental species, this result is similar to those of Hernandez and Marquez (1977), Neves et al. (2002) and Yang et al. (2019). Pathak, Sheih, Rajput, Bherdwaj, and Sharma (2013) reported that testicle size in donkeys was almost 5-fold higher than in mules. The testicle of the donkey was smaller than of the horse. In contrast, Chabchoub, Tibary, and Trimeche (2007) noted that the donkey's genitalia is larger than that of the horse under Tunisian conditions.

Chabchoub et al. (2007), Carluccio et al. (2013), Houssou et al. (2018) and Houssou et al. (2020) reported that factors affecting spermatogenesis in the stallion are the age and season. In our studies the testicle of the two equine species and the infertile hybrid were collected between March and April, so during breeding season from mature animals >7 years.

The result obtained for the epididymis and the vas deferens is reliable with the results of Hernandez and Marquez (1977), which indicate that the epididymis and the vas deferens of the mule have the same morphological characteristics as those of the horse and the donkey without spermatozoa.

Histology of testicle

The structural study has shown that there are all of the germ cells at different stages of division also we have noted spermatozoa. Therefore, spermatogenesis is normal in horses and donkeys. However, in the mule the seminiferous epithelium contains only a few spermatogonia and spermatocytes I, therefore, spermatogenesis stopped, the other stages are absent and replaced by empty spaces, which appear very clear. This result is similar to those of Chandley, Short, and Allen (1975).

Steiner and Ryder (2013) reported that the absence of germ cells in mule due to blockage during meiosis. The primary spermatocytes in the hinny could enter into synapsis but because of the failure of homologous chromosomes to pair (Han et al., 2016). They could not complete meiosis and therefore could not advance beyond the spermatocyte phase. Studies by Zong and Fan (1989) showed that irregular shaped spermatozoa, such as some with a diamond-shaped head and others with an abnormally long and thin neck were occasionally found in three year old hinnies.

The structural results obtained for interstitial tissue, indicated that in horses and mules the Leydig cell has a large rounded nucleus, but in the donkey the Leydig cells has a small nucleus. Pozor et al. (2017) reported that Leydig cells in the testicle of mature stallions contain a granular yellowish-brown pigment as well. The surface of the seminiferous tubes are voluminous in the horse and the donkey than in the mule these results are similar to the results of Hernandez and Marquez (1977), Neves et al. (2002) and Pathak et al. (2013). We have also carried out the enumeration of the seminary tubes on a few sections and we found that this number is greater in the mule compared to the horse and the donkey; this found is similar to that of Hernandez and Marquez (1977).

Neves et al. (2002), Costa et al. (2012) and Neves et al. (2014) that have been doing comparative and accurate testicular morphometric analysis in horse, donkey and mule, including structural evaluation of Sertoli and Leydig cells, the characterization of spermatogonial phenotype stem cells (SSCs). The authors have conclude that the structural results obtained for mule, horse and donkeys indicated that both Leydig and Sertoli cells were functionally normal in mule, strongly suggesting that mule seminiferous tubules are able to sustain complete development of spermatogenesis. Therefore, mules were considered potential candidates for transplants of SSCs originated from donkeys, horses, or even other large animals (Neves et al., 2002).

Histology of epididymis

We notice that in the horse the epididymal duct is separated at the level of the three segments (head, body and tail) while in the donkey these canals are united, in the mule are attached in the head but separated in the body and the tail. In the horse and donkey, the lumen is full of spermatozoa; on the other hand, in the mule we noticed the presence of a few spermatozoa. Chandley et al. (1975) and Han et al. (2016) have also noted the sperm poverty in the lumen of the body part of the epididymis in the hybrid.

Our morphometric results show that the height of the epithelium at the level of the body of the epididymis differs depending on the species. We also found the presence of folds in the epididymis tail in donkeys and mules and absence in horses. William, Bacha, and Bacha (2012) reported that in the horse, the lining of the duct in the tail region of the epididymis forms short, villuslike projections.

Histology of Vas Deferens

The animals represent the same structure of the vas deferens but with a histological peculiarity, the presence of spermatozoa in large quantities in the lumen of the duct in horses and in the donkeys, but in the hybrid we find few spermatozoa. Furthermore, in the donkeys, the ampulla of the vas deferens had a folded mucosa, similar result found by Abou-Elhamd, Salem, and Selim (2012) and Han et al. (2016). Some studies reported by Iannuzzi, Pereira, Iannuzzi, Fu, and Ferguson-Smith (2017) noted that the mules would they occasionally be able to produce a few spermatozoa. Canisso, Panzani, Miró, and Ellerbrock (2019) reported that mules display reproductive cyclic activity but are rarely fertile. However, there remains considerable doubt that the mules will ever be able to produce a sufficient quantity of sperm with the correct aptitude for fertilization. Our morphometric results of the length of folded in the vas deferens in the two species and the hybrid show that the height of the invaginations is longer in the horses than in the donkey while the mule is intermediate.

Conclusion

The two species (Horse, donkey) and the mule have significant differences in the area of seminiferous tubules. The concentration of spermatogenic cells, Leydig cells, Sertoli cells, epididymal ducts and vas deferens structures were significantly different. The main reason for the blockage of mule sperm may be the failure of homologous chromosome pairing, resulting in incomplete outline and incomplete meiosis. However, we have noted complete development of spermatogenesis in mule.

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