

Ultrastructural observation of the mule testis indicates normal function of somatic cells

E.S. Neves¹, H. Chiarini-Garcia, L.R. França²

Laboratory of Cellular Biology, Department of Morphology, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil 31270-901.

Abstract

The mule (*Equus mulus mulus*) is a domestic animal that results from the breeding of a male donkey (*Equus asinus*) to a female horse (*Equus caballus*). The number of metacentric and acrocentric autosomal chromosomes in horses and donkeys differs approximately by one-third, resulting in pairing failure of homologous chromosomes during meiosis. Probably for this reason, spermatogenesis in mules rarely advances beyond spermatocytes and leads to sterility in this hybrid equid. The results found in a recent study developed in our laboratory, comparing testis structure and function by using light microscopy in donkeys and mules, suggested that mules' seminiferous tubules are probably able to sustain complete spermatogenesis. In order to further investigate this hypothesis, we performed an ultrastructural analysis of Sertoli and Leydig cells in both mules and donkeys. The Sertoli cell tight-junction morphology and the morphofunctional integrity of the Sertoli cell barrier were also investigated in these species utilizing lanthanum as a tracer. The results showed that somatic cells of both donkeys and mules had similar structure and organelle morphology. This indicated that Sertoli and Leydig cell structure and function in mules were apparently normal and functional. The same was observed for Sertoli cell tight junctions and the integrity of the Sertoli cell barrier. Taken together, our results support the light microscopy data already obtained indicating that mules are potential viable candidates for transplantation of germ cells originated from donkeys, horses, and other equids.

Keywords: testis, ultrastructure, Sertoli cell, Leydig cell, mules, donkeys

Introduction

The male mule (*Equus mulus mulus*, 63 chromosomes) is a sterile hybrid domestic animal that results from the breeding of a male donkey (*Equus asinus*, 62 chromosomes) to a female horse (*Equus caballus*, 64 chromosomes; Benirschke *et al.*, 1962). The main cause of sterility in mules is related to the pairing failure of homologous chromosomes that occurs

during meiosis, probably because the number of metacentric and acrocentric autosomal chromosomes in horses and donkeys diverges approximately by one-third (Chandley *et al.*, 1974; 1975).

Early during development and before sexual differentiation, primordial germ cells, which are bipotential cells, migrate from the base of the allantois via the gut mesentery and populate the genital ridge, becoming gonocytes in this anlage where the primitive, undifferentiated gonads are beginning to form (Capel, 2000; de Rooij and Russell, 2000). At this time, the Sertoli cell, which presumably originated from the coelomic epithelium, expresses the male sex determining gene *Sry* in the short arm of the Y chromosome, thus playing a crucial role in directing testis differentiation. The other somatic cells of the testis (Leydig cells, peritubular myoid cells, endothelial cells, and fibroblasts) are thought to penetrate the genital ridge from the mesonephric region probably under the influence of factors produced by Sertoli cell precursors (Merchant-Larios and Moreno-Mendoza, 2001). Also important are the morphological interactions observed between germ cells, Sertoli cell precursors, and peritubular myoid cells that result in the formation of the seminiferous cords and represent the events necessary for the normal development of spermatogenesis (Yao *et al.*, 2002). In this regard, Sertoli cells in mammals and probably in all other vertebrates play a pivotal role in the differentiation and development of a functional testis. However, it should be mentioned that although the basic structure of the testis is highly conserved among vertebrates (Capel, 2000), specific characteristics of the testis structure might be found for a particular species. This aspect makes the mule and donkey an interesting model to perform comparative studies related to testis structure and function in both species, especially because *Sry* is considered to show great interspecific variability (Short, 1997).

Germ cell transplantation is a fascinating and powerful technique that has been largely utilized in the past decade in mammals for the purpose of investigating spermatogenesis and stem cell biology (Brinster, 2002; Dobrinski, 2005a; b; Khaira *et al.*, 2005; McLean, 2005). This technique also offers great potential for

¹Current address: Department of Anatomy, Federal University of Pernambuco, Recife, Brazil 50670-901

²Corresponding author: lrfranca@icb.ufmg.br

+55 31 3499-2816; fax: +55 31 3499-2780

Received: April 17, 2006

Accepted: June 12, 2006

studies involving biotechnology, transgenic animals, and the preservation of the genetic stock of valuable animals or endangered species (Brinster, 2002; Dobrinski, 2005a; b; Khaira *et al.*, 2005; McLean, 2005). However, the success and efficiency of germ cell transplantation depends on a recipient animal in which endogenous spermatogenesis is naturally absent or experimentally deprived (Brinster, 2002; Dobrinski, 2005a; b; Honaramooz *et al.*, 2005; McLean, 2005). Recently, we performed an extensive study comparing testis structure and function in donkeys and mules (Neves *et al.*, 2002) in which it was suggested that the seminiferous tubules in mules might be able to sustain complete spermatogenesis. Therefore, this species could be a potential candidate for transplantation of germ cells originating from donkeys and horses. In order to further investigate this hypothesis, we performed a careful and comparative ultrastructural analysis of Sertoli and Leydig cells in both mules and donkeys.

Materials and methods

Animals

Three sexually-mature donkeys (*Equus asinus*; 4-8 years of age) and 4 young adult mules (*Equus mulus mulus*; 3-3.5 years of age) were utilized in the present study. The donkeys belonged to the Pêga breed. They were donated by the Brazilian Pêga Donkey Association and were being utilized as breeders. Reproduction in this species is not seasonal (Gastal *et al.*, 1996); however, the animals were orchietomized during the equine-breeding period, which is from September to February in the south hemisphere. All mules were offspring of Pêga donkeys. Before surgery, all animals received a 0.8 ml, i.v. injection of Sedivet® (Boehringer De Angell) per 100 kg of body weight. All surgical procedures were performed by a veterinarian who followed approved guidelines for the ethical treatment of animals.

Tissue preparation

After the testes and epididymides were removed, blood was cleared from the testes by introducing 0.9% saline solution with heparin (125 IU/L) via a needle through the testicular artery. Subsequently, the testes were perfused-fixed by gravity-fed perfusion with 4% buffered glutaraldehyde for 25-30 minutes. After perfusion, testes were trimmed out from the epididymis and weighed and cut longitudinally by hand with a sharp knife. Tissue samples, measuring 1-3 mm in thickness, were taken near the tunica albuginea. Following glutaraldehyde fixation, the testes were diced into small pieces, placed into the same fixative for an additional hour, washed in phosphate buffer overnight, post-fixed with 1% osmium:1.25% potassium ferrocyanide mixture, dehydrated in ethanol,

and embedded in Epon 812-Araldite 502. Thin sections were then routinely prepared for light microscopy analysis. Ultra-thin sections from the previously selected areas of the testis were mounted on 200-mesh grids, stained with uranyl acetate and lead citrate, and examined using an EM-10 electron microscope (Zeiss, Oberkochen, Germany).

Study of the Sertoli cell barrier permeability

The protocol utilized to prepare the testis tissue was similar to that described in the literature (Neaves, 1973; Lewis and Knight, 1988; Hayat, 1993) with some modifications. Briefly, the testis was perfused-fixed with 4% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 1% lanthanum hydroxide. After being fixed, the testis tissue fragments, measuring 1-3 mm in thickness and taken near the tunica albuginea, were immersed in the same fixative for approximately 5 hours and then stored at 5°C in the buffer described above. Subsequently, these fragments were post-fixed with 1% buffered osmium containing 1% lanthanum hydroxide for 8 hours, washed in buffer, and then immersed in 0.5% aqueous uranyl acetate for 12 hours. Finally, the testis fragments were routinely prepared for transmission electron microscopic investigation as previously mentioned.

Results

Biometric data

The mean (\pm SEM) body weights of donkeys (249 ± 22 kg) and mules (262 ± 12 kg) investigated were similar. However, testis weight (\pm SEM) in mules (39 ± 7 grams) was nearly 5-fold lower than in donkeys (206 ± 9 grams).

Light microscopy evaluation of the testis

Figure 1 shows a panoramic view of the testis compartments in the donkey (Fig. 1A) and mule (Fig. 1B). Compared with donkeys (Fig. 1C), few germ cells were present in the seminiferous tubules of mule testes (Fig. 1D-E). A more careful histological evaluation showed that most of the seminiferous tubules in mules had a patent lumen, and in almost all of these tubules, spermatogonia and spermatocytes were present (Fig. 1D). Meiotic figures and/or secondary spermatocytes were eventually observed in these tubules, and early spermatids were rarely seen. In approximately one-third of the seminiferous tubules, only vacuoles of different sizes and shapes were observed in the seminiferous epithelium lined only by Sertoli cells and spermatogonia (Fig. 1E). Few tubular profiles presented only Sertoli cells (Fig. 1F), and in some, no lumen or vacuoles were observed (figure not shown). Almost half of the seminiferous tubules observed had apoptotic cells (presumably germ cells) in the epithelium (inset in Fig. 1E).

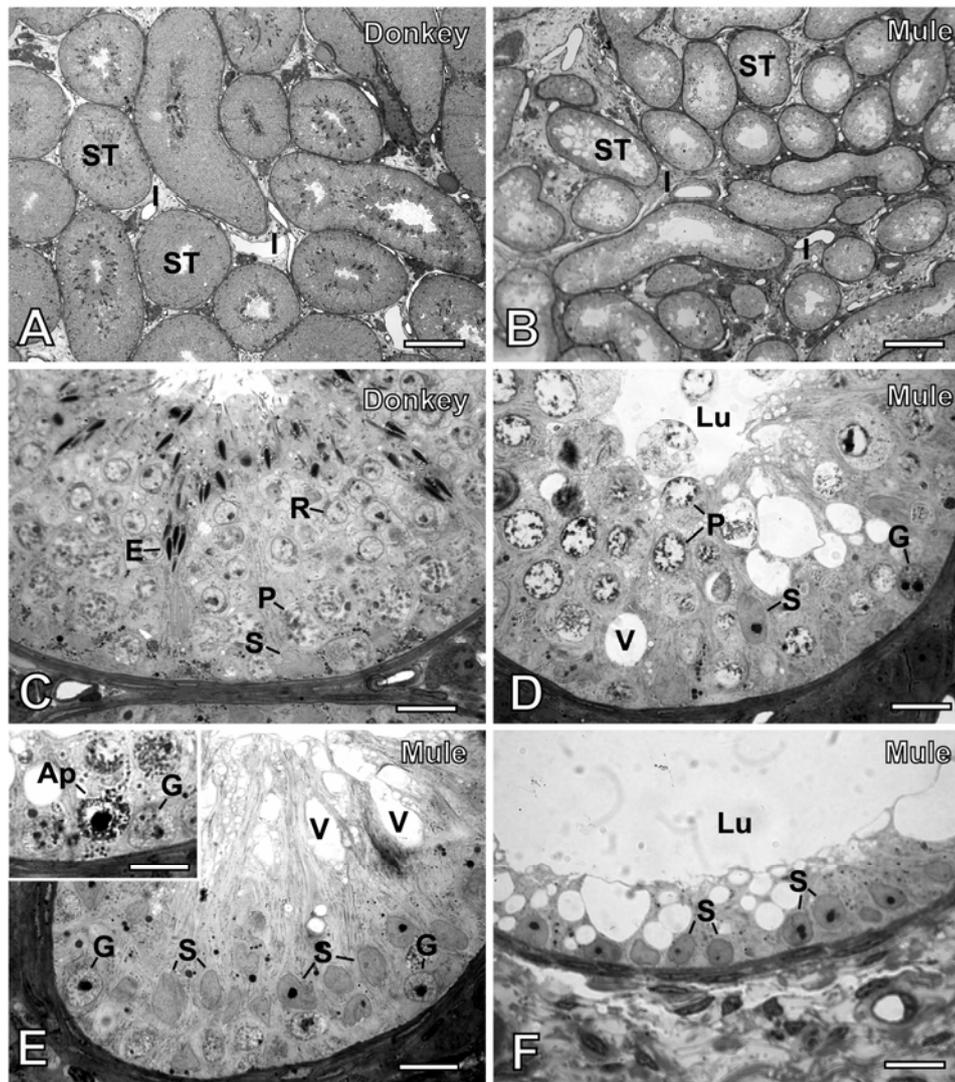


Figure 1. Light microscopy of the donkey and mule testis. Figure 1A and B shows a panoramic view of the testis compartments in the donkey and mule, respectively. Compared with donkeys (Fig. 1C), few germ cells were present in the seminiferous tubules of mule testes (Fig. 1D-E), and some tubules (Sertoli cell-only) were totally devoid of germ cells (Fig. 1F). Apoptosis was common in the seminiferous epithelium of mules (inset in Fig. 1E). Seminiferous tubules (ST); intertubular compartment (I); lumen (Lu); vacuoles (V); elongate spermatids (E); round spermatids (R); pachytene spermatocytes (P); spermatogonia (G); apoptosis (Ap); Sertoli cells (S). Bar = A and B, 150 μ m; C-F, 15 μ m; inset, 12 μ m.

Ultrastructure of Sertoli and Leydig cells

In both species investigated, Sertoli cells had a high number of organelles, predominantly mitochondria and smooth and rough endoplasmic reticulum. Mitochondria were elongated and showed few tubular cristae. In contrast with donkeys (Fig. 2A), Sertoli cells in mules (Fig. 2B) had a more heterochromatic nucleus

and a smaller and more condensed nucleolus.

Leydig cell ultrastructure in donkeys and mules is depicted in Fig. 3. Leydig cell morphology in both species was similar. In this regard, these cells had a round or oval shaped nucleus and a relatively large amount of smooth endoplasmic reticulum and mitochondria. This latter organelle was usually elongated, having tubular cristae and dense granules in the mitochondrial matrix.

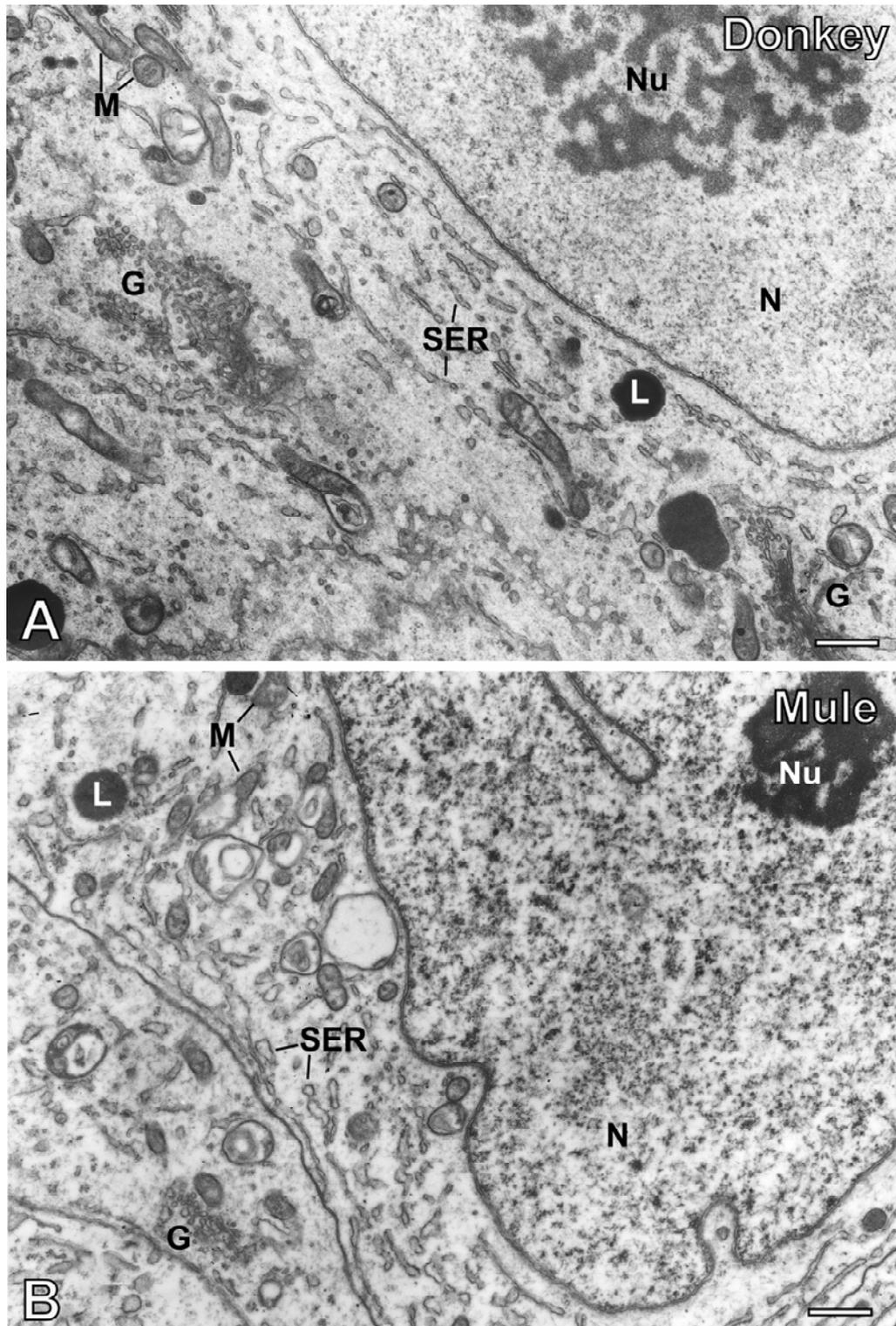


Figure 2. Sertoli cells ultrastructure investigated by transmission electron microscopy in the adult donkey (A) and mule (B). Observe that the Sertoli cell in the donkey has an euchromatic nucleus (N) and a conspicuous nucleolus (Nu). Smooth endoplasmic reticulum (SER), elongated mitochondria (M), Golgi (G), and lipid droplet (L) are commonly observed in the cytoplasm. In the mule, except for the presence of a relatively heterochromatic nucleus (N) and a smaller and more condensed nucleolus (Nu), the general morphology of the organelles such as smooth endoplasmic reticulum (SER), and mitochondria (M), and Golgi are similar to those described for the donkey. Bar = 0.5 μ m.

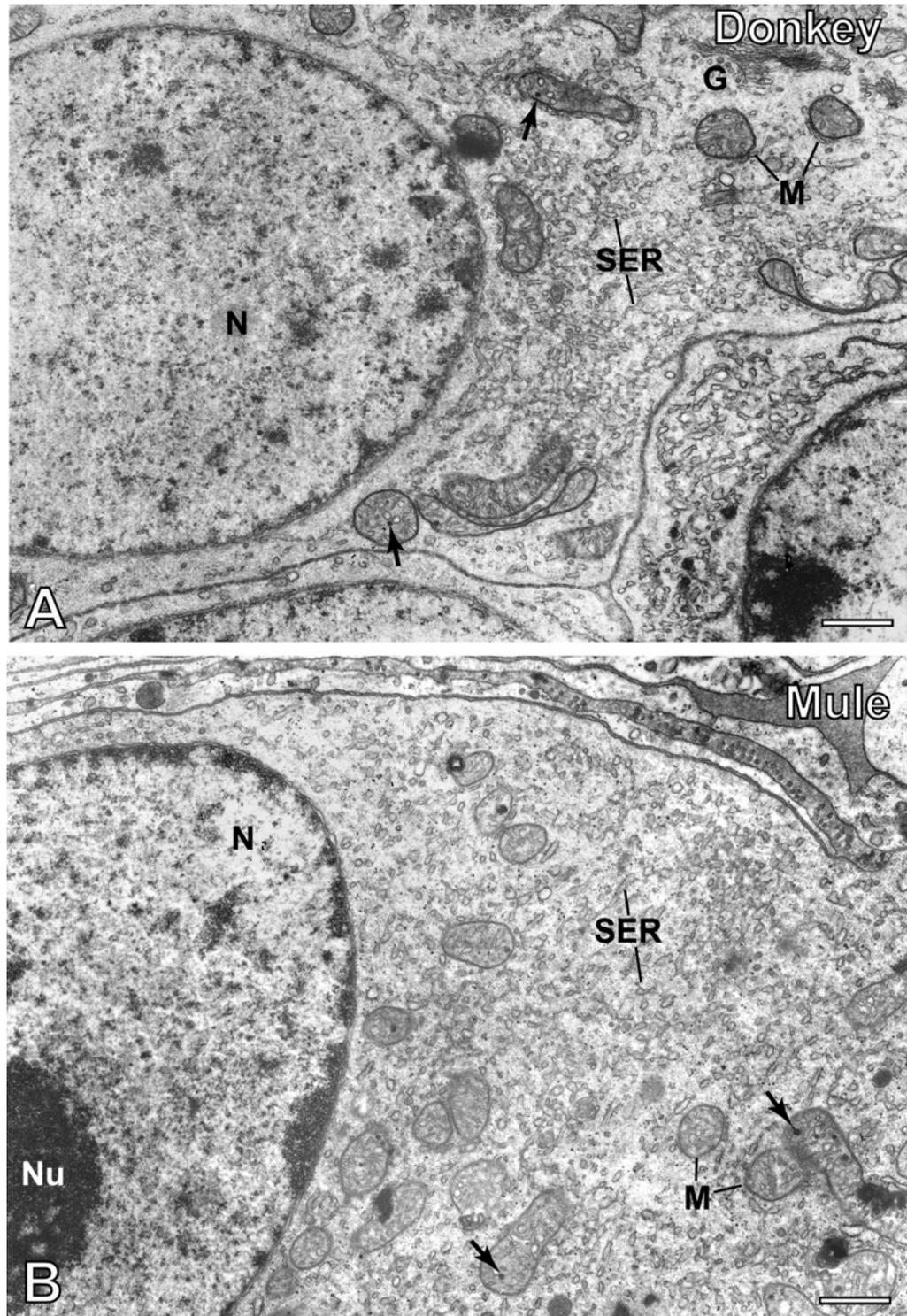


Figure 3. Leydig cell ultrastructure investigated by transmission electron microscopy in the adult donkey (A) and mule (B). The Leydig cell in the donkey has a relatively heterochromatic and round to oval-shaped nucleus (N). In the cytoplasm, elongated mitochondria (M) containing tubular cristae and a large amount of smooth endoplasmic reticulum (SER) are observed. Golgi (G) and electron-dense granules in the mitochondrial matrix (arrow) are also indicated. The general morphology of the Leydig cell in the mule is very similar to that described above for the donkey. In this regard, except for a conspicuous nucleolus (Nu), all other cell components mentioned for the donkey are present in the mule. Bar = 0.5 μ m.

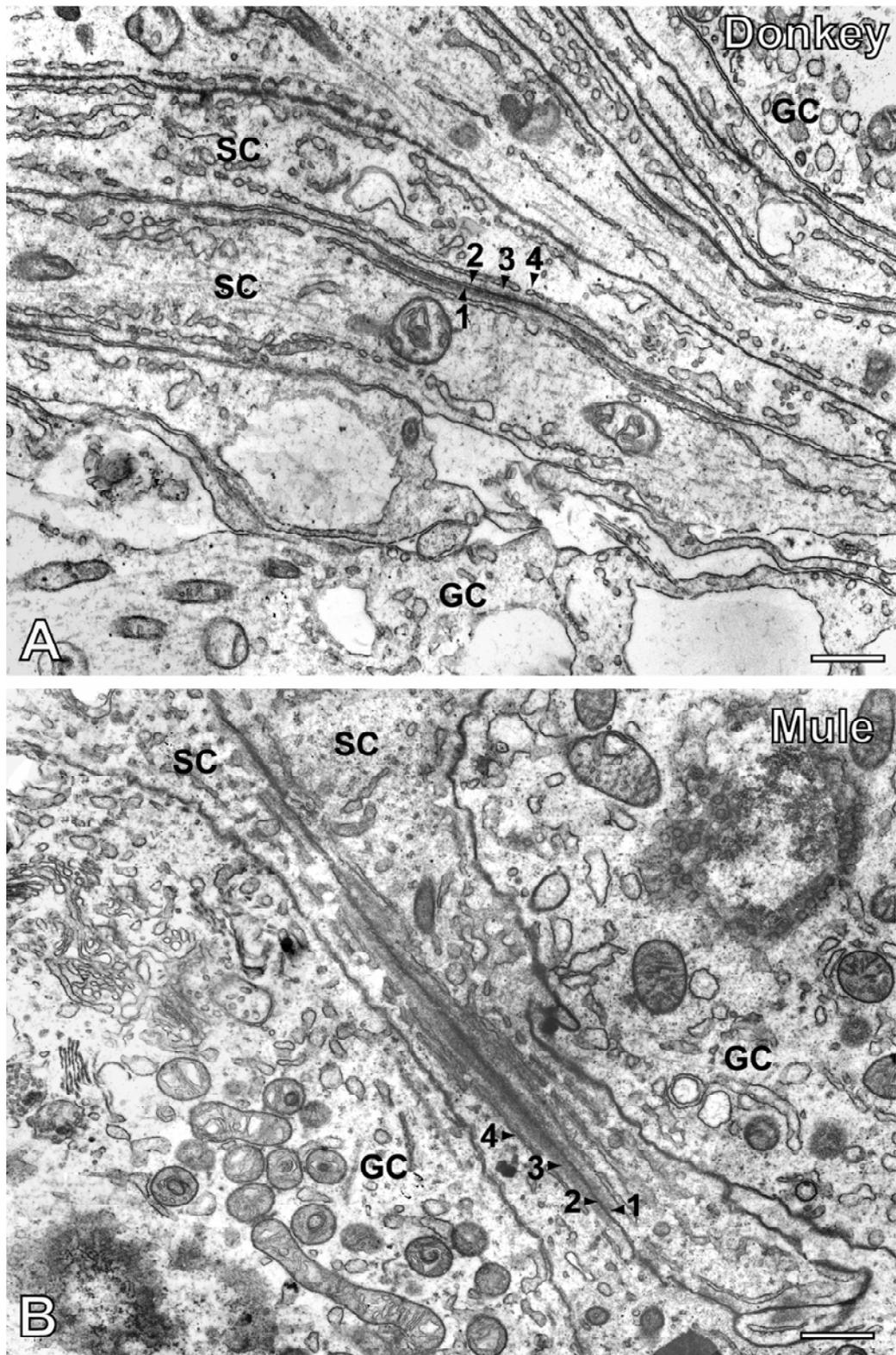


Figure 4. Sertoli cell (SC) ultrastructure investigated by transmission electron microscopy in the adult donkey (A) and mule (B). This figure depicts a Sertoli cell tight junction formed by the plasma membranes of two adjacent Sertoli cells (1 and 2) and actin filaments (3) surrounded or delimited by smooth endoplasmic reticulum (4). Germ cells (GC), near the Sertoli cells, are also indicated. Bar = 0.5μm.

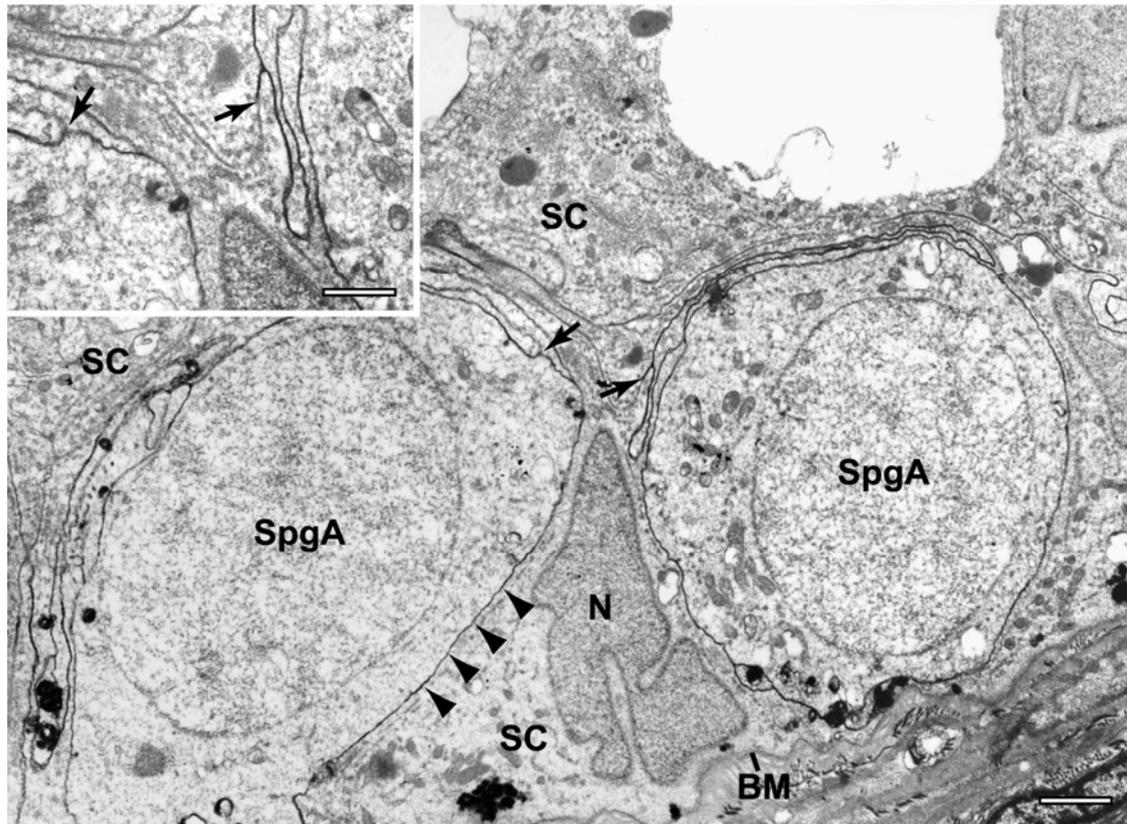


Figure 5. Sertoli cell ultrastructure investigated by transmission electron microscopy in the adult mule. Lanthanum was used as a tracer in order to investigate the Sertoli cell barrier functional integrity. The arrowheads indicate the presence of lanthanum in the intercellular space delimited by the Sertoli cell (SC) plasma membrane and the type A spermatogonia (SpgA) located in the seminiferous epithelium basal compartment. The arrows show the interruption of lanthanum progression (see also the inset at higher magnification), probably due to the Sertoli cell barrier (tight junctions). The Sertoli cell nucleus (N) and tunica propria basal membrane (BM) are also indicated. Bar = 1 μ m; inset, 0.5 μ m.

Sertoli cell barrier

As observed in Fig. 4, the tight junctions that are considered the morphofunctional basis of the Sertoli cell barrier were apparently normal in both donkeys and mules. The permeability and functional integrity of tight junctions were also investigated in adult mules utilizing lanthanum as a tracer. According to Fig. 5, the progression of this tracer between Sertoli cell plasma membranes was interrupted at the level of the tight junctions. This result strongly suggests that this very important aspect of the seminiferous epithelium was probably not altered in mules, providing an adequate environment for the development of germ cells.

Discussion

Testis structure and function in donkeys and mules were comparatively investigated in a recent study performed in our laboratory (Neves *et al.*, 2002). In this study, we showed that the percentage of seminiferous tubules containing spermatogonia and spermatocytes in mules was approximately 95%, and that in spite of

similar individual Leydig cell size, the total number of these cells in mules was only one-third of that observed in donkeys. However, the total number of Sertoli cells per testis, pachytene spermatocyte nuclear volume, and spermatogenic cycle length found for these two species were very similar. These results found for testis structure and function in mules, utilizing light microscopy investigation, suggested that the seminiferous tubules in this hybrid might be able to sustain complete spermatogenesis, thus making the mule a potential candidate for transplantation of germ cells originated from donkeys, horses, or other equids.

In the present study, we performed a careful ultrastructural investigation of Sertoli and Leydig cells. In particular, the ultrastructure of Sertoli cell tight junctions, as well as the functional integrity of Sertoli cell barrier, was investigated utilizing lanthanum as a tracer. The general morphological and ultrastructural characteristics of Leydig cells in mammals show great species variation (Russell, 1996). However, our results showed that Leydig cell ultrastructure in mules and donkeys were very similar. Specifically for mules, our



results are similar to studies developed by Hernández-Jáuregui and Monter (1977), suggesting that Leydig cell steroidogenic capacity is probably not affected in this species. This finding is of particular interest because it corroborates that the lack of complete spermatogenesis in mules is mainly due to the homologous chromosome pairing failure (Chandley *et al.*, 1974; 1975). In fact, although to our knowledge there is no scientific evidence for that in the literature, adult mules are well known for their very high libido.

Following the same trend found for Leydig cells, our data showed that Sertoli cell ultrastructure was very similar in mules and donkeys, and not altered in mules (Alvarenga, 1990). The same results were found for Sertoli cell tight junctions in donkeys and mules and for the Sertoli cell barrier integrity investigated in mules. Although some specific characteristics of Sertoli cell organelles might be found for different mammalian species (Russell, 1993; Russell and Brinster, 1996; Hess and França, 2005), Sertoli cell ultrastructural characteristics observed in the present study for mules and donkeys were similar to those generally described for mammals (Ross, 1977; Vogl *et al.*, 1993; Hess and França, 2005). Also, our findings corroborate what is suggested in the literature for hybrids that usually have a functional Sertoli cell barrier (Andersen-Berg, 1984).

All of the ultrastructural findings obtained in the present work reinforce that sterility in mules is probably caused by problems in the germ cell components of the seminiferous epithelium and not due to the somatic elements (i.e. Sertoli and Leydig cells) of the testis. The fact that Sertoli cells had more heterochromatic nuclei and smaller nucleoli in mules compared to donkeys is probably related to the functional status of these cells that are involved with much fewer germ cells. Also, the similar cyto-architecture found for Sertoli and Leydig cells in donkeys and mules in the present investigation suggest a possible role of the donkey Y chromosome in determining important structural and functional aspects of the mule testis (Neves *et al.*, 2002).

In summary, the ultrastructural results found for mules confirm our light microscopic findings, suggesting strongly that mule seminiferous tubules are able to sustain complete spermatogenesis. In this regard, mules are potential candidates for transplants of germ cells originated from donkeys, horses, or other equids and even other large animals. However, this possibility could only be confirmed or tested with the development of functional studies in which early germ cells are experimentally depleted, once in mules investigated in the present study most of the available niches are still occupied by spermatogonia.

Aknowledgments

The scholarship awarded to Elizabeth da Silveira Neves from the Brazilian Foundation (CAPES)

is appreciated. Financial support from the Minas Gerais State Foundation (FAPEMIG) and the Brazilian Research Council (CNPq) is gratefully acknowledged. Technical help from Rubens Miranda and Adriano Moreira is highly appreciated.

References

- Alvarenga FCL.** 1990. *Ultrastructural studies of spermatogenesis in mules (equus asinus x Equus caballus)* [in Portuguese]. Botucatu, Brazil: UNESP. Dissertation.
- Andersen-Berg K.** 1984. The blood-testis barrier in sterile blue fox-silver fox hybrids compared with that in normal foxes of both species. *Int J Androl*, 7:167-175.
- Benirschke K, Brownhill E, Beath MM.** 1962. Somatic chromosomes of the horse, the donkey and their hybrids, the mule and the hinny. *J Reprod Fertil*, 4:319-326.
- Brinster RL.** 2002. Germline stem cell transplantation and transgenesis. *Science*, 296:2174-2175.
- Capel B.** 2000. The battle of the sexes. *Mech Dev*, 92:89-103.
- Chandley AC, Short RV, Allen WR.** 1975. Cytogenetic studies of three equine hybrids. *J Reprod Fertil Suppl*, 23:365-370.
- Chandley AC, Jones RC, Dott HM, Allen WR, Short RV.** 1974. Meiosis in interspecific equine hybrids. I. The male mule (*Equus asinus* x *E. caballus*) and hinny (*E. caballus* x *E. asinus*). *Cytogenet Cell Genet*, 13:330-341.
- De Rooij DG, Russell LD.** 2000. All you wanted to know about spermatogonia but were afraid to ask. *J Androl*, 21:776-798.
- Dobrinski I.** 2005a. Germ cell transplantation and testis tissue xenografting in domestic animals. *Anin Reprod Sci*, 89:137-145.
- Dobrinski I.** 2005b. Transplantation. In: Skinner MK, Griswold MD (Eds.). *Sertoli cell biology*. London: Elsevier Academic Press. p.417-485.
- Gastal MO, Henry M, Beker AR, Gastal EL, Gonçalves A.** 1996. Sexual behaviour of donkey jacks: influence of ejaculatory frequency and season. *Theriogenology*, 46:593-603.
- Hernández-Jáuregui P, Monter HM.** 1977. Fine structure of mule testes: Light and electron microscopy study. *Am J Vet Res*, 38:443-447.
- Hayat MA.** 1993. Staining and related reagents. In: Stains and cytochemical methods. New York: Plenum Press. p.202-214.
- Hess RA, França LR.** 2005. Structure of the Sertoli Cell. 2005. In: Skinner MK, Griswold MD (Eds.). *Sertoli cell biology*. London, Elsevier Academic Press. p.19-40.
- Honaramooz A, Behboodi E, Hausler CL, Blash S, Ayres S, Azuma C, Echelard Y, Dobrinski I.** 2005. Depletion of endogenous germ cells in male pigs and goats in preparation for germ cell transplantation. *J*



Androl, 26:698-705.

Khaira H, McLean D, Ohl DA, Smith GD. 2005. Spermatogonial stem cell isolation, storage, and transplantation. *J Androl*, 26:442-450.

Lewis PR, Knight DP. 1988. Staining methods for sectioned material. In: Glaubert AM (ed.). *Practical Methods in electron microscopy, part I; cytological staining in electron microscopy*. Amsterdam: Elsevier Biomedical Press. p.62-65.

McLean DJ. 2005. Spermatogonial stem cell transplantation and testicular function. *Cell Tissue Res*, 322:21-31.

Merchant-Larios H, Moreno-Mendoza N. 2001. Onset of sex differentiation: dialog between genes and cells. *Arch Med Res*, 32:553-558.

Neaves WB. 1973. Permeability of sertoli cell tight junctions to lanthanum after ligation of ducts deferens and ductuli efferents. *J Cell Biol*, 59:559-572.

Neves ES, Chiarini-Garcia H, França LR. 2002. Comparative testis morphometry and seminiferous epithelium cycle length in donkeys and mules. *Biol Reprod*, 67:247-255.

Ross MH. 1977. Sertoli-Sertoli junctions and Sertoli-

spermatids junctions after efferent ductule ligation and lanthanum treatment. *Am J Anat*, 125:49-51.

Russell LD. 1993. Form, dimensions, and cytology of mammalian Sertoli cells. In: Russell LD, Griswold MD (Eds.). *The Sertoli cell*. Vienna, IL: Cache River Press. p.1-37.

Russell LD. 1996. Mammalian Leydig cell structure. In: Payne AH, Hardy MP, Russell LD (Eds.). *The Leydig cell*. Vienna, IL: Cache River Press. p.43-96.

Russell LD, Brinster RL. 1996. Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules. *J Androl*, 17:615-27

Short RV. 1997. The testis: the witness of the mating system, the site of mutation and the engine of desire. *Acta Pediatr Suppl*, 422:3-7.

Vogl AM, Pfeifer DC, Redenbach DM, Grove BD. 1993. Sertoli cell cytoskeleton. In: Russell LD, Griswold MD (Eds.). *The Sertoli cell*. Vienna, IL: Cache River Press. p.39-86.

Yao HH, Tilmann C, Zhao GQ, Capel B. 2002. The battle of the sexes: opposing pathways in sex determination. *Novartis Found Symp*, 244:187-198.