Anim. Reprod., v.11, n.4, p.517-525, Oct./Dec. 2014

Sertoli cell and spermatogenic efficiencies in Pêga Donkey (*Equus asinus*)

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Abstract

The donkey (*Equus asinus*) is a domesticated species from the *Equidae* family that is economically important and very well adapted to the arid regions of South America, Africa and Asia. However, except for a few studies in the literature related to testis structure and function in donkeys, to our knowledge there is no report regarding spermatogenic and Sertoli cell efficiencies in this equid species. Therefore, in the present study our main aims were to study these important parameters in Pêga donkeys. For this purpose, five sexually mature donkeys that had their testis perfused-fixed with buffered glutaraldehyde and routinely prepared for histological and stereological evaluations were investigated during the equine breeding period, which is from September to February in the South Hemisphere. The results found showed that, similar to most mammalian species already investigated, from the theoretical expected number, expressive germ cell loss occurred during the spermatogonial (~75%) and meiotic (~25%) phases of spermatogenesis, in such a way that, from each 10 spermatozoa expected to be formed from initial type A differentiated spermatogonia, only around 2 would be formed. However, mainly due to the higher Sertoli cell efficiency found for Pêga donkeys (~15 spermatids per Sertoli cell), spermatogenic efficiency (daily sperm production per testis gram) observed for this species (~42 million) is the highest observed for the domestic mammals already investigated, being ~110% higher than the value described in the literature for stallions.

Keywords: daily sperm production, morphometry, Sertoli cells, spermatogenesis, testis.

Introduction

The donkey (*Equus asinus*) is a domesticated species from the *Equidae* family. Because it easily adapts to the arid regions (Gastal *et al*., 1996), this species is economically very important in South America, Africa and Asia. Particularly in Brazil, the Pêga donkey is a valuable breed and its hybrids (mules) are frequently used to herd cattle, as a means of transportation, for equid sports and for pleasure (Gastal *et al*., 1996; Canisso *et al*., 2010). In the last three decades, several studies involving the reproductive physiology in donkeys have been performed, including those related to oestrus cycle (Henry *et al*., 1987), sexual behavior under different management systems (Henry *et al*., 1991, 1998; Lodi *et al*., 1995; Gastal *et al*., 1996; McDonnell, 1998, 2000), andrologic evaluations (Costa, 1991; Canisso *et al*., 2008) and seminal characteristics (Morais *et al*., 1994; Dowsett and Knott, 1996; Gastal *et al*., 1997; Canisso, 2008).

Aiming mainly to perform studies related to germ cells transplantation from fertile equid species (donors) to mules (natural infertile recipient), our research group has been doing comparative and accurate testicular morphometric analysis in donkeys and mules (Neves *et al*., 2002); including ultrastructural evaluation of Sertoli and Leydig cells (Neves *et al*., 2005), estimation of the spermatogenic cycle length (Neves *et al*., 2002) and, more recently, the characterization of spermatogonial stem cells (SSCs) phenotype (Costa *et al*., 2012). The ultrastructural results obtained for mules and donkeys indicated that both Leydig and Sertoli cells (including the Sertoli cell barrier) were functionally normal in mules, 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 other equid and even other large animals (Neves *et al*., 2005).

Daily sperm production (DSP) per gram of testicular parenchyma, which is a measure of spermatogenic efficiency in sexually mature animals, is very useful for species comparisons (Hess and França, 2007). In mammalian species investigated up to date, four to eighty million spermatozoa are produced daily per gram of testis tissue and usually species that have shorter spermatogenic cycle lengths have higher spermatogenic efficiency (Hess and França, 2007; Costa *et al*., 2010). However, the higher efficiency of spermatogenesis observed in most mammalian species results mainly from the combination of higher Sertoli cell support capacity for germ cells (Sertoli cell efficiency) and greater number of Sertoli cells per gram of testis (Hess and França, 2007).

Although there are already important information in the literature about the testis structure and function in donkeys (Neves *et al*., 2002, 2005; Costa *et al*., 2012), to our knowledge there is no report

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Received: May 12, 2014
Accepted: July 2, 2014

about spermatogenic and Sertoli cell efficiencies in this equid species. In this regard, our main objectives in the present study were to estimate these important parameters in sexually mature Pêga donkeys.

Material and Methods

Animals

Five sexually mature donkeys (*Equus asinus*; 4-11 years of age) from the Pêga breed were utilized in the present study. They were donated by the Brazilian Pêga Donkey Association and were being used as breeders. Although reproduction in this species is not seasonal in Brazil (Kreuchauf, 1984; Gastal *et al*., 1996), the animals were orchiectomized during the equine breeding period, which is from September to February in the southern hemisphere. Before surgery, all animals received i.v. injections of 0.8 mL of Sedivet (Boehringer De Angell, U.K.) per 100 kg of body weight. All surgical procedures were performed by a veterinarian and followed approved guidelines for the ethical treatment of animals. The protocol was approved by the Committee on Animal Experimentation-CETEA, Federal University of Minas Gerais (Permit Number: 056/11).

Tissue preparation

After the testes and epididymides were removed, the blood was cleared by introducing 0.9% saline with heparin (125 IU/L) via a needle through the testicular artery. Subsequently, they were perfused-fixed by gravity-fed perfusion with 4% buffered glutaraldehyde for 25-30 min. After perfusion, testes were trimmed out from the epididymides and weighed and cut longitudinally by hand with a sharp knife. Testis fragments were routinely processed and embedded in plastic (glycol methacrylate). Sections of 4 μm thickness were obtained and subsequently placed on glass slides and stained with toluidine blue for light microscopic investigations (Fig. 1).

Germ cell nuclear volume and number

Aiming to evaluate germ cells nuclear volume and number through the eight stages of the seminiferous epithelium cycle in donkeys, characterized according to the tubular morphology system (Neves *et al*., 2002), for each animal, they had their diameter measured and the number counted in ten round or nearly round seminiferous tubule cross-sections from each of the eight different stages of the seminiferous epithelium cycle. The following germ cell types were analyzed: type A undifferentiated spermatogonia (A_{und}); differentiated type A spermatogonia (A_1, A_2, and A_3); type B spermatogonia (B_1 and B_2); preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P), and diplotene (D) spermatocytes; and round (R) spermatids.

Germ cell nuclear volume was expressed in μm^3 and was obtained by the formula $(4/3)\pi r^3$, where r = nuclear diameter/2. The germ cells counts were corrected for section thickness and nucleus or nucleolus diameter according to Abercrombie (1946), as modified by Amann (1962). For this purpose, ten nuclear diameters per animal were measured for each cell type analyzed and cell ratios were obtained from the corrected cell counts done.

Sertoli cell number per seminiferous tubules cross-sections and Sertoli cell efficiency

For each animal, Sertoli cells nucleoli were counted in 10 round or nearly round seminiferous tubule cross-sections from the eight different stages of the seminiferous epithelium cycle. These counts were also corrected for section thickness and nucleolus diameter (Abercrombie, 1946; Amann, 1962). Twenty Sertoli cell nucleoli diameter were measured per stage and for each animal.

Daily sperm production

The DSP per testis and per gram of testis parenchyma (spermatogenic efficiency) was obtained according to the following formula: DSP = total number of Sertoli cells per testis x the ratio of round spermatids to Sertoli cells in stage 1 x stage 1 relative frequency (%)/stage 1 duration (days; França, 1992). This calculation was based on the assumption that there are no significant germ cell losses during spermiogenesis, particularly after spermatid nuclear elongation (Russell and Clermont, 1977). Therefore, the number of round spermatids counted at stage 1 of the seminiferous epithelium cycle in this species (Fig. 1C), just before they were elongating, was considered the number of potential spermatozoa released in the tubular lumen. The data related to the testis weight, total number of Sertoli cells per testis, the stage 1 relative frequency and stage 1 duration (days) were those already published from studies developed in our laboratory by Neves and colleagues (Neves *et al*., 2002).

Statistical analysis

All data are presented as the mean ± SEM. Analysis of variance (Newman-Keuls test) was done using the program Statistica 3.11 for Windows (StatSoft, Inc., Tulsa, OK). The significance level considered was $P < 0.05$.

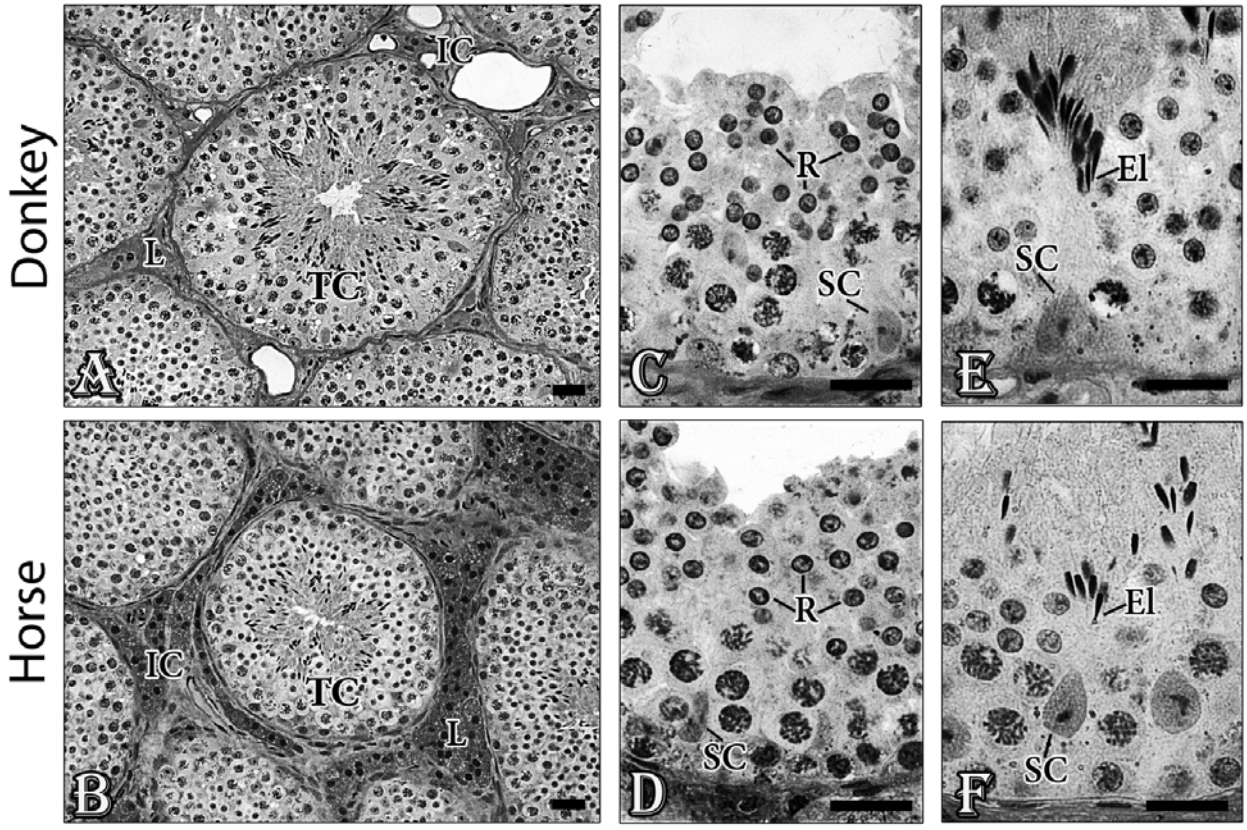

Figure 1. Donkey (A, C, E) and horse (B, D, F) testis parenchyma. Observe, at low magnification, the testis cytoarchitecture in donkey (A) and horse (B), illustrating the presence of tubular (TC) and intertubular (IC) compartment where Leydig cells (L) are located. The daily sperm production (DSP) was estimated counting the number of round spermatids (R) per Sertoli cell (SC) at the stage 1 of the seminiferous epithelium cycle (C, D). The number of elongated spermatids (El) per Sertoli cell, which is higher in donkeys (E) when compared to horses (F, Johnson and Thompson Jr, 1983; Johnson *et al*., 2000), is also illustrated in this figure. Scale bars = 25 µm.

Results

Germ cell nuclear volume and number

Germ cell nuclear volumes from type A undifferentiated spermatogonia to round spermatids are shown in Fig. 2A. Among the different spermatogonial types (type A and type B), the biggest volume (~700 μm^3) is observed in type A1 spermatogonia that gradually decreases its volume until type B2 (~310 μm^3) and preleptotene spermatocytes (~310 μm^3). In the meiotic phase, a substantial increase (~110%) occurs from preleptotene to diplotene spermatocytes. After the two successive meiotic divisions, a drastic nuclear volume reduction was noticed and round spermatids presented a nuclear volume approximately 6 fold smaller (~110 μm^3) than diplotene (Fig. 2A).The nuclear volume was very similar from early to late round spermatids (data not shown).

Due to four mitotic divisions, during the spermatogonial phase the number of spermatogonia per seminiferous tubules cross-sections increased approximately 4-fold (~3 to ~12) from type A_1 to B_2 (Fig. 2B). Considering that an 8 fold increase would be expected, very high germ cell loss occurred during this initial phase of spermatogenesis. Differently, in the meiotic prophase I (from preleptotene to diplotene), the germ cell number was rather constant (Fig. 2B). However, from the four theoretically expected spermatids to be formed per primary spermatocytes only approximately three were found, which means that about 25% of germ cells loss took place during the meiotic divisions (Table 1).

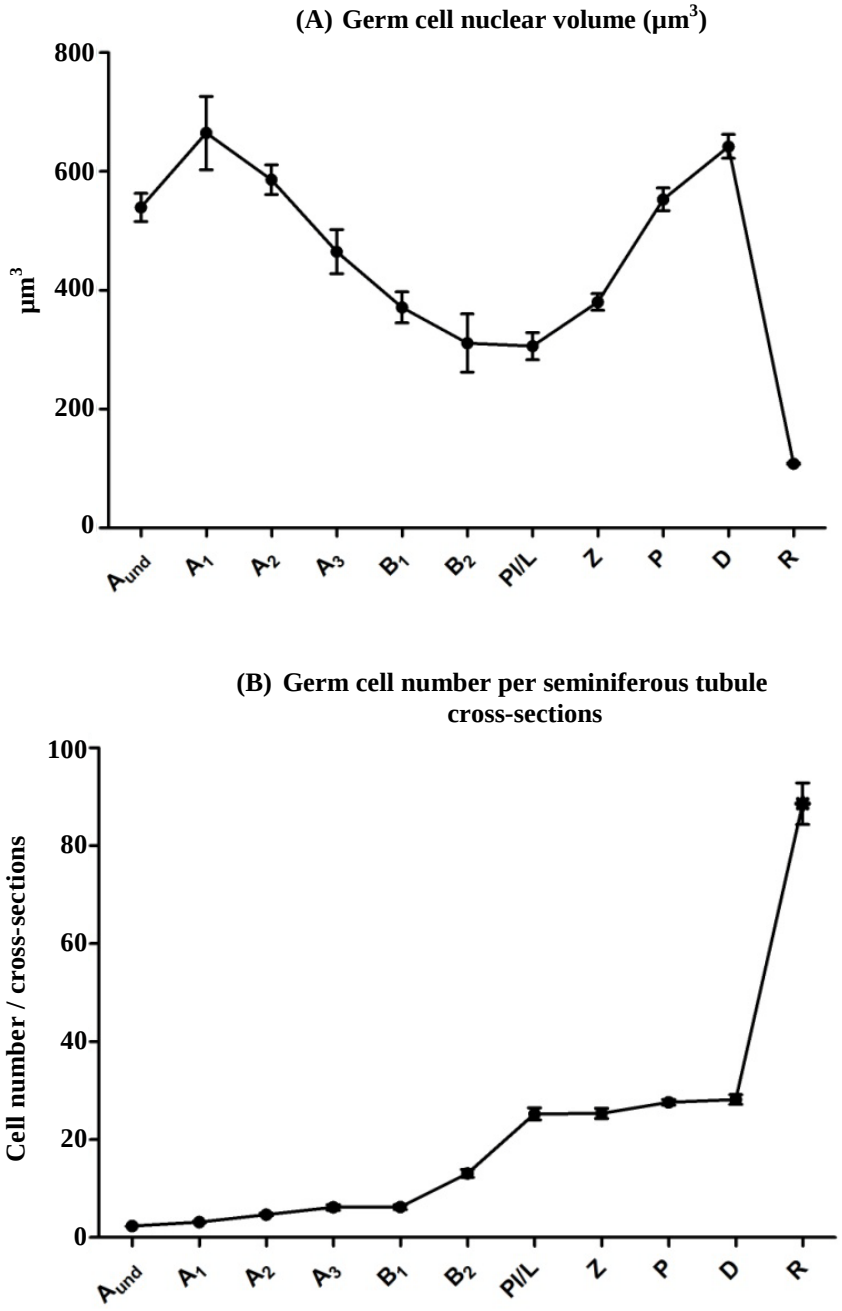

Figure 2. Nuclear volume (A) and number (B) of different germ cell types in donkeys. Aund, type A undifferentiated spermatogonia; A1, A2, A3, B1 and B2, type A and type B spermatogonia; Pl/L, preleptotene to leptotene primary spermatocyte, Z, zygotene spermatocyte; P, pachytene spermatocyte; D, diplotene spermatocyte; R, round spermatids.

Sertoli cell number and cell ratios

As observed in Fig. 3, the number of Sertoli cell nucleoli per seminiferous tubules cross-sections along the eight different stages of the seminiferous epithelium cycle was very similar, and this quite stable value confirms that the Sertoli cell is a reliable parameter to quantify and normalize germ cells counts (Fig. 3). The Sertoli cell efficiency in donkeys, estimated from the total number of spermatids per Sertoli cell at stage 1 of the seminiferous epithelium cycle, was 15.1 ± 2 (Table 1). From the cell counts at stage 1, Table 1 also shows that approximately 8 preleptotene spermatocytes were formed from type A spermatogonia (coefficient of efficiency of spermatogonial mitosis), whereas 26 spermatids resulted from each type A spermatogonia (overall rate of spermatogenesis).

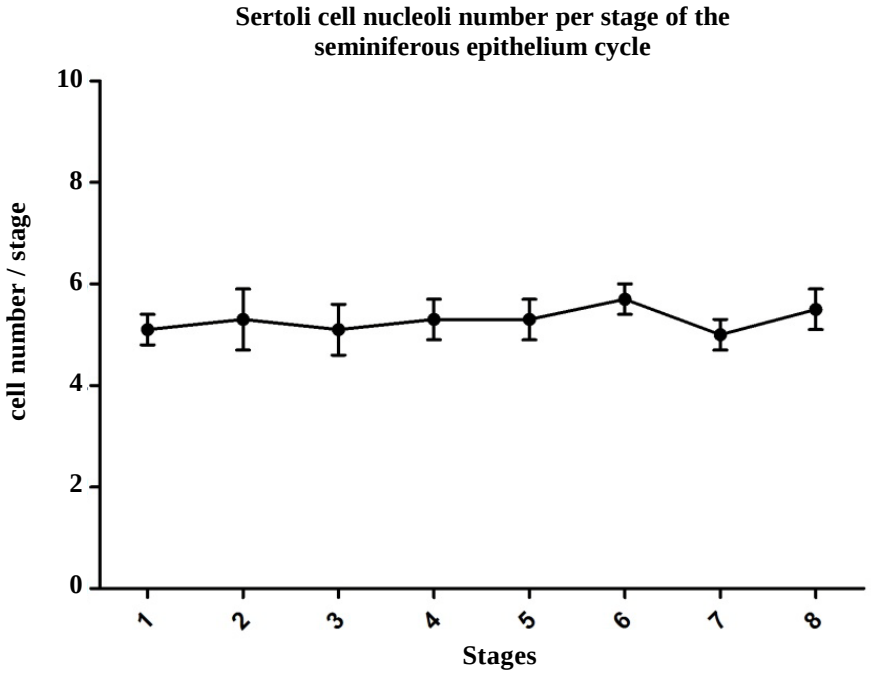

Figure 3. Number of Sertoli cell nucleoli across the eight different stages of the seminiferous epithelium cycle characterized according to the tubular morphology system (Mean ± SEM). Observe that the number of Sertoli cells along the eight stages is very similar (P > 0.05).

Table 1. Cell ratios and daily sperm production in Pêga donkeys (Mean ± SEM).

Parameters	
Preleptotene spermatocyte per type A spermatogonia[1]	8.2 ± 0.9
Round spermatids per pachytene spermatocyte (meiotic index)	3.0 ± 0.2
Overall rate of spermatogenesis[2]	26 ± 3.2
Round spermatids per Sertoli cell nucleoli (Sertoli cell efficiency)	15.1 ± 1.4
Daily sperm production per gram of testis ($x10^6$)	41.7 ± 5.0
Daily sperm production per testis ($x10^9$)	7.2 ± 0.9

[1]Coefficient of efficiency of spermatogonial mitosis.[2]Ratio of round spermatids per type A spermatogonia at stage 1 of the seminiferous epithelium cycle.

Daily sperm production

Table 1 also displays the data related to the number of sperm produced. As observed in this table, the daily sperm production per testis and per gram of testis in donkeys was approximately 7 billion and 42 million, respectively.

Discussion

To our knowledge, this is the first report describing the Sertoli cell and spermatogenic efficiencies in donkeys. Based on the number of spermatids found per Sertoli cell in this species, this cell can be considered one of the most efficient among mammals so far studied (Hess and França, 2007) . The association of this data obtained in the present work with the values previously found in donkeys for seminiferous tubules volume density in the testis parenchyma and for the cycle length (Neves *et al*., 2002), resulted in a very high spermatogenic efficiency for this species, this efficiency being the highest observed for domestic mammals already investigated (Hess and França, 2007).

Except for horses, in which the rate of germ cell apoptosis, Sertoli cell number and efficiency and daily sperm production are already available (Gebauer *et al*., 1974; Johnson and Neaves, 1981; Johnson and Thompson Jr, 1983; Johnson and Nguyen, 1986; Jones and Berndtson, 1986; Johnson and Tatum, 1989; Rodriguez-Martinez, 1992; Johnson *et al*., 1994, 2000; Blanchard and Johnson, 1997); there are few quantitative data in the literature related to testis structure and spermatogenesis for other members of the Equidae family (Trujillo *et al*, 1969; Hernández-Jáuregui and Márquez Monter, 1977; Nipken and

Wrobel, 1997; Neves *et al*., 2002, 2005). For instance, although Penzhorn and van der Merwe (1988) showed data related to the testis size and the onset of spermatogenesis in cape mountain zebra (*Equus zebra zebra*), there is no further description of the spermatogenic process in this species. In this context, in order to have a more comprehensive discussion, a table (see Table 2) compiling the data available in the literature for several important testis and spermatogenic parameters is shown for donkeys and horses, which are now the two better investigated domestic Equidae species in this regard.

The balance between proliferation and apoptosis plays a very important role in regulating the spermatogenic cells population in the seminiferous epithelium. Particularly, during the spermatogonial phase, the homeostatic mechanism that regulates apoptosis is considered density-dependent (a mechanism to limit germ cells to the number that can be supported by available Sertoli cells), limiting the amount of germ cells that enter in the meiotic phase (Huckins, 1978; De Rooij and Lok, 1987; Sharpe, 1994; De Rooij, 1998). In Pêga donkeys, analyses of the different types of spermatogonia numbers showed that, based on the theoretical final number expected for these cells, significant germ cells loss occurred during the spermatogonial phase. In this regard, only about eight primary spermatocytes in preleptotene were formed for each type A spermatogonia counted at stage 1. This value is very close to the one observed for adult horses (Jones and Berndtson, 1986, Costa *et al*., 2012). Considering that these donkeys and horses have five generations of differentiated spermatogonia (Amann, 1981; Chiarini-Garcia *et al*., 2009; Costa *et al*., 2012), the theoretical number expected of primary spermatocytes per each type A spermatogonia would be 32 cells. Therefore, confirming the assumption stated above, approximately 75% of germ cell loss was observed during spermatogonial phase in both donkeys and horses.

In order to eliminate cells with abnormal or aberrant chromosomes (Roosen-Runge, 1973; Sharpe, 1994), in a process now named checkpoints (Blanco-Rodríguez, 2001), at least one of four spermatids originated from a primary spermatocyte is expected to undergo apoptosis (França and Russell, 1998). Therefore, for most mammalian species already investigated, approximately 20-25% of germ cells loss occurs during the meiotic divisions (Roosen-Runge, 1973; França and Russell, 1998; Hess and França, 2007). In this regard, the values found in the present study for the meiotic index in Pêga donkeys are similar to other mammals, including horses (Berndtson *et al*., 1983; Jones and Berndtson, 1986; Johnson *et al*., 1994). In the Pêga donkey, the overall yield of spermatogenesis was approximately 26 round spermatids for each type A spermatogonia. This indicates that only approximately 20% of the theoretical number of spermatids (128) was produced; therefore, ~80% of germ cells loss occurs during the entire spermatogenic process in donkeys, which is very similar to horses (Jones and Berndtson, 1986). Once again and suggesting that spermatogenesis may be a very tightly regulated homeostatic process during phylogenesis, the data obtained for equids confirm those found in the literature for most mammals investigated so far (Huckins, 1978; França and Russell, 1998), where from each 10 spermatozoa expected to be formed from initial type A differentiated spermatogonia only 2-3 spermatozoa are produced.

Table 2. Comparison of several testis parameters in sexually mature donkeys and horses.

Parameters	Donkey[a]	Horse[b]
Testis weight (g)	166-219	117-213
Gonadosomatic index (%)[c]	0.15	0.12
Seminiferous tubule percentage	85	72
Coefficient of efficiency of spermatogonial mitosis[d]	1 : 8.2 (75)	1 : 8.3 (75)
Meiotic index[e]	3.1(25)	3 (25)
Overall rate of spermatogenesis[f]	1 : 26 (80)	1 : 26 (80)
Sertoli cell efficiency (spermatids per Sertoli cell)	15.1	8.7
Sertoli cells per gram of testis (million)	30	28
Spermatogenic cycle length (days)	10.5	12.2
Total duration of spermatogenesis (days)	47.2	54.9
Daily sperm production per gram of testis (million)	42	20

[a]Data obtained from Neves, 2001 and Neves *et al*., 2002.[b]Data obtained from several publications: Swierstra *et al*., 1974; Berndtson *et al*. 1983; Johnson and Thompson Jr, 1983; Jones and Berndtson, 1986; Setchell, 1991; Johnson *et al*., 1994, 2000.[c]Calculation of the gonad mass as a proportion of the total body mass. [d]Ratio of early primary spermatocytes per type A spermatogonia at stage 1of the SEC (percentage of presumptive germ cell loss during the spermatogonial phase in parenthesis).[e]Ratio of spermatids per primary spermatocytes (percentage of germ cell loss during meiosis in parenthesis).[f]Ratio of round spermatids per type A spermatogonia at stage 1 of the SEC (percentage of presumptive germ cell loss during spermatogenesis in parenthesis).

The number of spermatids per Sertoli cell is considered an accurate index to evaluate the Sertoli cell efficiency and function, being crucial in determining the magnitude of sperm production (Russell and Peterson, 1984; Sharpe, 1994; França and Russell, 1998; Hess and França, 2007)**.** In Pêga donkeys, the number of spermatids per Sertoli cell (~15) is one of the highest obtained for mammals (Russell and Peterson, 1984; Sharpe, 1994; França and Russell, 1998; Hess and França, 2007). In comparison to horses (Fig. 1), the number of spermatids per Sertoli cell in donkeys is 60% higher (Johnson and Thompson Jr, 1983, Johnson *et al*., 2000). Besides that, the germ cell pace (as expressed by the seminiferous epithelium cycle duration) in Pêga donkeys (Neves *et al*., 2002) is approximately 15% faster than the value observed for horses (Swierstra *et al*., 1974). According to the literature, the combination of short duration of spermatogenesis, high number of Sertoli cells per testis gram; high Sertoli cells support capacity, high seminiferous tubules volume density (%), and low germ cell loss during spermatogenesis results in a more efficient sperm production (Johnson, 1991; França and Russell, 1998; Hess and França, 2007). Therefore, taking into consideration all these aforementioned parameters (see Table 2), the DSP per gram of testicular parenchyma found in Pêga donkeys (42 million) was very high and about 100% larger than that observed for stallions (Johnson *et al*., 2000).

In summary, as shown in Table 2, several testis and spermatogenic parameters are very similar when donkeys investigated in the present study are compared to horses. However, confirming the literature, the noticeably higher spermatogenic efficiency found for donkeys resulted mainly from the higher number of spermatids per Sertoli cell (Sertoli cell efficiency) and, to a lesser degree, from the shorter duration of spermatogenesis and higher percentage of seminiferous tubules observed for this species.

Acknowledgments

The scholarships awarded to Elizabeth Neves de Melo from the Brazilian Foundation (CAPES) and to Guilherme Mattos Jardim Costa from CNPq are appreciated. Financial support from the Minas Gerais State Foundation (FAPEMIG), the Brazilian Research Council (CNPq) and the Pró-Reitoria de Pesquisa (PRPq-UFMG) is gratefully acknowledged. Technical help from Mara Livia dos Santos and Adriano Moreira is highly appreciated. Financial support: FAPEMIG and CNPq.

References

Abercrombie M. 1946. Estimation of nuclear populations from microtome sections. *Anat Rec*, 94:238-248.

Amann RP. 1962. Reproductive capacity of dairy bulls. III. The effect of ejaculation frequency, unilateral vasectomy, and age on spermatogenesis. *Am J Anat*, 110:49-67.

Amann RP. 1981. A review of anatomy and physiology of the stallion. *J Equine Vet Sci*, 1:83-105.

Berndtson WE, Squires EL, Thompson DL Jr. 1983. Spermatogenesis, testicular composition and the concentration of testosterone in the equine testis as influenced by season. *Theriogenology*, 20:449-457.

Blanchard TL, Johnson L. 1997. Determination of daily sperm production in stallion testes by enumeration of germ cells in homogenates. *Theriogenology*, 47:655-664.

Blanco-Rodriguez J. 2001. Mitotic/meiotic checkpoints and germ cell apoptosis. *In*: Robaire B, Chemes H, Morales CR (Ed.). *Andrology in the 21st Century*. Montreal, Canada: Medimond. pp. 173-184.

Canisso IF. 2008. *Sexual behavior, seminal parameters, and fertility of the semen freeze from donkeys (*Equus asinus*) Pêga breed* [in Portuguese]. Vicosa, MG: Federal University of Viçosa. Thesis.

Canisso IF, Souza FA, Carvalho GR, Guimarães JR, Silva EC, Lima AL. 2008. Some fundamental aspects of clinic andrologycal evaluation from donkeys (*Equus asinus*). *Rev Bras Reprod Anim*, 32:210-219.

Canisso IF, Carvalho GR, Morel MC, Guimarães JD, McDonnell SM. 2010. Sexual behavior and ejaculate characteristics in Pêga donkeys (*Equus asinus*) mounting estrous horse mares (*Equus caballus*). *Theriogenology*, 73:56-63.

Chiarini-Garcia H, Alves-Freitas D, Barbosa IS, Almeida FR. 2009. Evaluation of the seminiferous epithelial cycle, spermatogonial kinetics and niche in donkeys (*Equus asinus*). *Anim Reprod Sci*, 116:139-154.

Costa AJSA. 1991. *Andrologic evaluation of jacks Pêga breed* [in Portuguese]. Belo Horizonte, MG: Federal University of Minas Gerais. Thesis.

Costa GM, Leal MC, Ferreira CS, Guimarães DA, França LR. 2010. Duration of spermatogenesis and spermatogenic efficiency in 2 large neotropical rodent species: the agouti (*Dasyprocta leporina*) and paca (*Agouti paca*). *J Androl*, 31:489-499.

Costa GM, Avelar GF, Rezende-Neto JV, Campos-Junior PH, Lacerda SM, Andrade BS, Thomé RG, Hofmann MC, França LR. 2012. Spermatogonial stem cell markers and niche in equids. *PLoS One*, 7:e44091.

de Rooij DG, Lok D. 1987. Regulation of the density of spermatogonia in the seminiferous epithelium of the Chinese hamster: II. Differentiating spermatogonia. *Anat Rec*, 217:131-136.

de Rooij DG. 1998. Stem cells in the testis. *Int J Exp Pathol*, 79:67-80.

Dowsett KF, Knott LM. 1996. The influence of age and breed on stallion semen. *Theriogenology*, 46:397-412.

França LR. 1992. Daily sperm production in Piau boars

estimated from Sertoli cell population and Sertoli cell index. *In*: Proceedings of the 12th International Congress on Animal Reproduction and Artificial Insemination, 1992, The Hague, Netherlands. The Hague: ICAR. pp. 1716-1718.

França LR, Russell LD. 1998. The testis of domestic animals. *In*: Martínez F, Regadera J (Ed.). *Male Reproduction: A multidisciplinary overview*. Madrid, Spain: Churchill Communications Europe. pp. 197-219.

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.

Gastal MO, Henry M, Beker AR, Gastal EL. 1997. Effect of ejaculation frequency and season donkey jack semen. *Theriogenology*, 47:627-638.

Gebauer MR, Pickett BW, Voss JL, Swierstra EE. 1974. Reproductive physiology of the stallion: daily sperm output and testicular measurements. *J Am Vet Med Assoc*, 165:711-713.

Henry M, Figueiredo AEF, Palhares MS, Coryn M. 1987. Clinical and endocrine aspects of the oestrus cycle in donkeys (*Equus asinus*). *J Reprod Fertil*, 35:297-303.

Henry M, McDonnell SM, Lodi LD, Gastal EL. 1991. Pasture mating behavior of donkeys (*Equus asinus*) at natural and induced oestrus. *J Reprod Fertil*, 44:77-86.

Henry M, Lodi LD, Gastal MMFO. 1998. Sexual behaviour of domesticated donkeys (*Equus asinus*) breeding under controlled or free range management systems. *Appl Anim B*ehav, 60:263-276.

Hernández-Jáuregui P, Márquez Monter H. 1977. Fine structure of mule testes: light and electron microscopy study. *Am J Vet Res*, 38:443-447.

Hess RA, França LR. 2007. Spermatogenesis. cycle of the seminiferous epithelium. *In*: Cheng, CY (Ed.). *Molecular Mechanisms in Spermatogenesis*. Austim, TX: Landes Bioscience. pp.1-15.

Huckins C. 1978. The morphology and kinetics of spermatogonial degeneration in normal adult rats: an analysis using a simplified classification of the germinal epithelium. *Anat Rec*, 190:905-926.

Johnson L, Carter GK, Varner DD, Taylor TS, Blanchard TL, Rembert MS. 1994. The relationship of daily sperm production with number of Sertoli cells and testicular size in adult horses: role of primitive spermatogonia. *J Reprod Fertil*, 100:315-321.

Johnson, L, Neaves WB. 1981. Age-related changes in the Leydig cell populations, seminiferous tubules and sperm production in stallions. *Biol Reprod*, 24:703-712.

Johnson L, Thompson DL Jr. 1983. Age-related and seasonal variation in the Sertoli cell population, daily sperm production and serum concentrations of follicle-stimulating hormone, luteinizing hormone and testosterone in stallions. *Biol Reprod*, 29:777-789.

Johnson L, Nguyen HB. 1986. Annual cycle of the Sertoli cell population in adult stallions. *J Reprod Fertil*, 76:311-316.

Johnson L, Tatum ME. 1989. Temporal appearance of seasonal changes in number of Sertoli cells, Leydig cells, and germ cells in stallions. *Biol Reprod*, 40:994-999.

Johnson L. 1991. Spermatogenesis. *In*: Cupps PT (Ed.). *Reproduction in Domestic Animals*. New York, NY: Academic Press. pp.173-219.

Johnson L, Varner DD, Roberts ME, Smith TL, Keillor GE, Scrutchfield WL. 2000. Efficiency of spermatogenesis: a comparative approach. *Anim Reprod Sci*, 61:471-480

Jones L, Berndtson W. 1986. A quantitative study of Sertoli cell and germ cell populations as related to sexual development and ageing in the stallion. *Biol Reprod*, 35:138-148.

Kreuchauf A. 1984. Reproductive physiology in the jackass. *Anim Res Dev*, 20:51-78.

Lodi LD, Henry M, Paranhos da Costa MJR. 1995. Behavior of donkey jacks (*Equus asinus*) breeding horse mares (*Equus caballus*) at pasture. *Biol Reprod Monogr*, 1:591-598.

McDonnell SM. 1998. Reproductive behavior of donkey (*Equus asinus*). *Appl Anim Behav Sci*, 60:277-282.

McDonnell SM. 2000. Reproductive behavior of stallions and mares: comparison of free-running and domestic in hand breeding. *Anim Reprod Sci*, 60/61:211-219.

Morais RN, Mucciolo RG, Viana WG. 1994. Biologia reprodutiva de jumentos. II. Características fisicas e morfologicas do sêmen. *Braz J Vet Res Anim Sci*, 31:49-57.

Neves ES. 2001. *Estudo comparativo da estrutura do testículo e do processo espermatogênico em jumentos (*Equus asinus*) e burros* (Equus mulus mulus) [in Portuguese]. Belo Horizonte, Brazil: Federal University of Minas Gerais. Thesis.

Neves ES, Chiarini-Garcia H, França LR. 2002. Comparative testis morphometry and seminiferous epithelium cycle length in donkeys and mules. *Biol Reprod*, 67:247-455

Neves ES, Chiarini-Garcia H, França LR. 2005. Ultrastructural observation of the mule testis indicates normal function of somatic cells. *Anim Reprod*, 2:263-271.

Nipken C, Wrobel KH. 1997. A quantitative morphological study of age-related changes in the donkey testis in the period between puberty and senium. *Andrologia*. 29:149-161.

Penzhorn BL, Van der Merwe NJ. 1988. Testis size and onset of spermatogenesis in Cape mountain zebras (*Equus zebra zebra*). *J Reprod Fertil*, 83:371-375.

Rodriguez-Martinez H. 1992. Sperm production in the stallion. *Acta Vet Scand Suppl*, 88:9-28.

Roosen-Runge EC. 1973. Germinal-cell loss in normal metazoan spermatogenesis. *J Reprod Fertil*, 35:339-348.

Russell LD, Clermont Y. 1977. Degeneration of germ

cells in normal, hypophysectomized and hormone treated hypophysectomized rats. *Anat Rec*, 187:347-366.

Russell LD, Peterson RN. 1984. Determination of the elongate spermatid-Sertoli cell ratio in various mammals. *J Reprod Fertil*, 70:635-641.

Setchell BP. 1991. Male reproductive organs and semen. *In*: Cupps, PT (Ed.) *Reproduction in Domestic Animals*. San Diego, CA: Academic Press. pp. 22l-249.

Sharpe RM. 1994. Regulation of spermatogenesis. *In*: Knobil E, Neill JD (Ed.). *The Physiology of Reproduction*. New York, NY: Raven Press. pp. 1363-1434.

Swierstra EE, Gebauer MR, Pickett BW. 1974. Reproductive physiology of the stallion. I. Spermatogenesis and testis composition. *J Reprod Fertil*, 40:113-123.

Trujillo JM, Ohno S, Jardine JH, Atkins NB. 1969. Spermatogenesis in a male hinny: histological and cytological studies. *J Hered*, 60:79-84.