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## The quantification of testicular cells during the postnatal development in two Caviomorph rodents: the guinea pig (*Cavia porcellus*) and the cutia (*Dasyprocta agouti*)

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### ABSTRACT

The germinative, Sertoli and Leydig cells of two caviomorph rodents (*Cavia porcellus* and *Dasyprocta agouti*) were counted as well as the estimation of the total volume of the testis and the total volume of seminiferous tubules and interstitium in prepubertal, pubertal and adult animals. The number of spermatogonia, spermatocytes and spermatids cells increased during the pubertal phase in both rodents, notably the spermatid cells. The spermatocyte and spermatid slightly decreased in the adult of both rodents, but the increment in spermatogonia cells number was seen, mainly in cutias. The number of Sertoli cells increased in pubertal rodents, but in the adult the number reduced. Substantial number of Leydig cells was counted in pubertal and adult guinea pigs. In cutias, the number of Leydig cells increased in pubertal phase and decline in adults. The design-based stereological method has proven to be unbiased and reliable to be applied in reproduction studies.

**Key words:** Germinative cells, hystricomorpha, reproduction, stereology.

### INTRODUCTION

The guinea pig (*Cavia porcellus*) and the cutia (*Dasyprocta agouti*) are rodents that belong to the Hystricomorpha suborder and Caviomorpha infraorder. The geographical habitat of the guinea pig ranges from Colombia and Venezuela to

northern Argentina and that of the cutia ranges from Colombia to southern Brazil (Mackenzie 1972).

The guinea pigs are medium-sized rodent extensively used to support biomedical researches due to their similarities to the human immune system response (Padilla-Carlin et al. 2008, Welkos et al. 2015). The cutia has great importance to animal production as an alternative source of protein; it has been used as an experimental animal

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in reproduction studies with important application in wild animal conservation (Arroyo et al. 2014, Castelo et al. 2015).

The knowledge of the reproduction function is vital to establish the appropriate animal management to guarantee reproduction successful (França and Russell 1998).

The study of the testicular parenchyma is a useful tool to evaluate the spermatogenic capacity in normal, pathologic and experimental conditions (Cardoso 2009). Several studies have quantified the testicular cells using different techniques, as proposed by Bellvé et al. (1977), but most of them use histomorphometry (Nunes et al. 2013, De Melo et al. 2013, Morais et al. 2014). Thus, this work aims to estimate the number of testicular cells in two Caviomorph rodents during the postnatal development, applying design-based stereological techniques as an accurate tool.

## MATERIALS AND METHODS

### ANIMALS

This study used testes from nine guinea pigs (30, 45 and 90 days old), (body weights ranging from 248g to 582g) (n=3) and from nine cutias (5-8 months, 9-11 months and 12 months old), (body weights ranging from 1634g to 2735g) (n=3). For the bilateral orchiectomy, the anesthetic protocol was: ketamine 75-100mg/kg intraperitoneal (IP) and xylazine 10mg/kg IP. The experimental procedures were approved by the Ethical Committee of the Animal Experimentation of the College of Veterinary Medicine – University of São Paulo (FMVZ-USP) (protocol number 8735070714).

### QUANTITATIVE STUDY

#### *Te total volume of the testes $V(\text{ref})$*

The total volume of the testis was estimated dividing the fresh organ weight, in grams, by the

mean density of the testicular tissue:  $1.04\text{g/cm}^3$  (Mori and Christensen 1980).

#### *Sampling of the testes*

The right or left testis of guinea pig and cutia was randomly chosen and they were fixed by immersion in paraformaldehyde 4% for 24h at  $4^\circ\text{C}$ . Following the fixation, the chosen testis was cut in slices (3-mm-thick slices) (resulting in a total of 6-8 slices). Every second slice was chosen. The chosen slices were cut in bars (resulting in a total of 12-16 bars). Every second bar was chosen. And finally, the chosen bars were cut in blocks of different size (resulting in a total of 6-8 blocks) and these blocks were organised in a crescent-size arrangement, according to the principle of the Smooth Fractionator (Gundersen 2002). Every third or fourth block was chosen. The slices, bars, and fragments were sampled by the systematic, uniform and randomly (SUR) principle (Gundersen et al. 1999).

#### *Total number of sertoli cells, spermatogonia, spermatocyte, spermatid and leydig cells*

The sampled blocks were embedded in Araldite 502 (Electron Microscopy Sciences) and sections of  $0.5\mu\text{m}$  were exhausted cut in ultramicrotome (LEICA ULTRACUT UCT®). The sections were collected on slides and stained with Toluidine Blue. Every second or third section was chosen and the cell counting was performed in 1/250 of the sampled tissue section. The principle of the physical fractionator (Gundersen 1986) was used to estimate the total number of Sertoli cells, Leydig cells, spermatogonia, spermatocyte and spermatid cells. This principle is based on randomly sampling a preview known fraction of a structure and then counting the particles in this fraction. This method consists in multiplying the number of particles (Q) by the inverse of the sampled fractions: sfs (slices), sfb (bars), sfb (blocks), sfsc (sections) and

sfa (area). Two parallel physical sections, with a known distance, were used. The first section is the reference section and the second one, the look-up section. The distance between the “reference” and “look-up” sections was 4µm. The counting frame, with the total area ( $1.8 \times 10^6 \mu\text{m}^2$ ) was used to unbiased counting the particles.

*Volume density of seminiferous tubules  $V_v(t)$  and volume density of interstitium  $V_v(i)$*

The fractional volume of seminiferous tubules and interstitium was determined by point counting. Sections were sampled following SUR principle. On each sampled section a point system (distance between points was 125µm) were used. The total of points hitting the testis reference space ( $\Sigma\text{Pref}$ ) were counted, as well as the points hitting the seminiferous tubules ( $\Sigma\text{Pt}$ ) and the interstitium ( $\Sigma\text{Pi}$ ). The  $V_v(t)$  was estimated as:  $V_v(t) = (\Sigma\text{Pt}) / (\Sigma\text{Pref})$  and  $V_v(i)$  was estimated as:  $V_v(i) = (\Sigma\text{Pi}) / (\Sigma\text{Pref})$ .

*Total volume of seminiferous tubules ( $V_t$ ) and total volume of interstitium ( $V_i$ )*

The total volume of the seminiferous tubules ( $V_t$ ) was calculated multiplying the  $V_v(t)$  by the total volume of the testes  $V(\text{ref})$  and the total volume of interstitium ( $V_i$ ) was calculated multiplying the  $V_v(i)$  by the total volume of the testes  $V(\text{ref})$ .

STATISTICAL ANALYSIS

The data were analyzed by one-way ANOVA. The quantitative results were shown as mean followed by the coefficient of variation (CV). The difference between groups was considered significant when the p-value was less than 0.05. Tukey test was applied when differences were considered significant. The Minitab Express statistical software was used for the analysis.

RESULTS

TOTAL VOLUME OF TESTIS, SEMINIFEROUS TUBULES AND INTERSTITIUM

The mean values of the total volume of the testis ( $V_{\text{ref}}$ ) as well the fractional volume occupied by seminiferous tubules ( $V_t$ ) and interstitium ( $V_i$ ) were statistically different comparing the guinea pig groups. The  $V_{\text{ref}}$  ranged from 0.16 to 0.55  $\text{cm}^3$  ( $p < 0.01$ ), the  $V_t$  ranged from 0.13 to 0.50  $\text{cm}^3$  ( $p < 0.05$ ) and  $V_i$  ranged from 0.03 to 0.06  $\text{cm}^3$  ( $p < 0.05$ ). The percentual values of the fractional volume occupied by  $V_t$  ranged from 81% to 89% and the fractional volume occupied by  $V_i$  ranged from 19% to 11%, comparing prepubertal to adult guinea pig. In cutias, no difference between groups was detected for  $V_i$ . The  $V_{\text{ref}}$  ranged from 1.65 to 3.64  $\text{cm}^3$  ( $p < 0.05$ ), the  $V_t$  ranged from 1.15 to 3.22  $\text{cm}^3$  ( $p < 0.01$ ) and  $V_i$  ranged from 0.50 to 0.41  $\text{cm}^3$ . The percentual values of the fractional volume occupied by  $V_t$  ranged from 70% to 89% and the percentual values of the fractional volume occupied by  $V_i$  ranged from 30% to 11%. The age groups mean values are shown in Tables I and II.

TOTAL NUMBER OF GERMINATIVE, SERTOLI AND LEYDIG CELLS

The guinea pig germinative, Sertoli and Leydig cell numbers were different between groups. The Sertoli cells ranged from 2.82 to  $3.21 \times 10^6$  ( $p < 0.01$ ), spermatogonia ranged from 0.67 to  $1.14 \times 10^6$  ( $p < 0.05$ ), spermatocyte ranged from 3.54 to  $4.41 \times 10^6$  ( $p < 0.01$ ), spermatid ranged from 0.95 to  $3.21 \times 10^6$  ( $p < 0.001$ ) and Leydig cells ranged from 0.15 to  $0.63 \times 10^6$  ( $p < 0.01$ ). The cutia germinative cells were different between groups, however, no difference was observed in Sertoli and Leydig cells. Sertoli cells ranged from 19.80 to  $20.06 \times 10^6$ , spermatogonia ranged from 4.00 to  $4.86 \times 10^6$  ( $p < 0.05$ ), spermatocyte ranged from 9.50 to  $12.12 \times 10^6$  ( $p < 0.01$ ), spermatid ranged from 3.40 to  $11.42 \times 10^6$  ( $p < 0.001$ ) and Leydig cells ranged from 5.02 to

TABLE I

The mean values of the total volume of the testis (Vref), seminiferous tubules (Vt) and interstitium (Vi) in guinea pig.

Volume (cm <sup>3</sup> )	PP	P	A
Volume of testis (Vref)**	0.16 (0.15)	0.27 (0.11)	0.55 (0.35)
Volume of tubules (Vt)*	0.13 (0.20)	0.23 (0.11)	0.50 (0.37)
Volume of interstitium (Vi)*	0.03 (0.18)	0.04 (0.08)	0.06 (0.23)

Group differences: \* p<0.05; \*\* p<0.01.

$5.76 \times 10^6$ . The age groups mean values are shown in Tables III and IV and the seminiferous tubule cells (Figure 1).

## DISCUSSION

Our results showed that the increment in total volume of testes is closely related to the increase in the size of seminiferous tubules in cutia and in guinea pig. When comparing prepubertal to adult testes, the fractional volume occupied by the seminiferous tubules increased from 81% to 89% in guinea pig and from 70% to 89% in cutia.

According to Short (1997), the only way to increase the sperm production is by increasing the volume of seminiferous tubular tissue and testicular size, because the rate of sperm production per gram of testicular tissue is almost constant within the species. Zhengwei et al. (1997) showed that the number of germinative cells is strongly related to testicular volume.

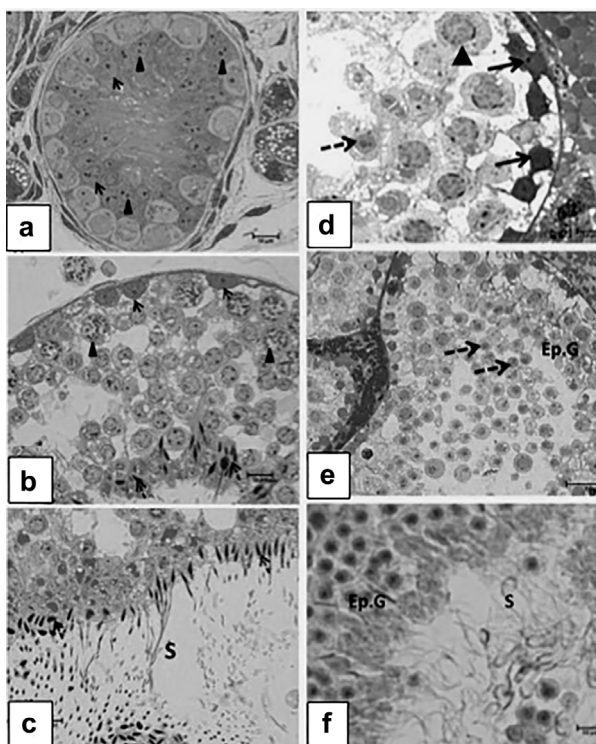
Considering the number of germinative cells in guinea pig and cutia, the most numerous type of cells was spermatocyte, followed by spermatid and spermatogonia. The number of spermatocytes increased 33% in pubertal guinea pigs and 29% in cutias. Bellvé et al. (1977) obtained cells from the seminiferous epithelium by sedimentation and they were able to identify spermatocytes in different phases 31%, 21% and 89% were respectively in leptotene, zygotene, and pachytene. Rodríguez

TABLE II

The mean values of the total volume of the testis (Vref), seminiferous tubules (Vt) and interstitium (Vi) in cutia.

Volume (cm <sup>3</sup> )	PP	P	A
Volume of testis (Vref)*	1.65 (0.50)	2.68 (0.14)	3.64 (0.05)
Volume of tubules (Vt)**	1.15 (0.46)	2.15 (0.15)	3.22 (0.07)
Volume of interstitium (Vi)	0.50 (0.60)	0.52 (0.13)	0.41 (0.10)

Group differences: \* p<0.05; \*\* p<0.01.



**Figure 1** - Pre-pubertal cutia, spermatocytes (arrowhead), Sertoli cells (filled arrows) (a); Pubertal cutia, spermatocytes (arrowhead), Sertoli cells (arrows), spermatids (dashed arrows) (b). Adult cutia, spermatid (dashed arrows), lumen of seminiferous tubule with spermatozoa (S) (c) Pre-pubertal guinea pig, sertoli cells (arrows), spermatocytes (arrowhead) and spermatids (dashed arrow) (d). Pubertal guinea pig, germinative epithelium (Ep.G) with spermatids (dashed arrows) (e). Adult guinea pig, germinative epithelium (Ep.G) with spermatozoa (S) (f). Toluidine blue.



TABLE III

The mean values of the total number of Sertoli cells, spermatogonia, spermatocyte, spermatid and Leydig cells in guinea pig.

Number of cells (10 <sup>6</sup> )	PP	P	A
Sertoli cells**	2.82 (0.06)	3.60 (0.05)	3.21 (0.09)
Spermatogonias*	0.67 (0.16)	0.87 (0.17)	1.14 (0.09)
Spermatocytes**	3.54 (0.04)	4.71 (0.06)	4.41 (0.04)
Spermatids***	0.95 (0.12)	3.37 (0.10)	3.21 (0.09)
Leydig cells**	0.15 (0.10)	0.38 (0.19)	0.63 (0.23)

Group differences: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

and Wettstein (2004) applying similar quantitative analysis reported 5.5% leptotene/zygotene spermatocyte of total cells in the guinea pig.

A peak of spermatid proliferation was observed in pubertal guinea pig and cutia. The number of these cells increased around 250% in both species and is coincident with the beginning of the production of spermatozoa. Slightly reduction of 5% in spermatid number was observed in adult animals from both species. This reduction might be related to the reduction of sperm production with age. In men, Jonhson et al. (1987) reported the reduction of early spermatid when evaluating the potential daily sperm production per gram of parenchyma.

In guinea pig, comparing prepubertal and pubertal animals, the number of spermatogonia increased approximately 30%, the same increase was observed when the pubertal animals were compared to the adult group. In cutias, however, the number of spermatogonia from prepubertal to pubertal ages increased only 2.5% and the increment of 18.5% was observed comparing the pubertal to adult animals. According to Okwun et

TABLE IV

The mean values of the total number of Sertoli cells, spermatogonia, spermatocyte, spermatid and Leydig cells in cutia.

Number of cells (10 <sup>6</sup> )	PP	P	A
Sertoli cells	19.80 (0.03)	20.90 (0.02)	20.06 (0.07)
Spermatogonias*	4.00 (0.07)	4.10 (0.09)	4.86 (0.06)
Spermatocytes**	9.50 (0.12)	12.25 (0.04)	12.12 (0.03)
Spermatids***	3.40 (0.12)	12.00 (0.10)	11.42 (0.09)
Leydig cells	5.02 (0.07)	5.98 (0.20)	5.76 (0.20)

Group differences: \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; No difference between groups was detected: Sertoli cells and Leydig cells.

al. (1996), the ration based on the most and the least advanced germ cell represents rates of cellular yield or degeneration, indicating the efficiency of spermatogenesis. Applying this concept to our results, the lowest ration between the spermatid/spermatogonia was observed in adult guinea pig and cutia showed poor cellular degeneration during spermatogenesis.

In our study, the Sertoli cells number increased 27% comparing prepubertal and pubertal testes, but decreased 12% comparing pubertal to adult one in the guinea pig. In cutia, the number of Sertoli cells remained almost constant comparing the groups and no statistical difference was observed. Progressive Sertoli cells deterioration or reduced in number may result in reduced sperm production (Johnson et al. 1987), unfortunately, we did not quantify directly the sperm production, but only the precursor cells.

The alterations in spermatogenesis, following the photoperiod, was investigated by Godoy Pieri et al. (2014). They observed that Sertoli cells of viscachas, captured in summer, had reduced capacity for supporting the developing round spermatids.

This seasonal rodent produced less sperm during the spring than the summer. Our study did not take into account the photoperiod to estimate the cell numbers and the testicular morphological structure but is suggestive that in the cutia and in the guinea pig, as *Hysticomorpha* rodents like viscacha, the spermatogenesis also follow it.

The Leydig cells increased approximately 150% in pubertal guinea pigs and around 19% in cutias and decreased 4% in cutia, but increased 65% in the guinea pig. Neaves et al. (1984) observed a reduction of 44% comparing the older men to the younger, however, Petersen and Pakkenberg (2000) observed no difference between young and old groups.

In conclusion, germinative cells increased during the pubertal phase in both rodents, as expected. Particular emphasis should be driven to the high proliferation of spermatid cells in pubertal phase and to the proliferation of spermatogonia in adult phase. The Sertoli cells number increased in pubertal guinea pigs and cutias, but the number reduced in adult animals. The Leydig cells doubled in pubertal guinea pigs and remained proliferating in adult phase. In cutias, the Leydig cells proliferating in puberty and slightly reduced in adults. The design-based stereological method has proven to be unbiased and reliable to be applied in reproduction studies.

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