

Histological and morphometric study of the epididymus of *Chinchilla lanigera* Grey under controlled conditions in captivity

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Date submitted: 12.03.2015. Date accepted: 06.05.2015

Summary

Chinchilla lanigera, native to the Andean Mountains of Perú, Chile, Bolivia and Argentina, is a specimen of great economic importance because of its fur. In mammals, spermatozoa originate in testes and are transported to the epididymis, where they undergo morphological and biochemical modifications known as sperm maturation, a basic step in the acquisition of their fertilizing ability. The aim of this work is the macroscopic and microscopic analysis of the epididymis of *Chinchilla lanigera* Grey and its sectorization based on a histomorphological study. The epididymis presents a clear segmentation into four regions: initial segment, caput, corpus and cauda. The epithelium lining the seminiferous tubules is pseudostratified, with principal cells with stereocilia and basal, clear, apical, narrow and halo cells. The histological analysis showed that principal and basal cells are the prevailing populations in all regions, also revealing narrow cells and the absence of clear cells in the initial segment. Each segment presents its different histological and morphometric characteristics, which supports the idea of the specific behaviour of each region, giving a segment-specific character to the process of sperm maturation in this species. No significant differences were found in the morphometric measurements or in the histological evaluation of the epididymis of samples collected in April and October. The fact that no differences were found between the samples collected during the two periods when the reproductive ability in nature is different suggests the importance of external factors in the control of the reproductive cycle of *Chinchilla lanigera*.

Keywords: *Chinchilla lanigera*, Epididymis, Captivity, Histology, Morphometric

Introduction

In mammals, spermatozoa, originating in the testes, are transported to the epididymis, where they undergo a series of morpho-biochemical modifications known as sperm maturation (Bedford, 1963, 1966; Bedford *et al.*, 1973). The functional maturation of spermatozoa

results from their exposure to the luminal environment of the epididymis, and is a fundamental step in the acquisition of their fertilizing ability (Bedford, 1965; Orgebin-Crist, 1968).

Numerous studies have shown that this organ is not only a place for sperm storage and transit (Yoshinaga & Toshimori, 2003) but also plays an active role in sperm remodelling (Axné, 2006). This action of the epididymis is performed in a region-dependent manner (Dacheux *et al.*, 2003) in which the different regions sequentially provide the components necessary for successful sperm–oocyte interaction.

Chinchilla lanigera is a rodent with seasonal reproductive cycles, the reproductive period being autumn–winter. Classified as an endangered species (Thornback & Jenkis, 1982; Glade, 1988), at present it exists almost exclusively in captivity for commercial purposes, with a low reproductive rate in these

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conditions. In later years the species acquired great economic importance with the commercialization of its fur and of breeding specimens. However, very little information is known about its reproductive biology, so the study of this animal is of great interest, particularly with respect to design of work techniques and protocols for the obtainment, handling and conservation of gametes.

With the progress of assisted reproduction techniques, knowledge of the processes involved in gamete formation and of the changes they experience during their preparation for fertilization and development becomes imperative as it will make possible successful *in vitro* fertilization. In this sense the study of the anatomy of the organs of the reproductive system is a necessary step for a better understanding of the biological mechanisms involved in the formation of a new individual.

The aim of our work is the microscopic analysis of the epididymis of *Chinchilla lanigera* Grey and its sectorization based on a histomorphological study under controlled temperature and photoperiod conditions.

Materials and methods

Animals

The animals used in this study were provided by a commercial hatchery. We selected 12 sexually mature male specimens from 11–12 months of age with a body mass of 600–650 g. All animals were housed under a 12 h dark–light cycle at a constant temperature of $22 \pm 1^\circ\text{C}$ and received water and food *ad libitum*. The samples were collected in April (autumn) or in October (spring) according to the time when specimens were slaughtered.

Tissue preparation

Epididymes were collected immediately after slaughter and fixed for 24 h in Bouin's solution and buffered formaldehyde at 4°C . After fixation, samples were washed in 70% alcohol and subjected to routine histological processing for embedding in Paraplast (Oxford Labware, St Louis, MO, USA). Sections ($4 \mu\text{m}$ thick) were stained with hematoxylin–eosin, toluidine blue pH 7 and Mallory's trichrome stain.

Morphometric analysis and photomicrography

The samples from each period of the reproductive cycle were sectioned in different regions for morphometric analysis. Ten preparations of each segment with two slices each per epididymis were analyzed.

Measurements were performed with an inverted Olympus CKX41 microscope at $\times 200$ and $\times 400$ magnifications. Photographs were analyzed with TSVIEW 5.0 CMOS software.

The relative distribution of the different cell types in all segments (initial, caput, corpus and cauda) of the epididymis was estimated by cell count in different sections of the duct. Only transverse epididymal sections were included in this study. Cells were counted in 10 sections per epididymis from each animal. The results of the cell count were corrected by applying the formula of Amann (1962) shown below, which provides a corrected value of the number of cells counted in histological sections:

$$N = \text{total number of cell types} \\ \times \frac{\text{section thickness}}{\text{section thickness} + \sqrt{(AD/2)^2 - (AD/4)^2}}$$

where AD is the average diameter of 10 nuclei from each cell type and N is the corrected number of cell types.

Statistical analysis

Means and standard errors were calculated for all data sets. Differences between groups were evaluated using one-way analysis of variance (ANOVA). A *P*-value of <0.05 was accepted as statistically significant.

Results

The epididymis is an organ formed by a single highly folded seminiferous tube that can be divided into four regions or segments: initial segment, caput, corpus and cauda. Based on the measurements of the morphological parameters, which are cited in Table 1, we determined five zones: zone I (initial segment), II (caput), III (corpus), IV and V (cauda), the last two corresponding to a proximal and distal localization respectively from the caudal region.

The epididymis is a highly vascularized tissue with medium caliber vessels and dense connective tissue in the intertubular space. The tubules are surrounded by layers of smooth muscle with varying thickness according to the region analyzed that become more prominent toward the caudal region.

As shown in Fig. 1, the epididymis is 4–5 cm long. The morphometric differences observed in the height of the epithelium, the length of stereocilia, the tubular and luminal diameter and the thickness of the muscle layer in all the histological regions studied are summarized in Table 1.

Table 1 Structural parameters in each epididymal region (mean values \pm SEM)

Parameter	Initial segment Zone I (μm)	Caput Zone II (μm)	Corpus Zone III (μm)	Cauda	
				Zone IV (μm)	Zone V (μm)
Epithelium height	43.68 \pm 4.28	41.18 \pm 2.49	39.48 \pm 3.76	22.32 \pm 2.05	18.62 \pm 2.18
Stereocilia length	21.32 \pm 2.05	19.14 \pm 0.92	11.32 \pm 1.69	1.11 \pm 0.27	1.07 \pm 0.27
Luminal diameter	122.50 \pm 7.69	137.73 \pm 11.69	153.21 \pm 8.62	634.51 \pm 21.43	716.55 \pm 18.76
Tubular diameter	207.92 \pm 9.22	216.29 \pm 17.30	199.95 \pm 6.54	652.61 \pm 8.31	738.79 \pm 15.32
Muscle wall thickness	11.51 \pm 1.19	12.24 \pm 0.78	15.35 \pm 1.73	21.06 \pm 0.64	23.16 \pm 0.39

SEM, standard error of the mean.

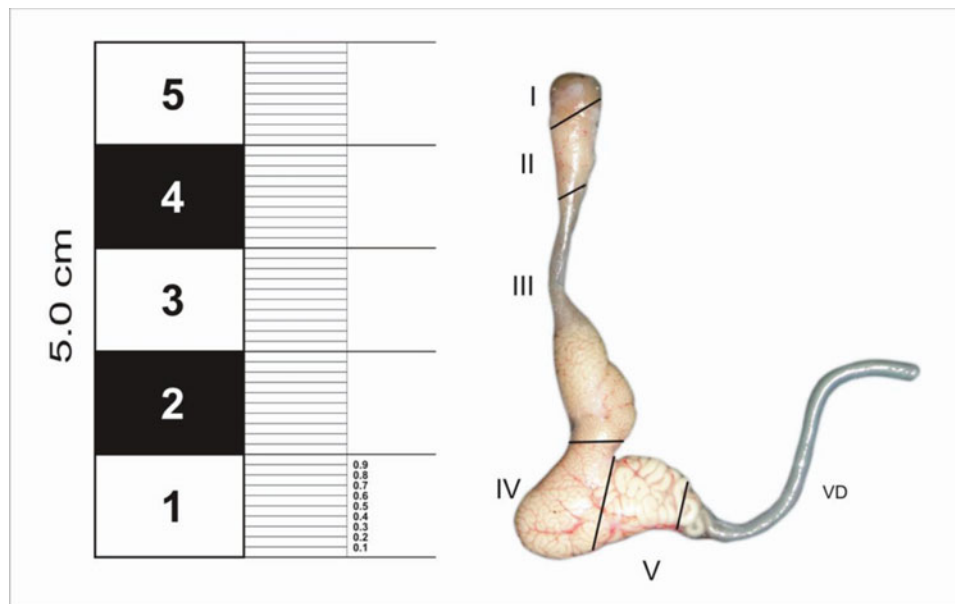


Figure 1 Macroscopic image of epididymis of *Chinchilla lanigera* in five differentiated zones: I (initial segment); II (caput); III (corpus); and IV, V (cauda). VD: vas deferens.

Histomorphometric evaluation

The epithelium lining the seminiferous tubules is pseudostratified, with principal cells (PCs) with stereocilia, basal (B), clear (C), apical (A), narrow (N) and halo (H) cells. [Figure 2](#) shows sections of all epididymal regions from samples collected in April (autumn) and October (spring). No significant differences among them were found in either the morphometric measurements or the histological evaluation (data not shown).

As shown in [Fig. 3](#), in all samples analyzed there were formations in the apical membrane of the cells corresponding to the apocrina secretion process in which epididymosomes are formed and later released into the tubular lumen. These membranous structures have been described in other mammalian species such as human ([Frenette et al., 2005](#)), mouse ([Rejraji et al., 2006](#)), ram ([Gatti et al., 2005](#)), chimpanzee ([Frohlich &](#)

[Young, 1996](#)), hamster ([Yanagimachi et al., 1985](#); [Legare et al., 1999](#)) and bull ([Frenette & Sullivan, 2001](#)).

Initial segment

In this segment there are tubules of smaller tubular and luminal size than in the rest of the epididymal duct ([Table 1](#)). A thin basal layer surrounded by myoid cells can be observed. The ducts are lined by a high columnar epithelium with different cell types: PC, B, N, A and H. The PCs have a higher percentage of relative distribution ([Fig. 4](#)), the spherical nuclei are located at the bottom of the epithelium. Vacuoles in their apical cytoplasm can also be seen. B cells, located on the basal layer, have an elongated nucleus parallel to it and scarce cytoplasm. N cells, found only in this segment, appear as thin intensely stained cells, extending from the basal layer to the tubular lumen,

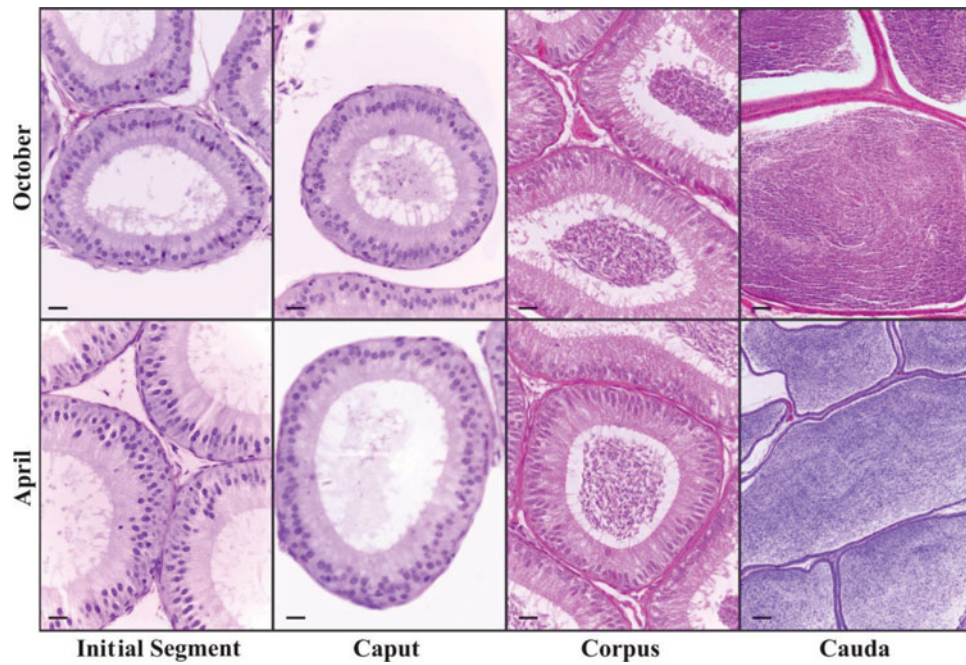


Figure 2 Microphotographs of the different regions of the epididymis of *Chinchilla lanigera* from samples collected in April and October. Hematoxylin–eosin stain. Initial segment, caput and corpus: scale bar, 22 μm ; cauda: scale bar, 82 μm .

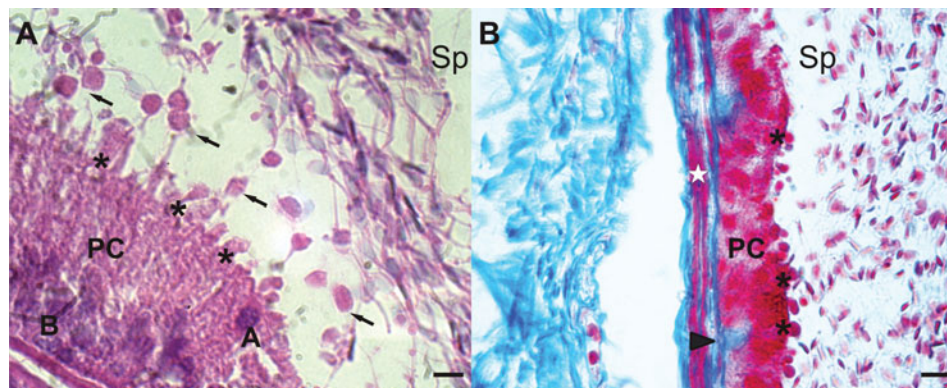


Figure 3 Microphotographs of regions of the epididymis showing apocrine secretory activity. (A) Caput: apical membrane of the cells with formation of epididymosomes (arrows) and vesicles released into the lumen (asterisks), H-E stain. (B) Cauda: the epithelium shows numerous forming vesicles (asterisks), Mallory's trichrome stain. A: apical cells; B: basal cells; PC: principal cells; Sp: spermatozoa; arrow: basal layer; white star: muscle layer. Scale bars, 8.2 μm .

with scarce development of the cytoplasm and with a nucleus in the middle part of the cell.

Caput

In the April and October period samples analyzed, transverse sections of the caput region showed a high columnar epithelium with the prevalence of PC (68.81%) and B (13.33%) over other cell types. The percentage of relative distribution is similar to the segment described above and there is also a thin basal layer. The stereocilia on the apical surface of the PC

in this region are still long compared with the rest of the epididymal duct. In contrast with the previous region, the PC has an elongated nucleus with a visible nucleolus but still retains its basal localization. The presence of numerous vacuoles in the apical cytoplasm is a characteristic that is still present in the epididymal head (Fig. 4).

B cell nuclei are perpendicularly oriented with respect to those of the PC. Muscle fibres surrounding the tubules are not prominent in this region. The tubular lumen increases its size and has a greater number of sperm.

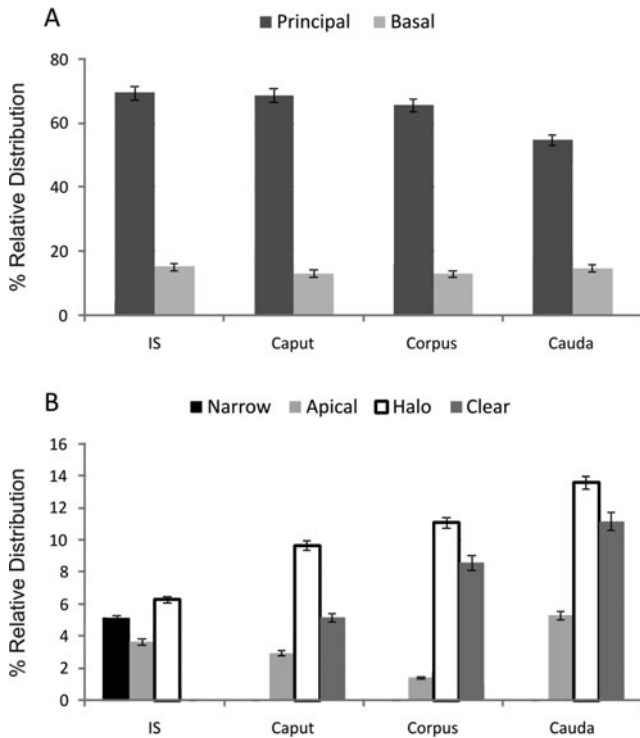


Figure 4 Relative distribution of the cell types present in each region of the epididymus. The values expressed represent the mean \pm SEM of 12 animals. IS, initial segment.

Corpus

In this segment there is a significant increase in the percentage of relative distribution of the halo cells, which are intraepithelial lymphocytes (11.1%), compared with the percentages found along the epididymus. As shown in Fig. 4B, there is an increase in clear cells together with a significant decrease in the percentage of relative distribution in apical cells (1.42%). The apical cell nucleus is located at half-height of the epithelium but, unlike N cells, this cell type is not in contact with the basal layer. The lining epithelium is shorter, the same as in the stereocilia. There is a concomitant increase in lumen diameter. The presence of sperm is more noticeable in this region and, from this segment onwards, contact of sperm with the lining of the epithelium becomes more frequent.

Cauda

The caudal region of the epididymus has a low pseudostratified cuboidal or cylindrical epithelium with very short stereocilia (Fig. 5). Principal and basal cells show no significant morphological changes and there is an increase in clear cells. Measurements at the height of the epithelium show a significant decrease compared with other zones of the epididymus. The basal layer shows no changes with respect to the

previous segments analyzed. In this portion of the epididymus we can see an increase in peritubular smooth musculature.

In this region there is a remarkable increase in the luminal and tubular diameters correlated with the presence of a dense mass of sperm. In addition, the data from the morphometric study show two well defined zones (IV and V), in which the zonal difference lies in the size variation of the tubular and luminal diameters and in the height of the epithelium (Table 1).

Discussion

The histology of the epididymus has been extensively studied in various mammalian species. The epididymus presents variations in its structure and function throughout the duct. However, the number of regions and their zoning depend on the species and on the classification criteria (Goyal, 1985).

On the basis of the results of the morphological study analyzed above and on the cell types identified and their relative distribution, the epididymus of *Chinchilla lanigera* shows a clear segmentation of the organ into four regions: initial, caput, corpus and cauda. Similar results were obtained by Aguilera-Merlo *et al.* (2005) for viscacha, by Serre & Robaire (1998) for Brown Norman rat, by Schön & Blottner (2009) for roe deer and for hamster by Vicentini & Orsi (1987).

In general terms, the histoarchitecture of the lining epithelium of the epididymus conserves the characteristics of the cell type described by other authors for other mammalian species. However, the presence of an initial segment has been described only for other rodents. In agreement with the findings in another hystricomorph rodent (Aguilera-Merlo *et al.*, 2005), our observations reveal the presence of narrow cells and the absence of clear cells only in the initial segment, in agreement with studies in adult rats (Adamali & Hermo, 1996).

These results suggest that the functions of this region would be protein degradation and the eventual internalization of other proteins for their later degradation (Hubbard, 1989; Murphy, 1991; Hermo *et al.*, 1996). Previous investigations confirm that narrow cells are in charge of acidifying the luminal environment, thus contributing to sperm quiescence (Cohen *et al.*, 1976; Au & Wong, 1980).

Principal and basal cells are the prevailing populations in all regions analyzed. The greatest length of stereocilia was found in the PCs of the head, thus contributing to luminal transit. They become shorter toward the caudal region, with the consequent increase

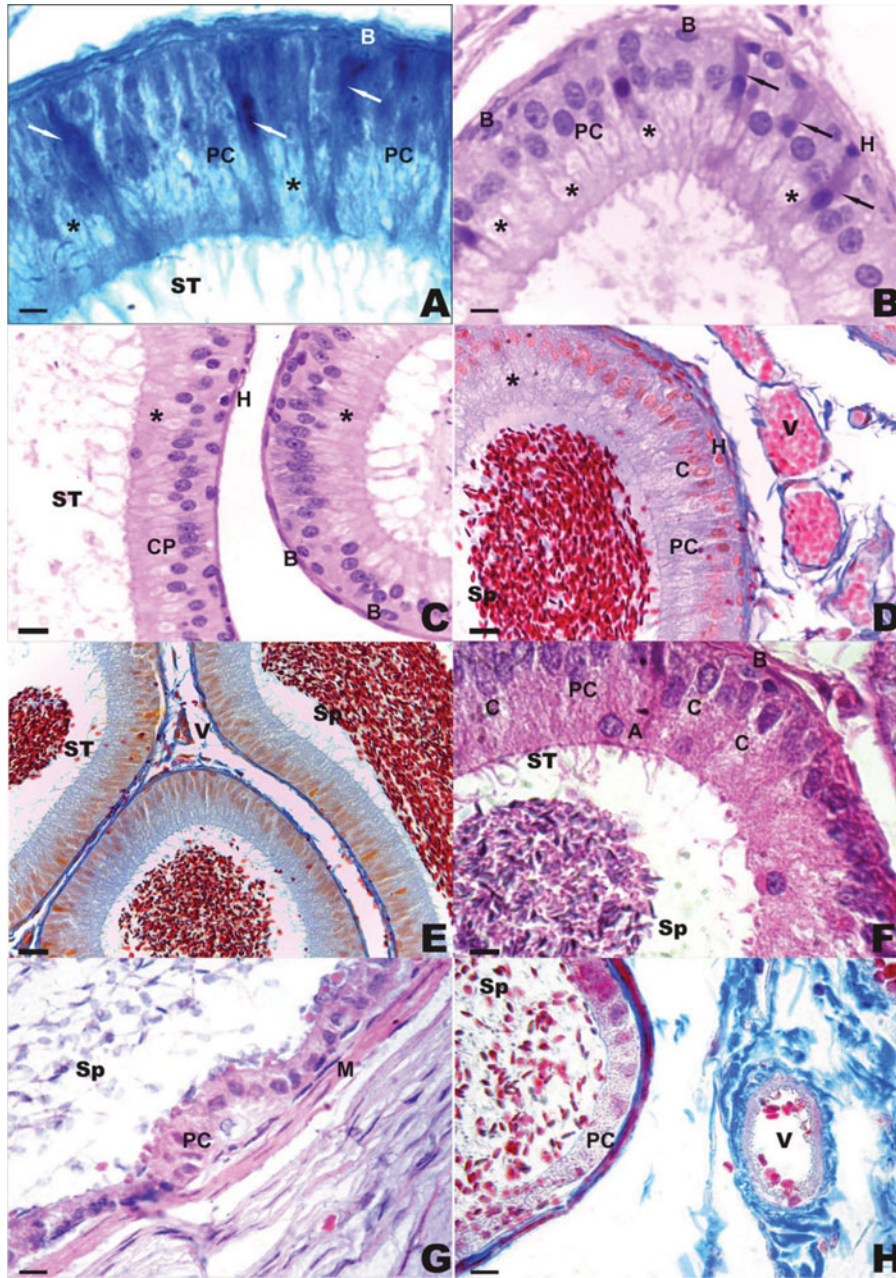


Figure 5 (A, B) Initial segment pseudostratified columnar epithelium. Intensely stained narrow cells (arrows) and numerous vacuoles (asterisks) in the apical cytoplasm. (A) Toluidine blue stain (TB). (B) Hematoxylin–eosin stain (H-E). (C, D) Caput: vacuoles and stereocilia are still noticeable, clear cells appear. (C) H-E stain. (D) Trichrome stain. (E, F) Corpus: numerous sperm appear in the tubular lumen and apical cells. (E) Trichrome stain. (F) H-E stain. (G, H) Cauda: cuboidal pseudostratified epithelium, halo cells and tubular interstitium with medium caliber vessels. (G) H-E stain. (H) Trichrome stain. A: apical cells; B: basal cells; C: clear cells; H: halo cells; PC: principal cells; Sp: sperm; ST: stereocilia; V: vessels. Scale bars: 22.01 μm (E); 35.4 μm (C); 82 μm (A, B, D, F–H).

in luminal content, both fluid and sperm, in a caudal direction. In caput and corpus, the cytoplasm of the PC is highly vacuolized, suggesting an absorption function. In contrast, epididymosomes release in the PC apical membrane makes these very special cells, where the secretion/absorption interplay is perfectly regulated.

In 1975 it was postulated that basal cells serve as stem cells of the epididymal epithelium (Hamilton, 1975). They were later considered as precursors to the PCs (Sun & Flickinger, 1982), but at present numerous gap junctions between basal cells and principal and clear cells are known to exist (Hejmej *et al.*, 2007; Alkafafy *et al.*, 2011). The important point here is the

degree of conservation among species, indicating the relevance of this intercellular junction for epididymal functioning.

Another cell type found from the caput to the cauda is the clear cell, similar findings have been reported for rats (Serre & Robaire, 1998). What is interesting about these cells is that they are species specific, as in gerbils they are found in the initial segment and in the cauda while in Golden hamster they appear only in the epididymal cauda (Beu *et al.*, 2001), no traces of them having been found in either pig (Orsi *et al.*, 1985) or monkey (Ramos & Dym, 1977). They have been implicated in the acidification of the luminal fluid by the secretion of protons, thus contributing to the quiescence of sperm motility in this region (Breton *et al.*, 1996; Blomqvist *et al.*, 2006; Pastor-Soler *et al.*, 2008).

Halo cells, proposed as lymphocytes or monocytes (Wang & Holstein, 1983), would play an immunological role in the epididymus. Later studies have demonstrated their active participation in the prevention of an autoimmune response (Beagley *et al.*, 1998; Serre & Robaire, 1999). In this work an increase is shown in the number of halo cells in the epididymal cauda compared with the initial segment. It seems likely that the increase in luminal content induces the increase in halo cell population.

Chinchilla lanigera is a species with seasonal reproductive cycles in wild life, in which the fall–winter period corresponds to the months of reproductive activity followed by gonadal quiescence in the summer (Weir, 1972; Neira, 1987; Rodriguez, 1988; Garcia *et al.*, 1989; Neira *et al.*, 1989).

In previous studies conducted with this species in captivity in conditions of natural ambient temperature and natural photoperiods, specimens showed noticeable morphometric changes in seminal vesicles (Orostegui *et al.*, 2000), bulbourethral glands and prostate (Cepeda *et al.*, 1999, 2006). These data are closely correlated with important decreases in serum testosterone levels during gonadal quiescence (Adaro *et al.*, 2002).

In our laboratory, however, in conditions of captivity in which external factors such as temperature, humidity and light/dark cycles were controlled all year round, we found no significant changes in epididymal histomorphology.

This is the first report of a histomorphometric study in this species with an assessment of the impact of certain controlled environmental factors on the epithelial lining.

In conclusion, each segment shows different histological and morphometric characteristics, which supports the hypothesis of a specific behaviour for each region, giving a segment-specific characteristic to the sperm maturation process in this species.

Moreover, the fact that no differences were found among the samples collected at two different periods when reproductive activity in nature is different suggests the importance of external factors in the control of the reproductive cycle in *Chinchilla lanigera*. Although this subject requires further study, it should be taken into consideration when analyzing the variables or factors that may have an influence on the control of the reproductive cycle in this species.

Acknowledgements

This work was supported by a grant from the Science Council of the National University of Tucumán (CIUNT).

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