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The ovary of *Lagostomus maximus* (Mammalia, Rodentia): an analysis by confocal microscopy

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ABSTRACT: *Lagostomus maximus* is a notable mammalian model for reproductive studies. Females have an extremely high ovulation rate, which is due to down-regulation of the follicular apoptosis pathway, which ensures a large pool of developing follicles. This large pool is supported by the convoluted anatomy of the mature ovary, whose germinal tissue is found in irregularly curved ridges throughout the cortex. Medullary tissue is restricted to a minimum. Lyso Tracker Red reconstruction under confocal laser scanning microscopy was used to recognize and measure all follicular stages from primordial to antral. Unlike most mammals in which early primordial follicles are just found in fetal life, the adult ovary shows regions packed with early primordial follicles. Follicle size ranged from 24 to 316 μm. We discuss the relationships of *L. maximus* follicles size with regard to other species of mammals and propose that the physiology of the adult viscacha ovary obeys to a neoteny process in the evolution of this species.

Introduction

The number of germ cells in female mammals may be determined before birth and may not be renewable (Edson *et al*., 2009). In female mammals' gametogenesis, apoptosis is a feature of the oocytes during fetal development. The loss of germ cells by apoptosis during the fetal stage varies from 60 to 85% depending on the species. Women will reach adulthood having a loss of more than 99% of germ cells, and only 0.1% of germ cells produced during embryo development will reach maturation and ovulation. Therefore, more than 99% of follicles present at birth will follow the course of the apoptotic pathway (the woman is an extreme case of germ cell loss, reviewed by Hussein, 2005). This fact is reflected by follicular atresia in all mammalian species studied (Kierszenbaum, 2006). The ovary of most rodents behaves essentially in this way.

The ovary is an endocrine organ and apoptosis leading to follicular atresia is under hormonal control. Hormonal control is a complex system of cellular signaling. Follicular development and the ovulation rate are under genetic control and are determined by complex interactions between molecular signals and the ovarian tissue (Fabre *et al.,* 2006; Sugino and Okuda, 2007). The number of mature oocytes released during

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one reproductive cycle varies between species, and is defined and limited to a fixed number for each species. The ovulation rate is 1-2/cycle in primates, deer and goats, while it may be 4 or more than 15/cycle in cats, dogs, pigs and rodents (Telfer, 2004; McNatty *et al*., 2005). However, the ovulation rate of the plains viscacha *Lagostomus maximus* lies outside these ranges (400-800/cycle). This extremely high ovulation rate and the continuity in folliculogenesis (the adult ovary always contains primordial follicles and all stages of the growing follicles) were first described by Weir (1971a,b). Females of several mammalian species ovulate an oocyte number greater than the number of fetuses that are given birth, but none reaches the ovulation rate of the plains viscacha (Jensen *et al*., 2006). Several factors may be contributing to maintaining the high number of healthy follicles and the ovulation rate in this species, but the regulation of the apoptotic pathway seems to be the direct cause of the high ovulation rate in *L. maximus* (Jensen *et al*., 2006, 2008). The ovary of *L. maximus* also shows abundant interstitial tissue with a large amount of lipids, which are considered precursors for steroidogenesis (Gil *et al*., 2007). Based on the hypothesis that the anatomy of the adult ovary should accompany the effect of the presence of a large number of follicles, we focused our work on the analysis of the ovarian morphology by using a confocal microscopy method (Zucker *et al*., 1998, 2000; Zucker and Jeffay, 2006).

Materials and Methods

Animals and sample collection

Five adult *L. maximus* females (weighing higher than 2 kg) were obtained from the Estación de Cría de Animales Silvestres (ECAS), Buenos Aires province, Argentina. Animals were maintained in the laboratory under the conditions described by Weir (1970) and were treated in accordance with international welfare standards (Canadian Council on Animal Care, http://www.ccac.ca). Animals were anesthetized by intramuscular injection of ketamine hydrochloride (Holliday Scott S.A.) and xylazine hydrochloride (Richmond Laboratories, Veterinary Division) and then sacrificed by intracardiac injection of Euthanyle (sodium pentobarbitone and sodium diphenylhydantoin (Brouwer S.A.) and the ovaries were removed and processed as described below.

Routine histology

Histological analysis was performed on hematoxylin-eosin stained paraffin 5-µm sections of paraformaldehyde-fixed ovaries.

Laser confocal microscopy

Staining, dehydration and clearing of the ovaries were performed as described by Zucker *et al.,* (1998) and Zucker *et al.,* (2000). Briefly, ovaries were submerged in LysoTracker Red (5 µM) for 30 minutes at 37°C and were then carefully washed with Hanck's buffered salt solution and fixed overnight (4°C in 4% paraformaldehyde). Samples were dehydrated with methanol. Then were cleared with a solution of 1 part benzyl alcohol to 2 benzyl benzoate by volume. The pieces were transferred to slides with a 1-mm thick and 10-mm diameter cavity. The slides were sealed with a cover slip and maintained in a dark and wet chamber at 4ºC until microscopic analysis in a confocal microscope (FluoView FV300, acquisition software FluoView version 3.3; Olympus, Japan). The emission wavelength used was 543 nm.

Follicle measurements and stages

Follicle measurements were made using 40x and 100x objective lenses. The image processing software was from Nikon Corporation (EZ-C1 free viewer, silver version 3.00 Build 502 and by ImageJ 1.38 X Wayne Rasband, NIH, US, public domain). Illustrations were obtained with Adobe Photoshop CS2, 1990-2007, Adobe System Inc. Follicles were classified as primordial, primary, secondary, preantral (follicles at an early stage of antrum formation) and antral on the basis of granulosa cell number, theca layers, number of follicle cell layers, antrum formation and the general morphology of follicles according to Oakberg (1979) and Gougeon (1996).

Statistics

Follicle size was determined on confocal microscopy images. The data were analyzed by the test of Kruskal and Wallis (1952), using the software provided by InfoStat (InfoStat version 2008, Group InfoStat, FCA, developed by Universidad Nacional de Córdoba, Argentina).

Chromosomes

Chromosomal analysis was performed using the routine technique for obtaining metaphase chromosomes from bone marrow. Standard procedures were followed to determine the diploid (2n) and fundamental number (FNa = number of autosomal chromosome arms).

Results

Routine (Fig. 1) and laser confocal microscopy using the fluorescent dye Lysotracker Red (acidophilic) (Figs. 3-4) allowed us to recognize a convoluted microanatomy of the mature viscacha ovary, whose germinal tissue was found in irregularly curved ridges throughout the cortex. Medullary tissue was restricted to a minimum.

A total of 409 follicles was observed and none was atretic. Primordial follicles were by far the most abundant, with a much smaller number of primary, secondary, preantral and antral follicles (Fig. 2). Mean follicle sizes were: primordial, 24.5 μm; primary, 68.7 μm; secondary, 127.6 μm; preantral, 256.3 μm; and antral 315.9 μm (Table 1). The frequency and distribution of follicles are shown in Figure 2. The three-dimensional aspect of the ovary and these follicles is shown in Figures 3 and 4.

Three homogeneous groups were detected by statistical analysis according to follicle size (primordial, primary+secondary, and preantral+antral) but which were statistically different between them ($P<0.05$; Fig. 4).

Chromosome analysis was performed to establish that animals used for this study were genetically wild-type. The karyotype showed 2n= 56; FNa = 106. Both the number and morphology of chromosomes were as described by Wurster *et al.* (1971), Hsu and Benirschke (1971) and Vidal *et al.* (1973). Also, an autosomal pair with secondary constrictions, typical for this species, was observed. No chromosomal abnormalities or mutations were seen.

Discussion

Oogenesis in most mammals is a complex process that begins and ends during female embryogenesis. The rate of ovulation and folliculogenesis in mammals is a well-known process, but yet there are many questions

TABLE 1.

Follicular developmental stages. Follicle and oocyte diameters from normal ovaries of adult viscacha, *Lagostomus maximus*.

Follicle stages (N)	Follicle diameter (range) (μm)	Mean ± SD (μm)	Oocyte diameter (range) (μm)	Mean ± SD (μm)
Primordial (21)	21.32 - 30.7	24.5 ± 2		
Primary (11)	38.7 - 90.3	68.7 ± 19	18.6 - 50.3	34.7 ± 11.4
Secondary (21)	83.3 - 185.7	127.6 ± 27.8	31.7 - 75.8	53.7 ± 11.6
Preantral (14)	179.6 - 319.2	256.3 ± 49.2	51.4 - 71.7	62.0 ± 5.7
Antral (7)	238.6 - 391.7	315.9 ± 50.5	59.6 - 73.3	64.9 ± 7.6

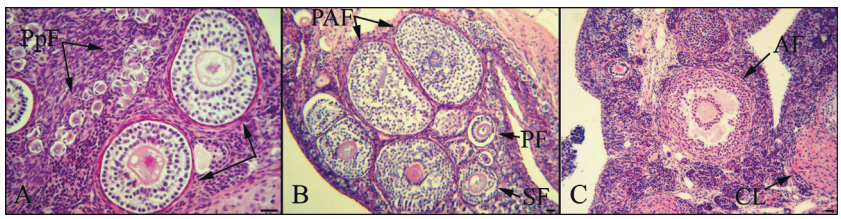


FIGURE 1. Some features of the adult ovary in *Lagostomus maximus* (hematoxylin and eosin). All stages of follicular development were recognized from primordial to antral, but no atretic follicles were seen. A: Numerous primordial follicles and two preantral follicles. B: Primary, secondary and preantral follicles. C: An antral follicle and a small corpus luteum are surrounded by abundant interstitial tissue. Abbreviations; PpF: primordial follicle, PF: primary follicle, SF: secondary follicle, PAF: preantral follicle, AF: antral follicle, CL: corpus luteum. Scale bar represents 50 μm.

to be answered. Females reach reproductive maturity with a fixed number of oocytes that is supposedly characteristic or unique for each species. However, the debate of whether there is formation of gametes after birth in female mammals started in the 1920's and has not yet concluded (Tilly *et al.*, 2009). For instance, there are examples of mitosis in germ cells in adult female prosimians, primates and *Mus* sp. (Telfer, 2004).

In rodents, an order with significant genetic variability, the range of variation in the ovulation rate is wide between species, but not more than 20 oocytes are released per cycle, with the single exception of the plains

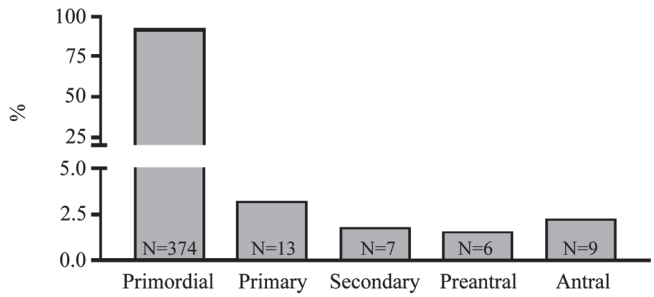
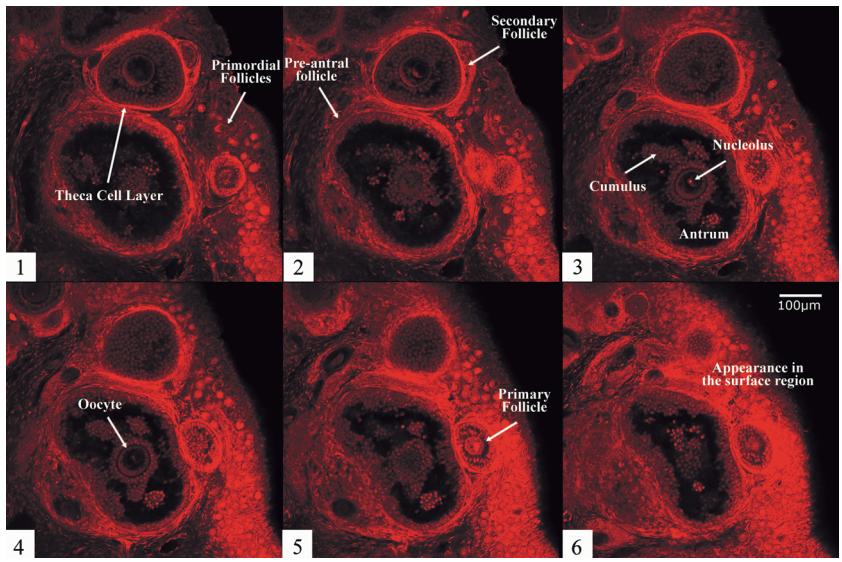


FIGURE 2. Frequency of follicle stages in ovaries of adult *Lagostomus maximus*.

FIGURE 3a. Six sequential confocal images (approximately 20 mm apart) from a small area where several follicles in different stages of development were seen (the preantral label refers to a follicle at an early stage of antrum formation).



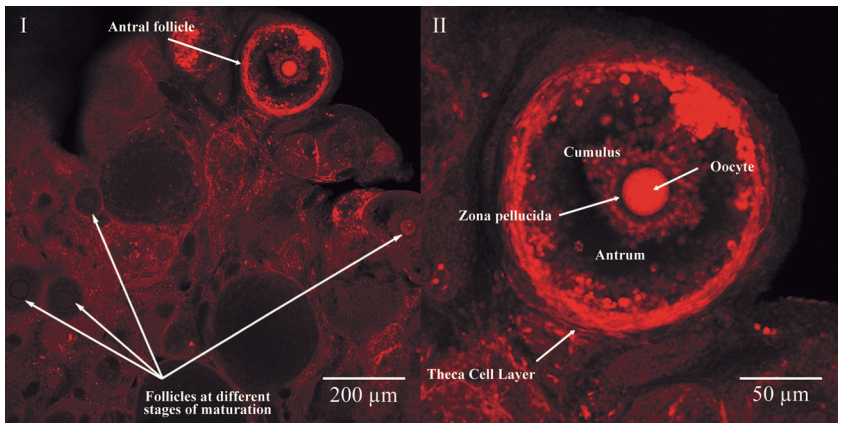


FIGURE 3b. I. A confocal image of several follicles in various stages of maturation and close to the ovarian surface. II. Detail of an antral follicle.

viscacha. In mammals, follicle morphology and proliferation are strongly regulated and are characteristic for each species (Edson *et al*., 2009). These known facts were recently illustrated in a comparative study between mouse, hamster, pig and human led by Griffin *et al*. (2006), which showed that follicle morphology, size, oocyte diameter and granulose cell proliferation are strongly regulated and are fixed genetically. Furthermore, the species showed significant differences in the mentioned parameters but the latter were specific for each species. Likewise, both ovulation rate and litter size are under genetic control (Fabre *et al.,* 2006) and, in most mammalian species studied so far, the apoptotic pathway leads to follicular atresia in the ovary (Jensen *et al.,* 2006; Palumbo and Yeh, 1994).

Follicular morphology in *L. maximus* is similar to that in other mammals but the size of follicles and germ cells is lower than in other species such as mice, hamsters, pigs and humans (Griffin *et al.*, 2006). Three other facts are notable in the adult viscacha ovary which deviate from the general rule: they present and conserve a large number of primordial follicles; females have a high ovulation rate (Weir, 1971b); and no follicular atresia is observed. The latter phenomenon has been related to the overexpression of antiapoptotic genes such as *BCL-2* (Jensen *et al.,* 2006, 2008).

The ability to produce and maintain such number of follicles in the ovary must be anatomically supported. We have noticed that the morphology of the viscacha ovary shows a pattern different from that seen in other rodents and most mammals, because of the great and lobated development of its cortical tissue, which we are interpreting as an adaptation to support the amount and type of follicles that were observed. Authors such as Tilly *et al.* (2009), and Johnson *et al.* (2005), have suggested that the source of germ cells during adulthood would be the bone marrow. In the ovary of *L. maximus*, stem cells from bone marrow seem not needed to maintain ovarian function, since germ cells are abundant. Given that ovulation rate in mammals is a response to hormonal induction (hormones are the stimulus for ovulation within a complex process), the hormonal signaling pathway is essential for ovarian function in mammals (Fabre *et al*., 2006). In order to understand in deep how the viscacha ovary is functionally normal (with no atresia), it will be important to investigate the hormonal signaling in relation to apoptosis in the ovary.

Most important, the number and proportion of follicular types in the viscacha ovary remind those found in immature human beings and mice (Moniruzzaman and Miyano, 2010). The process of paedomorphosis (which includes neoteny) is common in developmental biology (Raff and Kaufman, 1983) and we think that the retention of immature features of the ovary in adult viscachas may be interpreted as neoteny and that can be related to the down-regulation of the metabolic pathways of apoptosis. Our hypothesis of neoteny in *L. maximus* may be in agreement with the fact that adult ovaries from species with prosimian ancestral features have some germ cells in mitosis (Telfer, 2004).

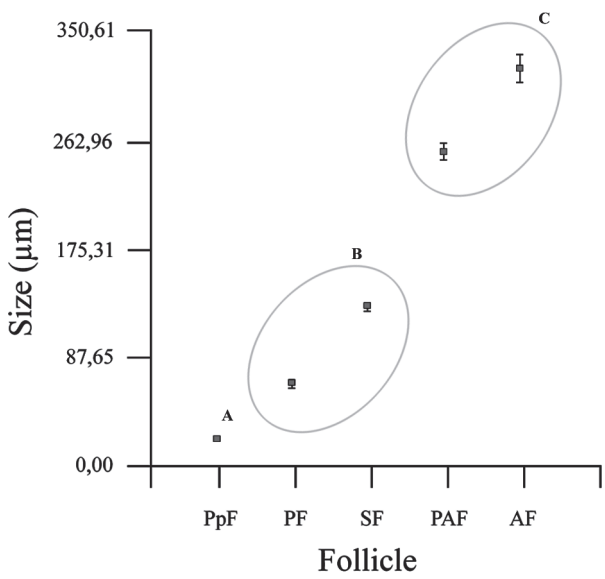


FIGURE 4. Three homogeneous groups, labeled A, B and C, were found significantly different between them. A, primordial follicles; B, primary+secondary, and C, preantral+antral.

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