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Ovarian function of the algerian wild Libyan jird, *Meriones libycus* during seasonal reproductive cycle: histological and immunohistochemical expression

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Summary. *Meriones libycus* (Libyan jird), a nocturnal Saharan rodent, is characterized by a seasonal reproductive cycle with a short active phase (spring and early summer) and a long resting period (late summer, autumn, winter). Histological and immunohistochemical techniques were performed in order to study the seasonal variations in mature ovaries. During the breeding season, the ovary showed a continuous cyclical activity, the various stages of folliculogenesis from primordial to preovulatory follicles were observed; broken follicles and corpora lutea were also observed. During sexual quiescence, the ovarian cycle was interrupted; anovulation was observed without any corpus luteum. Non mature antral follicles entered the atretic process. Steroid and steroidogenic enzyme activities were studied using indirect immunohistochemistry. 17ß-estradiol, progesterone, testosterone hormones and P450 aromatase (P450 arom) were detected in the different components of the ovary and in various stages of healthy and atretic follicles during the seasonal reproductive cycle. Our results indicate that during ovarian folliculogenesis in breeding season steroids hormone and P450 arom present important activities. In comparison with the resting period, steroidogenesis and steroidogenic enzyme activity became less pronounced in the healthy preantral follicle; it seemed that steroid biosynthesis was reduced and could be involved in the stimulation and maintenance of the ovarian structural integrity in early follicle development. In conclusion, the histological and immunohistochemical seasonal variations of ovaries in *Meriones libycus* support the hypothesis that seasonal fluctuations are indirectly involved in regulating reproduction, inducing significant changes in both ovarian morphology and its hormonal function.

Key words: Ovaries, Folliculogenesis, Rodents, Sahara, Reproduction, Histology, Immunohistochemistry, *Meriones libycus*

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Introduction

Reproduction in mammals consumes much energy, requiring a period of reproduction coinciding with the time of year that is most adequate to the habitat. However, the most rodent species also breed at specific times of the year in order to ensure the survival of their offspring. They are born and raised when food availability is high and ambient temperature comfortable. There is evidence that seasonal variations in reproductive activity occur in Saharan rodents in the field (Khammar and Brudieux, 1984, 1987, 1991; Zaime et al., 1992; Gernigon et al., 1994; Gernigon-Spychalowicz, 1995) but very little information is available about environmental factors (food availability, rainfall, temperature, photoperiod) controlling these changes. Seasonal cycles of fertility and infertility are often accompanied with behavioral and physiological responses (Krishna and Bhatnagar, 2011). When conditions for reproduction become unfavorable, the stoppage of reproductive activity was demonstrated by

structural and functional atrophy of reproductive organs, which is correlated with a decreasing in hormone production (Young et al., 2000; Aguilera-Merlo et al., 2005, 2009; Schradin, 2008; Salverson et al., 2008; Shahed and Young, 2008). In male *Meriones libycus*, several studies about seasonal variations and hormonal cycle of reproductive organs have been published (Belhocine and Gernigon-Spychalowicz, 1994, 1996; Belhocine et al., 1996, 2007, 2010; Belhocine, 1998; Mataoui, 1999; Mataoui et al., 2006). In contrast to the male, few data about physiological, structural and functional aspects in reproductive activity of females are avaible. Structural and ultrastructural variations of the reproductive organs were studied during estrus cycle (Smaï et al., 1996; Smaï, 1998). The aims of the present study were to examine the histological seasonal variations of the ovary and to localize steroid hormones (17ß estradiol, progesterone, testosterone) and steroidogenic enzyme (P450 aromatase) in follicles, in order to clarify their involvement in the regulation of ovarian function during breeding and non-breeding seasons when Saharan ambient conditions for reproduction become unfavorable.

Materials and methods

Animals

Meriones libycus (Lichtenstein, 1823) is a nocturnal, herbivorous and granivore Gerbilidae rodent, indigenous to the northwestern Algerian Sahara. The breeding period of Libyan jird is short (spring, earlier summer) with a long resting season (late summer until late winter) (Belhocine and Gernigon-Spychalowicz, 1994). Wild adult females captured near Beni-Abbes region (30°7'N and 2°10'W) were seasonal, spontaneously ovulating polyestrous mammals. 20 adult females were used; 12 were captured during active season and 8 females were in quiescent sexual period.

Estrous cycle determination

Determination of sexual state of animals was monitored with vaginal smears. Estrous cyclic stages were determined twice a day with examination of vaginal cytology at early morning and late evening; the smear was obtained by inserting a cotton stick saturated with distilled water into the vagina. Cells from the vaginal cavity and walls were stained with Giemsa. Histological characteristics of the cells on proestrus (round, nucleated cells), estrus (cornified cells), metaestrus (round nucleated cells, cornified cells, leukocytes) and diestrus (predominance of leukocytes) were identified with light microscope (Smaï, 1998).

Histology

At each phase of the estrous cycle in breeding and resting seasons, the females were euthanazied at late evening because of their nocturnal activity (18h 00- 20h 00 in active season and 17h 00- 19h 00 in resting season); ovaries were removed and fixed in Bouin's solution or in buffered 10% formalin; the ovaries were dehydrated in a series of ethanol of increasing degree (70%, 95%, 100%); they were then embedded in paraffin. Sections (5μm thick) were cut with Leitz vertical microtome and mounted on normal slides for histological study or on "Super Frost" glass slides for immunohistochemistry analysis. After dewaxing and dehydratation, the sections were stained with Romeis's azan, Masson's trichrome and Heindenhein's azan (Gabe, 1968; Exbrayat, 2000).

Immunohistochemistry

Expression of steroid hormones (17ß estradiol, progesterone, testosterone) and steroidogenic enzyme (Cytochrome P450 aromatase) was detected by indirect immunohisto-chemistry using streptavidin-biotin-peroxydase complex (ABC) (Hsu et al., 1981) with the Dako LASB2 kit. Following dewaxing in xylene and hydratation of slides with distilled water, sections were rinsed in PBS, surrounded with "Pap-pen" and then treated for 5 min with hydrogen peroxide diluted at 3% in PBS (PBS-H_2O_2 at 3%) in order to eliminate activity of endogenous peroxidase, followed by 15 min rinsing. Non-specific binding of the secondary antibody was blocked by incubation of sections with normal goat serum. Sections were then incubated for 30 min at room temperature with primary antibodies, polyclonal rabbit anti-17ß estradiol (1/200) (Chemicon), polyclonal rabbit anti-progesterone (1/200) (Biomeda AbCys), polyclonal rabbit anti-testosterone (1/50) (Abcam), and monoclonal mouse anti-human cytochrome P450 aromatase (1/100) (AbCys). The slides were washed 3 times in PBS for 5 min and incubated with secondary biotinylated antibody directed against the species in which primary antibody was preparated for 30 min at room temperature. After rinsing 3 times in PBS for 5 min, slides were incubated with a streptavidin-biotin-peroxydase complex for 30 min, and washed thoroughly. Immunoreactivity was visualized in red with amino-ethyl-carbazole (AEC), or in brown with diaminobenzidine (DAB). Sections were counterstained using Mayer's hematoxylin and mounted in aqueous medium (Crystal mount). Tissue sections were analyzed at 40, 100, 400, 1000 x magnification under light microscope Nikon Eclipse E400 and photographed with a Nikon digital camera DxM1200.

Control sections

In negative controls, sections were incubated with PBS instead of the primary antibody.

Results

Histological and immunohistochemistry seasonal variations in Libyan jird ovaries (Table 1)

Ovaries were active in spring and early summer and

quiescent from later summer until late winter.

Histological description of ovaries

During adult life, the ovary of *Meriones libycus* was covered with a single epithelium with cubic or columnar cells. It was divided into cortex, with healthy and atretic follicles, and medulla composed of loose fibrous connective tissue, blood and lymphatic vessels, and nerves. At breeding season, (Fig. 1a,b) the ovary showed a continuous cyclical activity. Primordial to Graafian follicles were observed together, as well as ruptured follicles and corpora lutea. At resting season, (Fig. 1c,d) anoestrus dominated. The ovary was characterized by healthy primordial and primary follicles located at the periphery of cortex. Very few preantral follicles grew to become large antral follicles. Follicles did not mature, and became atretic. Follicular atresia was more pronounced compared to the active season. Ovulation did not occur; broken follicles and corpora lutea were not observed.

Table 1. Immunoreactivity status of steroid hormones and steroidogenic enzyme localization and intensity of immunostaining in adult *Meriones libycus* ovary during seasonal reproductive cycle.

Healthy follicles types/Antibodies		Breeding season	Resting season
Primordial follicle	P	-/+	-/+
	E	-/+	+
	T	+	-
	P450arom	-/+	+
Intermediate follicle	P	+	+
	E	+	+
	T	+	-
	P450 arom	+	+
Primary follicle	P	+	+
	E	+	+
	T	+	-
	P450 arom	++	+

Healthy follicles types/Antibodies		Breeding season		Resting season	
		Follicular compartments			
		Gr	Ti	Gr	Ti
Preantral follicle	P	+	++	+	+
	E	+++/++	++	+	+
	T	+	+	-	-
	P450 arom	++	++	++	++
Antral follicle	P	++	+++		
	E	+++	++		
	T	+	++	Non-existent	
	P450 arom	++	++		
Large antral follicle	P	++	+++		
	E	+++	-/++		
	T	+	+++	Non-existent	
	P450 arom	+++	-/++		

Not detected: -; low staining: +; intense staining: ++; very intense staining: +++; Gr: granulosa; Ti: Theca interna; P: progesterone; E: 17ß estradiol; T: testosterone; P450 arom: P450 aromatase.

Ovarian folliculogenesis

In both periods, histological analysis presents the same morphological characteristic in ovarian follicular. Immunohistochemical examination indicated that whatever the phase of estrous cycle during the active season, and stages of growth, healthy follicles always showed the same immunohistochemical features for estradiol, progesterone, and P450 arom. Except for in the estrus, metaestrus and early diestrus, the healthy follicles presented negative immunoexpression for testosterone. Immunoreactivity of all steroid hormones and steroidogenic enzyme investigated was cytosolic; except for in the proestrus phase, 17ß estradiol was detected in both the nucleus and cytoplasm of some cells of the granulosa.

Primordial follicles. Numerous primordial follicles were generally observed on the cortex of ovary. They were not developed and consisted of a primary oocyte (stopped on prophase of first meiotic division) encapsulated with flattened squamous granulosa cells (Fig. 2a). In transitional or intermediary follicles, the oocyte was surrounded by a mixture of flattened and cubic granulosa cells (Fig. 2b).

Primary follicles. These follicles constituted a single layer of cubic granulosa cells surrounding the oocyte. The small primary follicles were characterized by 6-12 cubic granulosa cell layers surrounded by a lamina basalis. In the large primary follicle, the oocyte was surrounded by 20- 28 granulosa cell layers, accompanied by the development of an outer encapsulating sheath derived from the stroma, constituting the undifferenciated theca folliculi (Fig. 2c). Immunohistochemical analysis showed that during the breeding period, some primordial, intermediary and primary follicles revealed a slight immunostaining to progesterone (Fig. 2d), testosterone (Fig. 2e,f), 17ß estradiol (Fig. 2g) and P450 arom (Fig. 2h,i). During the resting season, similar immunostaining for progesterone, 17ß estradiol and P450 arom was shown in these follicles, but a negative testosterone immunoresponse (Fig. 2j,k) was observed. Secondary follicles or preantral follicles consisted of oocytes, with a large nucleus (or germinal vesicle), surrounded with more than two layers of granulosa cells. Zona pellucida appeared thick; the theca folliculi remained still undifferenciated at this stage (Fig. 3a). As the preantral follicle enlarged (Fig. 3b) the proliferating granulosa cells expressed a mitotic activity; follicular cells continued to divide and the number of layers increased to form a stratified epithelium 2 to 12 cell layers thick. Theca folliculi differentiated on both theca interna and theca externa. In these follicles, during breeding season, the theca interna showed a clear immunohistochemical signal for progesterone, contrarily to granulosa cells which were weakly immunolabeled (Fig. 3c). During the resting period, progesterone immunoexpression was reduced in

both granulosa and theca cells (Fig. 3d). In the same season, immunoreactivity of testosterone was diffuse and slight (Fig. 3e) but no signal was expressed in resting period (Fig. 3f). During the active period, estradiol (Fig. 3g) and P450 arom (Fig. 3i) were highly expressed in both granulosa and theca cells; their signal was low during sexual quiescence (Fig. 3h,j). 17ß estradiol and P450 arom immunoreactivities were also observed in the oocyte cytoplasm of all growing follicles. Except in some large antral follicles, oocyte cytoplasm presented negative immunoexpression of P450 arom.

Tertiary follicles or antral follicles. The large preantral follicle continued to enlarge and reached early antral stage with the formation of multiple fluid-filled intercellular spaces; these spaces enlarged and coalesced into a single larger crescent cavity, the antrum. Oocytes became eccentric and surrounded with granulosa cells, both forming the cumulus oophorus. Granulosa cells surrounding the zona pellucida constituted the corona radiata. The peripheral granulosa cell layer observed near the lamina basalis formed the mural granulosa cells. The theca interna cells rapidly enlarged and became polyhedral or spindle-shaped, and finally formed an epithelial layer of 3 or 4 cells layers. These cells were enmeshed in a reticular and fibrous network and contained a plexus of capillaries and lymphatics. The liquor folliculi contained in the antrum increased in volume when ovulation approached (Fig. 4a). During active season, in smallest and largest healthy antral follicles, progesterone (Fig. 4b) and testosterone (Fig. 4c) were highly expressed in theca interna, but immunostaining was low in granulosa cells; estradiol (Fig. 4d) and P450 arom (Fig. 4e) expressed a strong immunoexpression in both granulosa and theca interna cells. However, within the granulosa layer a faint

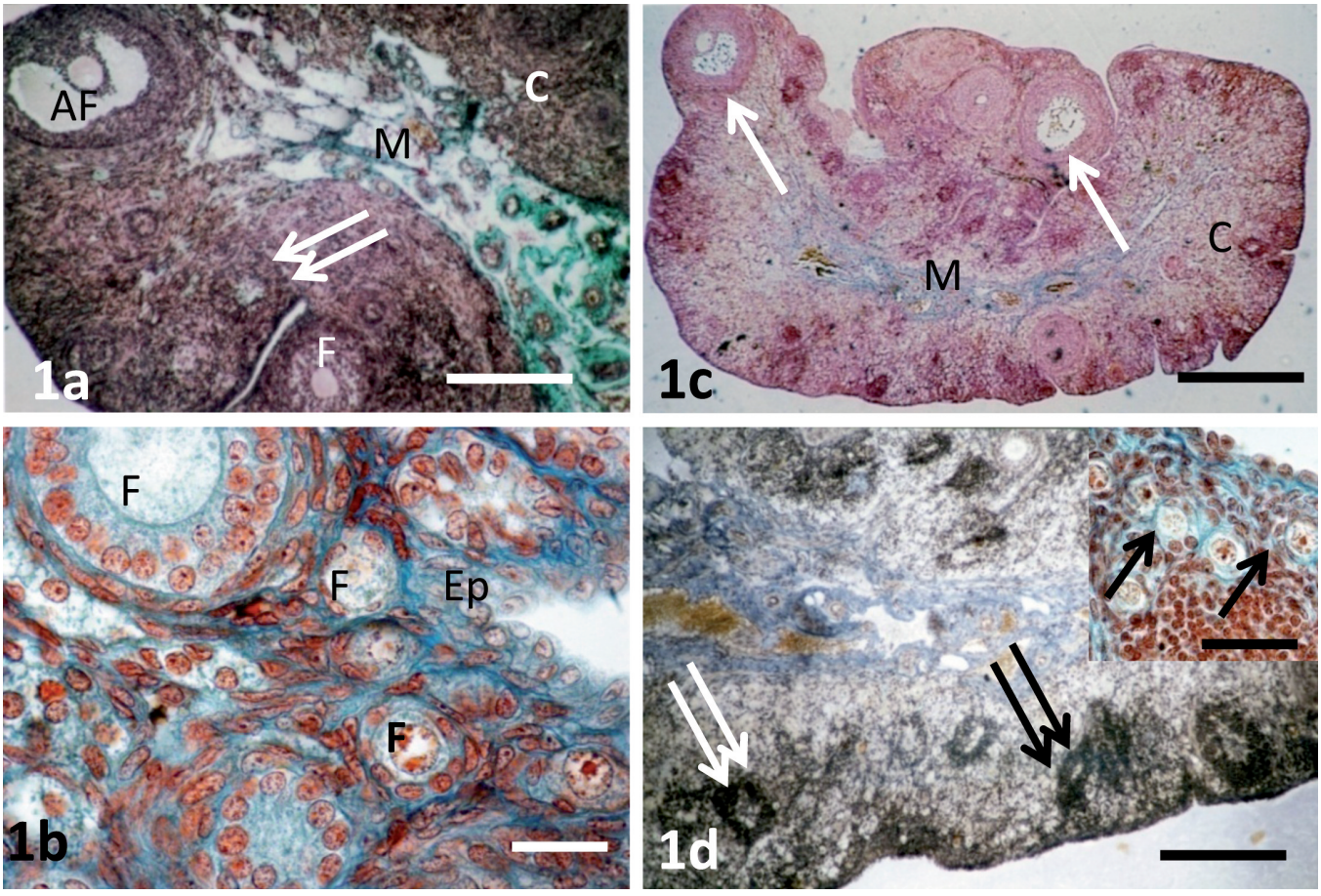

Fig. 1. General views of adult wild *Meriones libycus* ovaries during the seasonal cycle of reproduction. **1a, 1b.** In breeding season (spring and onset of summer) the ovary showed two regions: cortex (C) contained many healthy growing follicles (F) (detail in **b**) and secondary atretic follicles (double arrows). Medulla (M) with an important loose fibrous connective tissue and blood vessels. **1c, 1d.** In resting period (end of summer until late winter) the ovary exhibited different stages of non developed follicles, antral atretic follicles (arrow), an abundance of secondary atretic follicles (double arrows) (d), healthy primordial and intermediate follicles (arrow) (insert) . 1a: Masson's trichrome; 1b: Romeis's azan; 1c: Heindenhein's azan; 1d: Hematoxylin-Heindenhein's blue; insert: Romeis's azan. Legends: AF: antral follicle; C: cortex; Ep: epithelium surface; F: Healthy follicles; M: Medulla; arrow: antral atretic follicles double arrows: secondary atretic follicles. Bars: 1a, 1d, 200 μm; 1b, 10 μm; 1c, 500 μm; insert in 1d, 10 μm.

gradient of staining for estradiol and P450 arom in the cytoplasm was observed: immunolabeling decreased from the strongly stained peripheral granulosa to the granulosa cells located near the antrum. In other antral follicles, estradiol (Fig. 4f) and P450 arom (Fig. 4g) immunoexpression was typically observed in the granulosa cells; theca interna remained unmarked. In these follicles, the granulosa expressed the cytosolic and nuclear labeling of estradiol; the nuclear signal was observed in the cumulus cells and some of them were unstained (Fig. 4h). During resting phase, all the antral follicles which did not mature underwent atresia. Following ovulation, immunoreactivities for progesterone, estradiol and P450 arom were strong in both luteinized granulosa cells and theca interna cells of the corpus luteum (Fig. 5a,b). Negative controls without primary antibodies were devoid of immunolabeling.

Histological and immunohistochemistry of atretic follicles during seasonal reproductive cycle (Table 2)

In breeding and quiescent sexual periods various stages of follicle atresias were observed and exhibited the same histological signs. Some different immunohistochemical features were detected during the reproductive seasonal cycle. Indeed, two types of atretic follicles were present in ovaries of *Meriones libycus*.

Type A: Atresia predominated in secondary follicles. The first sign of the degeneration involved prominent

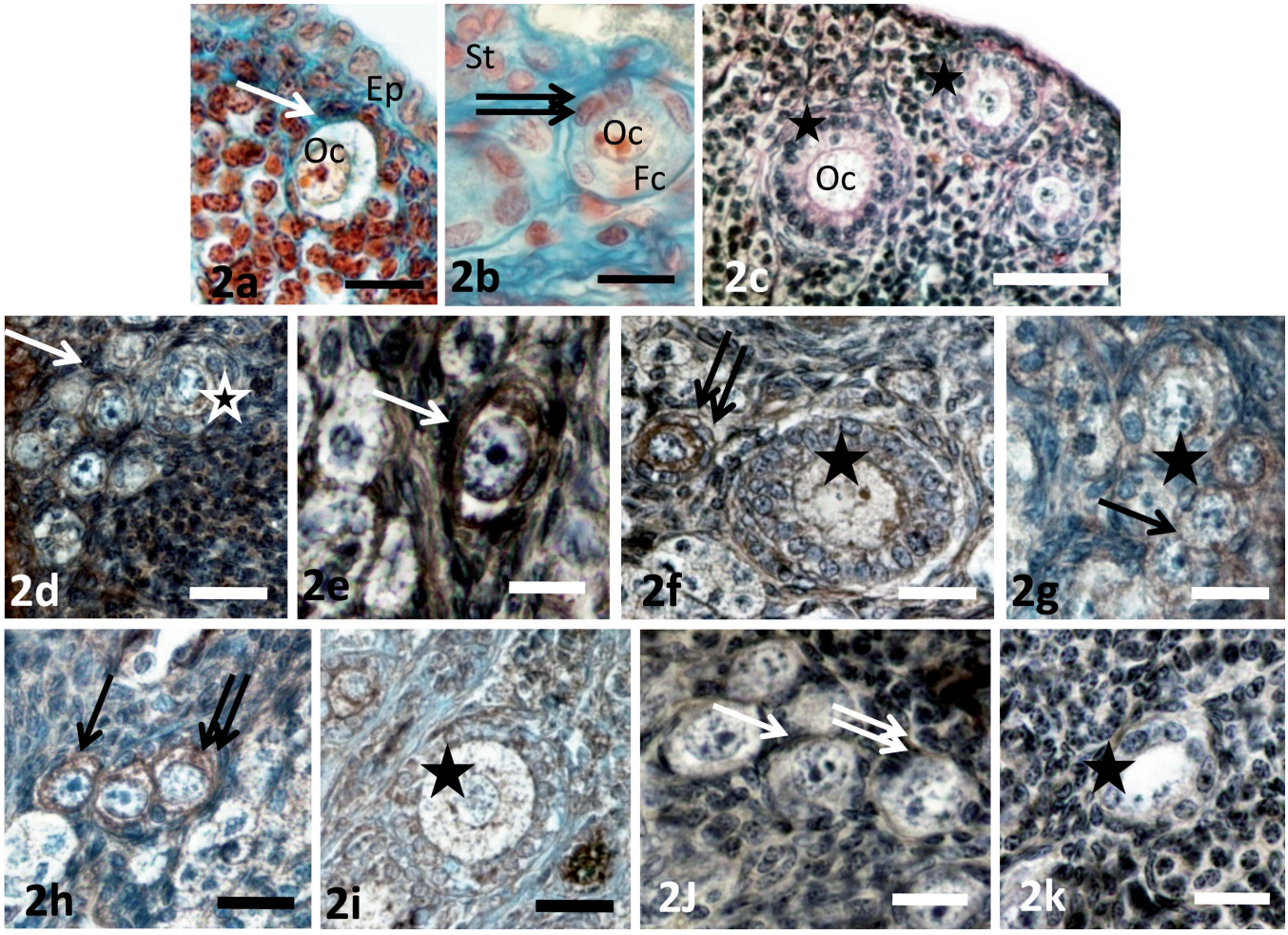

Fig. 2. Histology and immunohistochemistry of nongrowing follicles (primordial, intermediary, primary) in adult *Meriones libycus* ovaries during seasonal reproductive cycle. **2a.** Primordial follicle (arrow) localized on the peripheral cortex. Primary oocyte (Oc) surrounded with a few flattened follicular cells. **2b.** Intermediary follicle (double arrows). **2c.** Primary follicles of different size (asterisk). **2d.** Visualization of progesterone with immunohistochemistry in both periods. **2e, 2f.** Visualization of testosterone with immunohistochemistry in active season. **2g.** Visualization of estradiol with immunohistochemistry in both periods. **2h, 2i.** Visualization of P450 arom with immunohistochemistry in both periods; signal is weak in different non-growing follicles. **2j, 2k.** during sexual quiescence follicles exhibited a negative signal to testosterone 2a,b. Romeis's azan; 2c: Masson's trichrome. 2d-k: immunohistochemical results. Legends: Ep: surface epithelium; Fc: Follicular cells; Oc: Oocyte; Primordial follicle (arrow); Intermediary follicle (double arrows); Primary follicles (asterisk). Bars: 2a, 2b, 2d-2k, 10 μm; 2c, 30 μm.

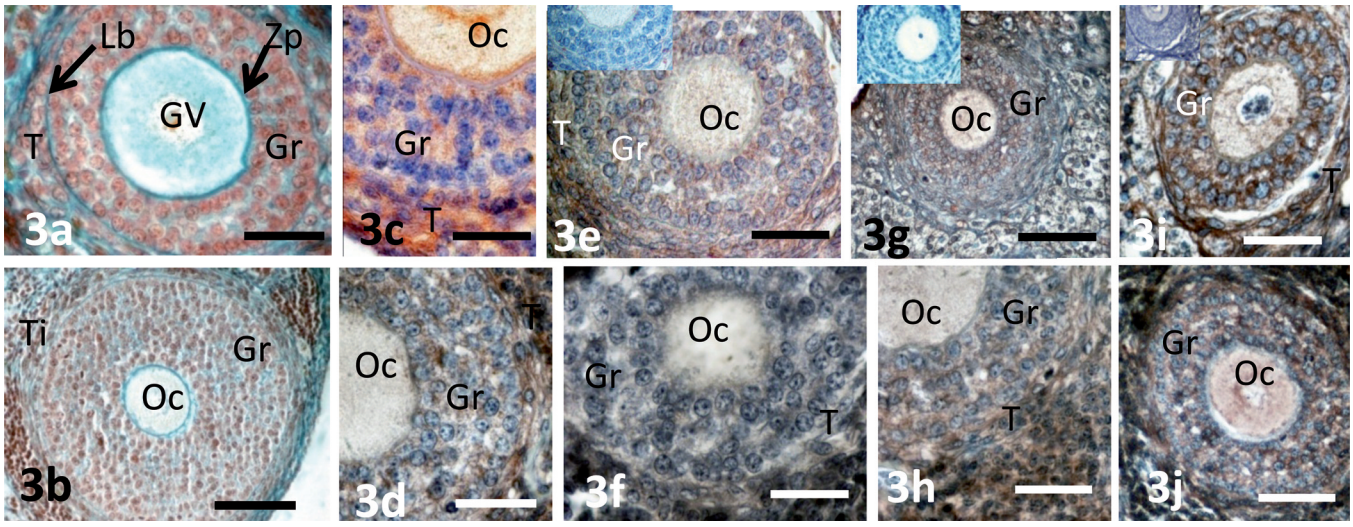

Fig. 3. Histology and immunohistochemistry of growing follicles (preantral, antral follicles) in adult *Meriones libycus* ovaries during seasonal reproductive cycle. Small **(3a)** and large secondary follicles **(3b)**. **3c.** An obvious immunomarquage of progesterone in the theca cells (T); the granulosa (Gr) appeared weakly positive during breeding period. **3d.** Labeling with progesterone in resting phase; immunoexpression decreased in both granulosa (Gr) and theca cells (T). **3e.** Labeling with testosterone in breeding phase; a diffuse and slight signal was found in both granulosa and theca cells. **3f.** negative staining with testosterone in resting season. **3g.** Intense labeling of secondary follicle with estradiol in both granulosa (Gr) and theca cells (T) in active season. **3h.** labeling with estradiol in resting season showing weaker signal than in breeding season. 3i: with P450 arom, an important immunolabeling of granulosa cells and theca in the active period was observed. 3j: immunoreactivity of P450 arom is attenuated in resting season. 3a, 3b: Romeis's azan; 3c-j: immunohistochemical results. Gr: granulosa; GV: germinal vesicle; Lb: Laminas basalis; Oc: oocyte; T: Theca; Ti: Theca interna. Bars: 3a, 3 d-3j, 10μm; 3b, 3g, 25 μm; 3c, 2.1 μm.

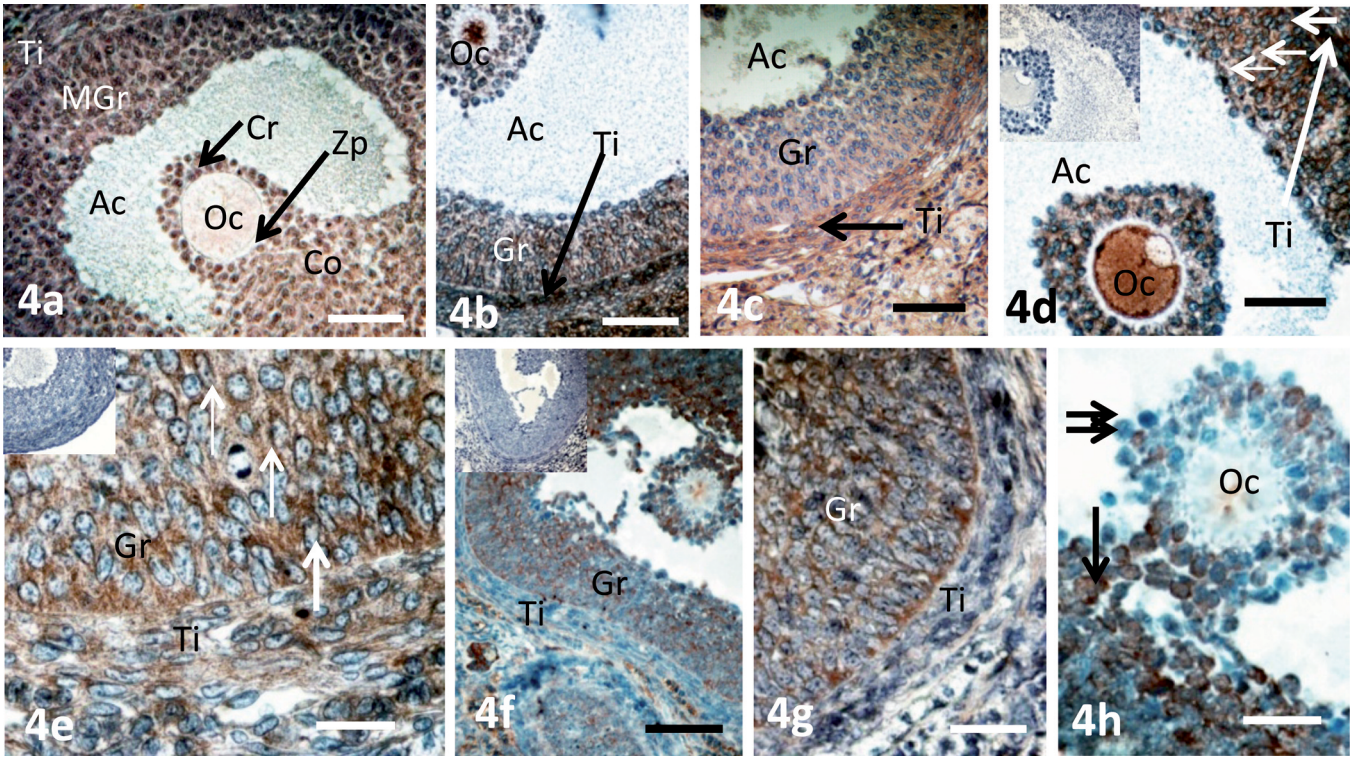

Fig. 4. Histology and immunohistochemistry of growing follicles (preantral, antral follicles) in adult *Meriones libycus* ovaries during seasonal reproductive cycle. **4a.** Healthy large antral follicle with primary oocyte (Oc) surrounded by thick zona pellucida (Zp), cumulus oophorus (Co), mural granulosa (MGr), and conspicuous differentiation of the theca on both theca interna (Ti) and theca externa. **4b.** Labeling with progesterone in breeding season; theca interna (Ti) showed a high immunohistochemical signal; immunostaining is low in the granulosa cells (Gr). **4c.** Visualization with testosterone in active phase; strong signal located in the theca interna (Ti); immunoexpression remained weakly diffuse in the granulosa cells (Gr). **4d.** Staining with estradiol in breeding season; positive signal involved both granulosa cells (Gr) and theca interna (Ti). Note the faint gradient of label in the granulosa layer: the strong immunostaining being from the granulosa cells (arrow) collected near the lamina basalis to those closer to the antrum (arrow). **4e.** with P450 arom in breeding season; granulosa cells are intensely immunostained (Gr); theca interna also showed a positive signal; Note the same gradient of P450 arom was observed in the granulosa layers (arrows). Labeling of another large antral follicle in active period with estradiol **(4f)**; with P450 arom **(4g)**; cytoplasm immunoresponse found only in the granulosa cells (Gr); the theca interna (Ti) showed negative signal. **4h.** nuclear signal was observed in the cumulus cells (arrow), some of them were unstained (double arrows). 4a: Masson's trichrome. 4b-h: immunohistochemical results. Ac: Antral cavity; Co: Cumulus oophorus; Cr: Corona radiate; Gr: granulosa; MGr: Mural Granulosa cells; Oc: oocyte; Ti: Theca interna. Bars: 4a, 50 μm; 4b-4d, 4f, 25 μm; 4e, 4h, 10 μm.

apoptotic changes in oocyte and zona pellucida (Fig. 6a), whereas alterations of the granulosa cells were secondary. In these cells, many apoptotic bodies were observed and gradually lost the cell-to-cell bond; the lamina basalis became thick and fibrous (Fig. 6b). In the last stage of secondary stage of atresia, granulosa cells completely disappeared (Fig. 6c) and were replaced with connective tissue. During both active and resting seasons, in these follicles, immunohistochemical study revealed positive staining for estradiol and progesterone in the non apoptotic granulosa cells; theca cells became fibrous, showing a signal in both steroid hormones investigated (Fig. 6d). Testosterone appeared slightly or non-existent in the granulosa cells, but theca cells were strongly labeled (Fig. 6e). Compared to the resting period, these follicles exhibited a negative immunoreactivity to testosterone. In both periods, immunoexpression to P450 arom was displayed more intensely in granulosa and theca cells (Fig. 6f) than the signal corresponding to estradiol, progesterone and testosterone.

Table 2. Immunolabelling variations of steroid hormones and steroidogenic enzyme localization and intensity of stain in atretic follicles of adult *Meriones libycus* ovary during seasonal reproductive cycle.

Atretic follicles types/Antibodies		Breeding season		Resting season	
		Gr	Ti	Gr	Ti
Secondary follicle	P	+	-/+	+	+
	E	+	-	+	-
	T	-/+	+	-	-
	P450 arom	++	++	++	+
Early antral follicle	P	++	++	++	++
	E	++	+	++	++
	T	+	++	-	-
	P450 arom	+++	++	++	++
Mid antral follicle	P	-	+	-	-
	E	++	-	++	+
	T	+	++	-	-
	P450 arom	+	+/-	+	-/+
Late antral follicle	P		-		-
	E		-		-
	T		-		-
	P450 arom		-		-

Not detected: -; low staining: +; intense staining: ++; very intense staining: +++; Gr: granulosa; Ti: Theca interna; P: progesterone; E: 17ß estradiol; T: testosterone; P450 arom: P450 aromatase.

Type B: Atresia on antral follicle was most conspicuous, typified by distinctive degenerative changes in the granulosa cell layers. In the beginning they showed almost unchanged oocyte and zona pellucida. The degeneration in the granulosa cells layers occurred progressively.

The early stages of atresia were characterized by thin condensed granulosa cell layers, the presence of few degenerated nuclei in mural granulosa cells usually are adjacent to the antral cavity, presence of little cellular debris in follicular fluid. The lamina basalis was intact (Fig. 7a). Immunohistochemical analysis indicated that in the antral atretic follicles, intensity of labeling decreased progressively when stages of atresia advanced until the interruption of steroid hormones and P450 arom biosynthesis. However, in the early antral atretic follicle, during breeding and resting phases, steroid activity and P450 arom biosynthesis continued. The immunohistochemical features of estradiol, P450 arom, progesterone and testosterone resembled those observed in the healthy early antral follicles. The gradients of staining for progesterone, estradiol and P450 arom (Fig. 7b) within granulosa layer were reversed in comparison

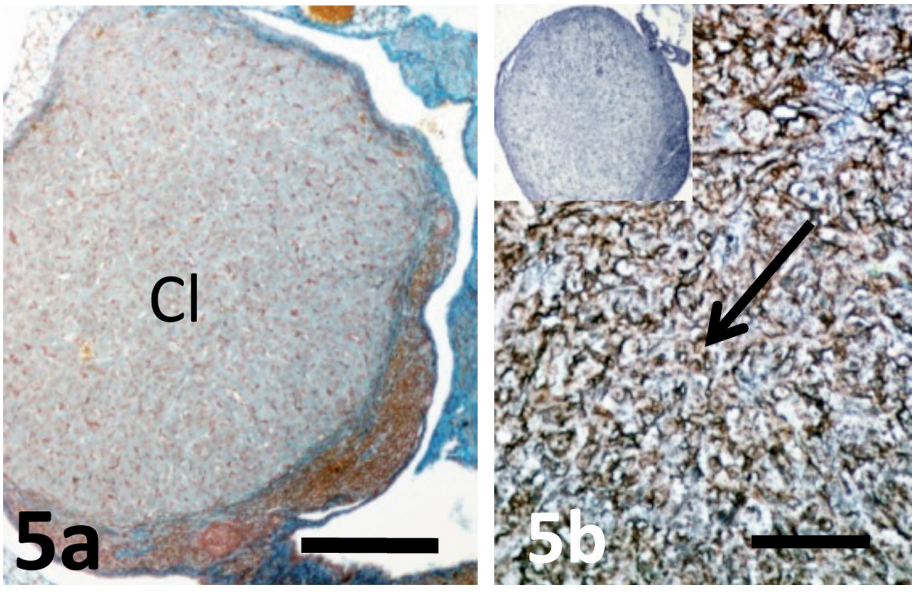

Fig. 5. Histology and immunohistochemistry of growing follicles (preantral, antral follicles) in adult *Meriones libycus* ovaries during seasonal reproductive cycle. **5a.** Corpora lutea (Cl) formation following ovulation. **5b.** Positive immunoreactivity with estradiol of corpus luteum; the same immunohistochemical signal was found with P450 arom and progesterone. 5a: Romeis's azan; 5b: immunohistochemical results. Bars: 5a, 100 μm; 5b, 10 μm.

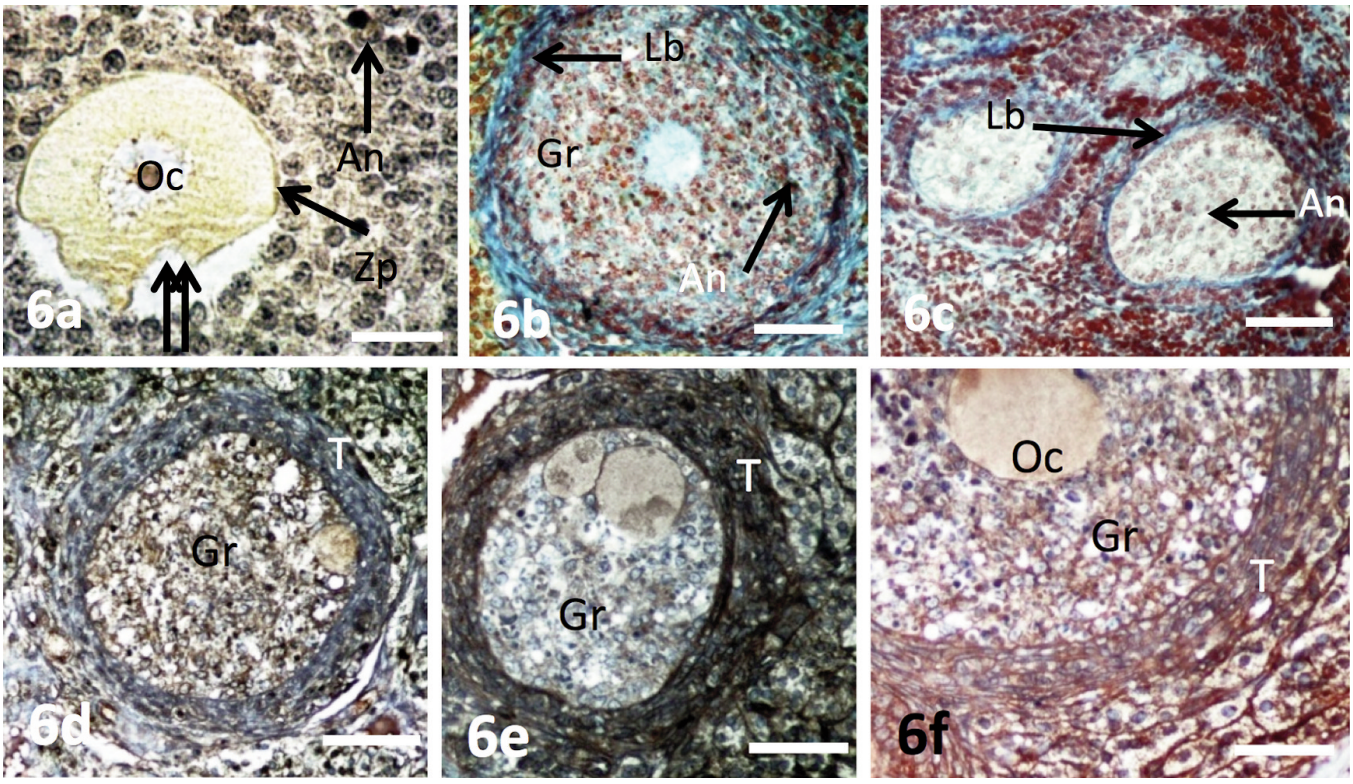

Fig. 6. Histology and immunohistochemistry in several atretic follicles in adult *Meriones libycus* ovaries during seasonal reproductive cycle. Atretic secondary follicles with early and late atresia. **6a.** Early retraction of the whole oocyte-zona pellucida (double arrows). **6b.** Advanced atresia: presence of many apoptotic nuclei (An) in granulosa (Gr) with fibrous lamina basalis (Lb). **6c.** In late atresia, granulosa (Gr) completely disappeared, and few apoptotic nuclei remained. **6d.** Vizualisation of estradiol and progesterone with immunohistochemistry in active and resting periods; signal is weak in non apoptotic granulosa cells (Gr); signal is negative in theca cells (T). **6e.** Visualization of testosterone during active season; immunostaining is absent or very weak in the granulosa cells; it is positive in theca cells (T). **6f.** Visualization of P450 arom in active and quiescent seasons; an important immunostaining was observed in both non apoptotic granulosa cells (Gr) and theca cells (T). 6a: Masson's trichrome. 6b, 6c: Romeis's azan. 6d-f: immunohistochemical results. Lb: Lamina basalis; Oc: Oocyte; Zp: Zona pellucida. Bars: 6a, 10 μm; 6b-6f, 25 μm;

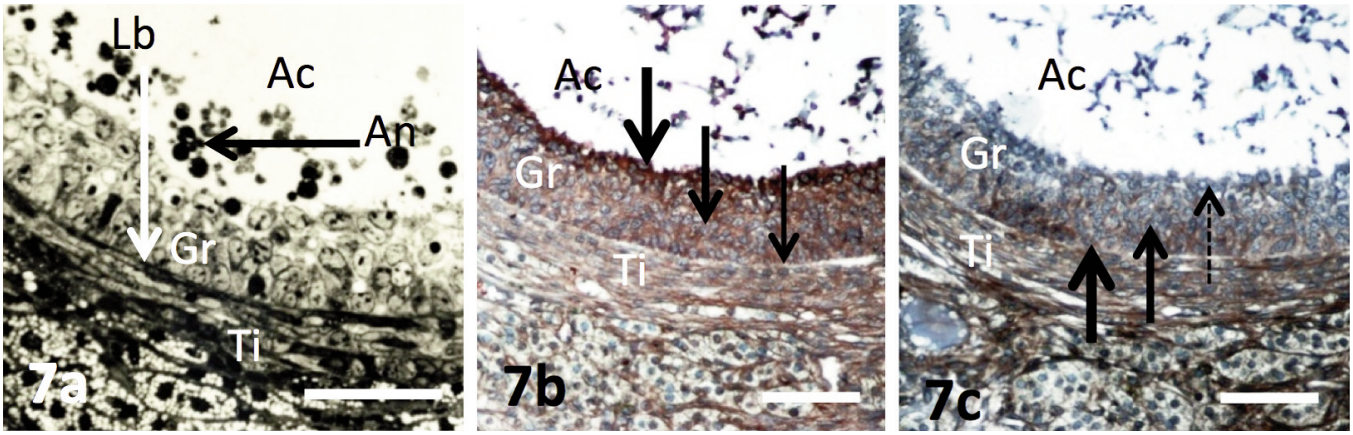

Fig. 7. Histology and immunohistochemistry in several atretic follicles in adult *Meriones libycus* ovaries during seasonal reproductive cycle. Atretic antral follicle. **7a.** In early atresia, note the thin condensation of granulosa cells (Gr) and pyknotic and apoptotic nuclei (An). Lamina basalis (Lb) is intact. **7b.** Visualization of progesterone with immunohistochemistry; the same signal of staining for estradiol and P450 arom was observed, note that gradients of staining for those antibodies within the granulosa layer were reversed (arrows) in comparison with healthy antral follicles. **7c.** Visualization with testosterone, a positive immunoreactivity manifests during active season with reverse gradient of staining (arrows); in quiescent period those follicles showed a negative signal to testosterone. 7a: Semithin section, Methylen blue-AzurII. 7b, 7c: immunohistochemical results. Ac: Antral cavity; Lb: Lamina basalis. Bars: 7a, 30 μm; 7b-7c, 25 μm.

with healthy antral follicles; indeed the immunoexpression of the early antral atretic follicle was more important in antral granulosa cells than mural granulosa cells, became lessened. At this stage, contrarily to testosterone, the mural granulosa showed a positive signal, but the antral granulosa exhibited a heterogeneous cell population with faint and/or the absence of staining (Fig. 7c). During resting season, and in early diestrus, estrus and metaestrus, all the atretic follicle stages did not show positive labeling for testosterone, comparable to what was expressed in late diestrus and proestrus phases.

In the mid-atretic follicle, histological examination showed that mural granulosa and cumulus cells were dissociated and disorganized; the number of pyknotic granulosa nuclei was maximal. In antral cavity, cellular debris was abundant. Sometimes lamina basalis was no longer observed. Theca interna lost distinct layered structures and exhibited morphological changes with some degree of hypertrophy (Fig. 8a). At this stage, in both periods, signals for progesterone, estradiol and P450 arom became very weak in the degenerative granulosa cells and theca interna (Fig. 8b). Testosterone immunoreactivity appeared strong in the theca interna during active season (Fig. 8c).

In the late atretic follicle, granulosa cells completely degenerated and were eliminated. However, theca interna was enlarged (Fig. 9a). At this stage, in both periods, signals for progesterone, estradiol, testosterone and P450 arom were negative (Fig. 9b). No immunoreaction was detected in any of the negative controls prepared by omission of the primary antibody.

Discussion

Major functions of the mammalian ovary are the

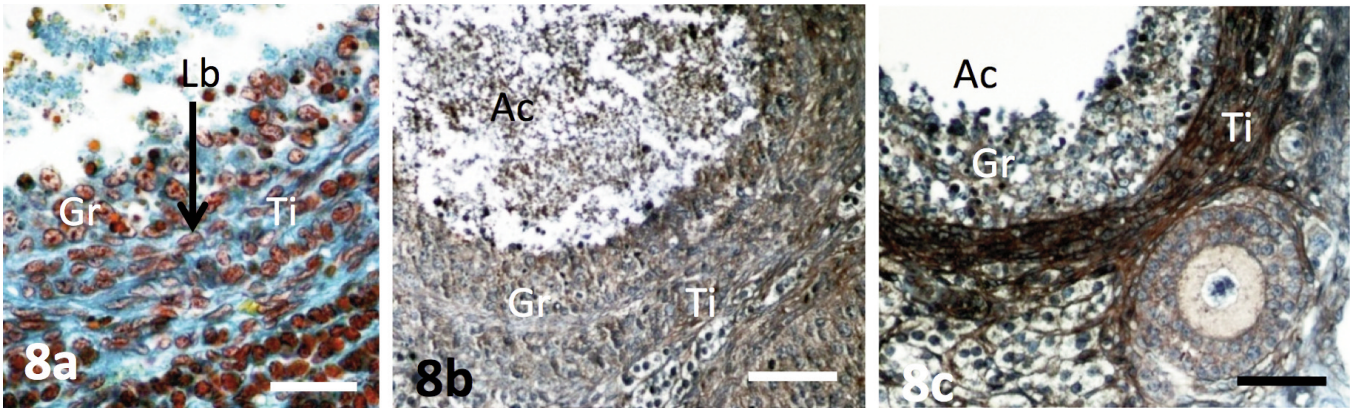

Fig. 8. Histology and immunohistochemistry in several atretic follicles in adult *Meriones libycus* ovaries during seasonal reproductive cycle. Mid-atretic antral follicle. **8a.** The lamina basalis (Lb) disappeared; loosened or dissociated granulosa cells (Gr). **8b.** Visualization of progesterone with immunohistochemistry; the signal is very weak in degenerative granulosa cells and enlarged theca interna; the same immunohistochemical signal was observed for estradiol and P450 arom in active and quiescent periods. **8c.** Visualization of testosterone with immunohistochemistry during active season; the label is strong in the theca interna (Ti); in non apoptotic granulosa cells, a positive signal manifests; during resting season, testosterone is not detected. 8a: Romeis's azan. 8b, 8c: immunohistochemical results. Ac: Antral cavity; Lb: Lamina basalis. Bars: 8a, 10 μm; 8b-8c, 24 μm.

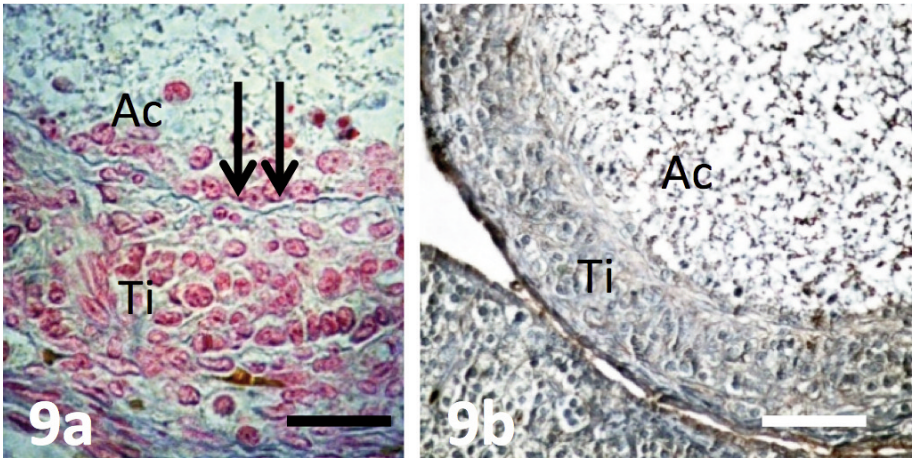

Fig. 9. Histology and immunohistochemistry in several atretic follicles in adult *Meriones libycus* ovaries during seasonal reproductive cycle. Late antral follicular atretic. **9a.** Granulosa completely disappeared (double arrows); maximal hypertrophy of thecal cells (Ti). **9b**. Granulosa layer is completely eliminated; no label with different antibodies was observed in enlarged theca interna (Ti). 9a: Romeis's azan. 9b: Immunohistochemical results. Ac: Antral cavity. Bars: 9a, 10 μm; 9b, 25 μm.

differentiation and release of the mature oocyte for fertilization, and successful propagation of the species. The structural unit and functional basis of the ovary is the ovarian follicle, which consists of three major cell compartments: the oocyte, the inner granulosa cells and the outer thecal cells. Follicles develop through primordial, primary and preovulatory stages, after which ovulation occurs and residual follicular cells luteinize to form corpora lutea (Fauser and Van Heusden, 1997). Specific morphological and functional changes are observed during this development (Hirshfield and Midgley, 1978; Roy, 1994). Regulation of the proliferation, cytodifferentiation and atresia, associated with folliculogenesis, are controlled by circulatory feedback between the hypothalamic-pituitary axis and the paracrine/autocrine factors originating from the follicle itself (Hirshfield, 1991; Greenwald and Roy, 1994; Zeleznik and Benyo, 1994; Richards et al., 1995; Gougeon, 1996). Our present histological observations indicate that adult female *Meriones libycus* is a seasonally breeding animal and shows significant changes in ovarian morphology. In spring and early summer, the reproductive function is restored. The ovary is characterized by cyclical activity; after ovulation, broken follicles and corpora lutea are observed. Various stages of folliculogenesis developing from primordial to preovulatory follicles present similarity to that described in mouse (Allen, 1922; Pederson and Petters, 1968), rat (Singh and Gouraya, 1993), hamster (Norman and Greenwald, 1971), Mongolian gerbil (Norris and Adams, 1974), sand rat (*Psammomys obesus*) (Boubekri, 1998; Boubekri et al., 2007) and woman (Familiari et al., 1989; Gougeon, 1996). The contrary occurs during a long period of quiescence, the ovary is characterized by interrupted cycle; anovulation is observed without any corpus luteum; antral follicle that does not mature, becomes atretic. The information regarding histological description in the ovary at this period is very limited. In mammalian species submitted to seasonal variations of reproductive activity, Thibault and Levasseur (2001) demonstrated that gametogenesis was suppressed during sexual period; at this phase, the atrophy of ovaries is observed and folliculogenesis is blocked at primordial follicle stage. According to Delost (1968), a comparable result is found in many wild non hibernant rodents. *Psammomys obesus*, a diurnal Saharan rodent sharing the same habitat with *Meriones libycus* showed a very short resting season in summer (Khammar, 1987). At this period, folliculogenesis seems normal but neither broken follicle nor corpora lutea are observed in the ovaries and follicular development is slowing down (Boubekri, 1998). In *Meriones libycus*, it is therefore possible that during the quiescent phase, seasonal anovulation, and the presence of all antral follicles which did not ovulate, are controlled by environmental factors which may cause effects on the activity of the hypothalamic-pituitary-gonadic axis. However, Shanas and Haim (2004) demonstrated that under an effect of water restriction, the ovaries of golden spiny mouse (*Acomys russatus*) exhibited an increased number of atretic follicles, and the uteri and body masses decreased significantly. Compared to female Shaw's Jird, neither photoperiod nor free-water deprivation affected reproductive status (El-Barky et al., 1999). Desert environments are unpredictable; therefore, opportunism is more likely to be a strategy adopted by desert rodents to regulate their reproductive function.

The ovarian sex steroidogenic pathway gives rise to three major classes of steroid hormones: progestins, androgens and estrogens. In *Meriones libycus* ovaries, the steroidogenesis and aromatase presence were examined by immunohistochemical (IHC) approach. In the breeding season, 17ß estradiol, testosterone and P450 arom were detected within the granulosa and theca cells in all the healthy follicles. In preantral follicles, the presence of both estradiol and P450 arom in all cells, as well as testosterone in thecal cells only, suggested that estradiol was implicated in paracrine regulation to modulate follicular growth at this stage. In some large antral follicles, theca interna displayed a strongly immunolabeling of testosterone; in rat antral follicles, theca interna is the site of follicular androgen output, which is utilized for conversion to estrogens (Tetsuka et al., 1995). In these follicles, immunopositivity of estradiol and P450 arom in the cytoplasm of the granulosa cell layer, and their absence in theca interna, show a functional differentiation associated with cellular cooperation in the biosynthesis of estradiol; however, the presence of nuclear immunoreactivity in some cells of the granulosa probably demonstrates an autocrine action. Our observations were supported by data from human (Tamura et al., 1992), rat (Ishimura et al., 1989), hamster (Tsuri et al., 1992) and sand rat (Boubekri et al., 2004, 2009; Boubekri, 2010). Our study showed a gradient of immunoreactivity for P450 arom decreasing from the peripheral granulosa to antral granulosa cells. These observations are consistent with the data of Ford and Lunstra (1992), Irian and Hodgen (1992) and Tsafriri and Adhasi (1994) who found that granulosa cells collected near the lamina basalis produced more estradiol than those close to the antrum; indeed, these findings coincide with our results showing a comparable gradient of immunoexpression of estradiol within the granulosa layer in *Meriones libycus*. This heterogeneous labeling of estradiol in those cells is also found in the sand rat (Boubekri et al., 2009; Boubekri, 2010). Similar heterogeneity in granulosa populations of P450 arom was noted in cow (Roberts and Echternkamp, 1994) and rat (Kasson et al., 1985). In other antral follicles, the estradiol and P450 arom were found both in granulosa cells and theca interna. Similar observations were reported by Boubekri et al. (2004, 2007, 2009), Boubekri (2010) in sand rat, Lautincik et al. (1994) and Shores and Hunter (1999) in pig, Matsuda et al. (1994) in mouse and hamster. The immunoreactivity of P450 arom in theca interna would suggest the involvement of this compartment in estradiol biosynthesis. It is possible that the thecal estradiol contribution is vital to ensure the

synthesis of a sufficient quantity of estradiol to trigger the LH surge. Our current data also showed estrogen and P450 arom labeling in cytoplasm of oocytes in *Meriones libycus* in all follicle stages, suggesting that oocytes produced estrogens, in accordance with Suzuki et al. (1984) and Tamura et al. (1992) who showed respectively the presence of estrogen in hamster oocytes and P450 arom in human oocytes, while Nekola and Nalbandov (1971) suggested that oocyte secreted factors that inhibited luteinization of granulosa cells. Since granulosa cells lack the ability to produce estrogen in early developing stage, it is assumed that estrogen produced from oocytes is necessary in the early stage of follicular and oocyte development. In the large preantral follicle, according to Magoffin (1991), initiation of progesterone biosynthesis was demonstrated by an intense immunopositivity in the theca cells and a slight labeling of progesterone in granulosa. In growing antral follicle, progesterone remained weak in the granulosa layer. According to the works of Kamada et al. (1997), progesterone regulates the action of estrogen through the decrease of estrogen receptor and down regulation of its own receptor in granulosa cells. In these follicles progesterone immunoexpression was obviously exhibited in the theca interna; this finding suggested that cells of theca became functional and that progesterone secretion is the most important of the corpus luteum steroids (Richards et al., 1986). Indeed, *Meriones libycus* corpus luteum secretes both estradiol and progesterone. Our results are consistent with data from Boubekri et al. (2009) and Boubekri (2010) in the sand rat. In comparison with the healthy preantral follicle in resting phase, the immunopositivity of estradiol, progesterone and P450 arom became less pronounced in both granulosa and thecal cells. It seems that the steroid biosynthesis was reduced and exerts a paracrine action in these follicles.

Recent studies have suggested that follicular atresia, a degenerative process in the mammalian ovary mediated by apoptosis (Kaipia and Hsueh, 1997) and regulated by hormones (Hsueh et al., 1994), was chararacterized by the internucleosomal fragmentation of cellular DNA (Billig et al., 1993). In these follicles, the granulosa layer had thinned considerably and became disorganized and dissociated, and apoptotic bodies were abundant. The lamina basalis was interrupted with the progression of atresia; the granulosa layer was virtually absent, and thecal cells hypertrophied. Similar observations were done in the mouse (Byskov, 1974), rat (Peluso et al., 1980; Gondos, 1982; Navarro et al., 2005), hamster (Greenwald, 1973), guinea pig (Rawson et al., 1979), bovin (Yang and Rajamahendran, 2000), and human (Yuan and Giudice, 1997). Previous data indicated that in atretic and cystic follicles of pigs (Kyan et al., 1996) and rat (Machell et al., 2000; Baravalle et al., 2006), the loss of granulosa cell adhesion disrupted contact between cells and promoted apoptosis (Peluso et al., 1996). Also, Kidder and Mhaw (2002) and Gittens et al. (2003) demonstrated that the structural integrity of ovarian folliculogenesis and production of fertilizable oocytes depended on gap junctional intercellular communication within both the developing and mature follicles; indeed, an impairment in gap junction or reduced number of them caused by absence of FSH (Amsterdam and Rotmensch, 1987; Hsueh et al., 1994) resulted in extrusion of the oocyte from its original follicular structure (Cortvrindt et al., 1997). Therefore, the previous findings in mouse (Cortvrindt et al., 1997; Baker and Spears, 1997), rat (Chun et al., 1996; McGee et al., 1997), and human (Abir et al., 1997) showed that FSH participate in maintaining healthy oocyte growth and follicular structure, also inhibiting apoptosis. Braw et al. (1981), Tsafriri and Braw (1984) showed that abolition of gonadotropin either by hypophysectomy or pentobarbital treatment, resulted in atretic changes in large antral follicles. In this sense, the most probable explanation for the seasonal anovulation and all non-ovulated antral follicles cell proliferation and differentiation would be either changes on the levels of circulating gonadotropins or modifications of the intraovarian microenvironment needed for a normal development. However, an immunohistochemical study carried out of pituitary gonadotropins in adult female wild *Meriones libycus* (unpublished data, Smaï et al.) demonstrated that seasonal variations of both FSH and LH cells were correlated with the variations of gonadic activity during the seasonal reproductive cycle: an important reduction in number of these cells was detected in resting phase compared to the active season; it seems that in sexual quiescence of *Meriones libycus*, a decrease of both FSH and LH levels in plasma, influenced by environmental factors, probably did not attain a significant level of activity. Consequently, it prevented ovulation and caused cell death in follicles. Our present study indicates that in both periods the intensity of labeling in the antral atretic follicles decreased progressively when stages of atresia advanced until the interruption of steroid hormones and P450 arom biosynthesis; indeed, in late atresia, theca interna remains the most resistant portion and morphological alterations may not occur until granulosa cells are affected. We suggest that theca cells, when they are associated with another compartment like the oocyte or extracellular matrix, may play an important role in antral follicular development and in controlling granulosa cell apoptosis. Yada et al. (1999) and Tadjima et al. (2002) demonstrated in bovine that theca cells regulate the fate of granulosa cells through the follicular maturation process by secreting paracrine factors that suppress apoptosis.

In summary, during the seasonal reproductive cycle, the ovarian function of *Meriones libycus* changes with environmental conditions. In resting season (from the end of summer until winter), food and hydrous resources are scarce, seasonal anovulation was observed, and all non-ovulated antral follicles became atretic; follicular cell deaths were numerous. Immunohistochemical examination indicates that in breeding season (spring

and beginning of summer), when food is abundant and temperature comfortable, steroid hormones and P450 arom present important activities in comparison to the resting phase.

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