Original Article

Studying the Dynamic Process of Rat Ovary Through Histological Sections and Cell Culture

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ABSTRACT

Background: Primordial follicles, which are the structural units of the ovary and represent the ovarian reserve, continue their development by entering into paracrine, endocrine, and cell-cell interactions with the structures and cells in the niche.

Objectives: To examine the development of cells and structures in the ovary in cell culture medium and histological sections and observe the effects of the niche they formed on folliculogenesis.

Methods: Three 3-week-old as the first group for cell culture, and three 8- to 10-week-old (early reproductive period) and three 12- to 14-month-old (late reproductive period) Wistar albino rats as the second and third groups for histologic sections were included in our study. To prepare histological sections, ovarian tissues of rats in the early and late breeding periods were excised and passed through routine histological tissue process steps. Additionally, ovarian surface epithelium proliferating together with ovarian stromal cells in explant cell culture was isolated and multiplied.

Results: Ovarian surface epithelium and stromal cells were observed to proliferate in cell culture. Primordial follicle-like structures were observed between the surface epithelial cells. Histological examination revealed periovary adipose tissue around the ovary, separated from the surface epithelium by a thin fibrous sheath. It was observed that primordial follicles were located in the tunica albuginea layer. The fibroblast-like mesenchyme in the tunica albuginea of the corpus luteum formed after ovulation thickens this layer by the proliferation of cells and creates a suitable environment for developing primordial follicles. Also, the tubules of the rete ovarii, which emerge during the formation of the genitourinary system in the embryological period, continue to exist in the ovaries and mesovarium in rats in the early and late reproductive period.

Conclusion: The presence of tubules of the rete ovarii in the ovary and mesovarium during the reproductive period may indicate that these structures are also important in folliculogenesis.

Keywords: Epoophoron structures, Folliculogenesis, Graaf follicle, Ovary, Rete ovarii

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Introduction

The ovary consists of the cortex and medulla layers. The folding of the ovary during the embryonic period contributes to the formation of these layers. The border between the medulla and cortex is not clear. The surface of the ovary is a single-layered cuboidal epithelium and pseudostratified epithelium in some areas. In some other areas, it is paved with squamous epithelium, and a basement membrane is underneath. The cellular layer, the germinal epithelium, continues with the mesothelium outside the ovary (Auersperg et al., 2001; McKey et al., 2022). Tunica albuginea, an irregular tight connective tissue layer, is located between the surface epithelium and cortex, and there are fibroblast-like mesenchyme cells within the layer. The tunica albuginea also contains a significant amount of type I and type III collagen fibers (Lind et al., 2006; Can, 2014).

Ovarian follicles are located in the stroma of the cortex. Follicles, as the structural units of the ovary, form a microenvironment for oocytes (Ross & Pawlina, 2006). The development of follicles in the ovary occurs with cell-cell interactions, endocrine, autocrine, and paracrine factors, especially follicle-stimulating hormone (FSH) secreted from the pituitary gland. The most common type of follicle in the ovary is the primordial follicle, which represents the ovary's reserve. Primordial follicles contain a primary oocyte surrounded by a single row of squamous follicle cells. After puberty, primordial follicles are selected and begin to grow. They continue to develop as primary, secondary, and then Graaf follicles (Ünal & Seçme, 2022). The medulla layer, surrounded by loose connective tissue, has blood, lymph vessels, and nerves (Kinnear et al., 2020). In rats, parts of the ovary have a thin fibrous sheath with fibroblasts exactly next to the surface epithelium. This fibrous sheath is located between the surrounding adipose tissue (peri-ovarian adipose tissue) and the ovarian surface epithelium. It has been reported that peri-ovarian adipose tissue provides the development of follicles in the ovary. It has been shown that this tissue is important for the functions of the ovary. Humans have no periovarian adipose tissue (Yang et al., 2018; Zhu et al., 2020; Zhang et al., 2021; Ünal & Seçme, 2022).

Ovulation is the process of releasing the secondary oocyte from the Graaf follicle. After ovulation, the basement membrane is destroyed, and the follicle's granulosa and theca interna cells rearrange to form a temporary gland called the corpus luteum (Ross & Pawlina, 2006).

The ovary is a dynamic complex organ that develops follicles according to estrus stages. We aim to examine the development of cells and structures in the ovary in cell culture medium and histological sections and to observe the effects of the niche they formed on folliculogenesis.

Materials and Methods

Three 3-week-old Wistar albino rats as the first group for cell culture, and three 8- to 10-week-old and three 12- to 14-month-old Wistar albino rats as the second and third groups for histologic sections were included in our study. For cell culture, prepubertal rats were selected that had not yet entered the estrous cycle and in which antral follicles had not been seen. Experimental steps of the study were carried out using the Pamukkale University Experimental Surgery Application and Research Center and Cell Culture Laboratory infrastructure of the Histology-Embryology Department.

Cell culture process

The ovaries of three prepubertal (3 weeks old) female rats were collected under sterile conditions. First, the surrounding adipose tissue was excised under a stereo microscope. Then, the ovarian tissue was divided into small pieces in a petri dish, and an explant cell culture was created. In mixed cell culture, surface epithelial cells proliferating with ovarian stromal cells were locally labeled, and these cells were isolated by applying local trypsinization.

After the adherent of the ovarian surface, epithelial cells proliferated and became confluent (70%-80%); the cells were removed with trypsin enzyme 0.25% (Hyclone, USA) and inoculated into two new flasks with a complete medium. The complete medium contains Dulbecco's modified Eagle's medium (DMEM) (Capricorn Scientific, Germany), fetal bovine serum (FBS) (Capricorn Scientific, Germany), and penicillin-streptomycin (Pan Biotech, Germany). Since the cells proliferated rapidly, the flasks' media were changed daily, creating optimal culture conditions. To evaluate the viability of proliferating cells, they were stained with Trypan blue, and the number of cells was determined with the Neubauer improved counting chamber. All these stages were done using an inverted microscope (CKX41 Olympus, Japan).

Preparing tissue sections and taking histological images

Ovarian tissues of rats in the early breeding period (8-10 weeks old) and rats in the late breeding period (12-14 months old) were excised, and routine histological tissue process steps were performed. Serial sections with 5-µm thickness were taken from paraffin blocks by a microtome. Images of ovarian tissue sections stained with hematoxylin-eosin and Masson's trichrome were taken under a light microscope (BX51 Olympus, Japan).

Results

Cell culture

Cells began to migrate approximately 3 days after tissue fragments were taken from the ovary. On the seventh day, ovarian stromal cells and surface epithelial cells (mixed cell culture) filled the petri dish. The morphological appearance of the ovarian stromal cells was observed to be fibroblast-like cells, and the ovarian surface epithelium was in the form of cobblestone. In addition, primordial follicle-like structures were observed among the ovarian surface epithelial cells (OSE) (Figures 1 and 2). Counting under phase contrast microscopy revealed that 1.5×10^6 and 2×10^6 OSE were grown in the culture dishes.

Histological view

The ovarian surface epithelium was single-layered squamous epithelium in some areas, single-layered cuboidal epithelium in others, and stratified in some places. While the ovarian surface epithelium generally develops as a single-layered squamous and cuboidal epithelium in early breeding period rats (second group), it is stratified cubic epithelium in some regions in late breeding period rats (third group) (Figure 3). Also, the peri-ovarian adipose tissue adjacent to the ovary's surface epithelium forms the oocytes' macro environment (Figure 4).

When the corpus luteum was formed from the ovule Graaf follicle, it was seen that the volume of this structure increased considerably. However, at this time, the surface epithelium is flat, with few in cell number, and the tunica albuginea, located under the surface epithelium, is quite thin. Later, it was observed that fibroblast-like mesenchyme cells in the surface epithelium and tunica albuginea proliferated and showed a concentric arrangement by increasing the amount of collagen fibers they contained. The squamous surface epithelium proliferates and turns into cuboidal epithelium in some places. The tunica albuginea layer thickens, new vascular structures (capillary vessels, venules) are formed and restructured, and primordial follicles are seen in this reconstructed tunica albuginea layer (Figures 5 and 6).

The expanding tunica albuginea layer adjacent to the corpus luteum provides a favorable environment for developing follicles in this region. In addition, primordial follicles were found not only in the tunica albuginea of the corpus luteum but also in the tunica albuginea layer of the entire ovary. In some areas, the tunica albuginea makes crypt-like extensions into the cortex with the ovarian surface epithelium. Primordial follicles were also observed in these regions in the sections. In some cases, after ovulation, some of the ovarian surface epithelium invaginates into the cortex region and continues its existence with parts of the tunica albuginea. Primordial and primary follicles, or inclusion cysts, were also seen in these structures.

It was observed that spiral arterioles were common in the whole ovary (cortex and medulla layer). Also, it has been seen that the follicles developing in some corpus luteum structures form a cystic appearance in some places (Figure 7).

The rete ovarii found in the ovaries exist in both early-breeding period rats and late-breeding period rats (Figure 8). In the medulla layer of the ovary, vascular structures (small arteries-arterioles and small veins), adipose tissue (adipocyte cells), and primordial-primary follicles were observed, albeit very rarely.

As a result of apoptosis of granulosa cells in atretic follicles that cannot continue their development, the theca interna layer dominates. These structures, which are turned into the thecal glands, are located in the cortex and medulla layer. Apart from these, they have been seen in the medulla layer of the ovary in structures called ganglia, which belong to the autonomic nervous system (Figure 9).

Discussion

Primordial germ cells originate from the epiblast, migrate along the primitive streak, and embed in the endoderm cells in the wall of the yolk sac near the allantois in the third week. In the fourth week, they move along the dorsal side of the hindgut mesentery with ameboid movements and reach the primitive gonads at the beginning of the fifth week, where they are called oogonium. The primary sex cords extend from the coelomic epithelium into the mesenchyme. Later, the cells in the primary

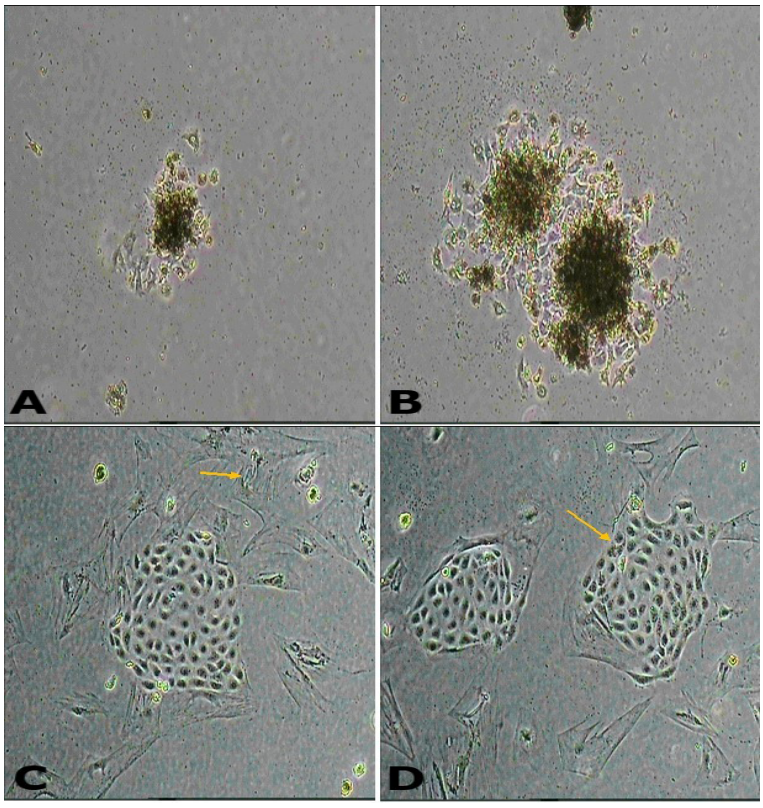

Figure 1. Migration of cells from tissues (A and B) and ovarian stromal cells and ovarian surface epithelium (C and D) (X10)

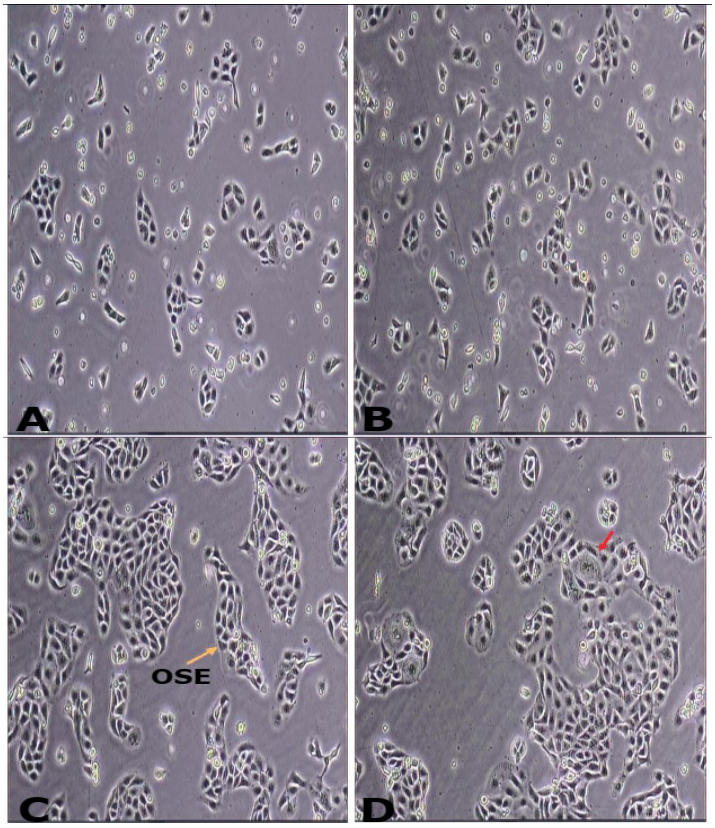

Figure 2. Proliferation of ovarian surface epithelium (A, B, and C) and primordial follicle-like formed between cells (D) (X10)

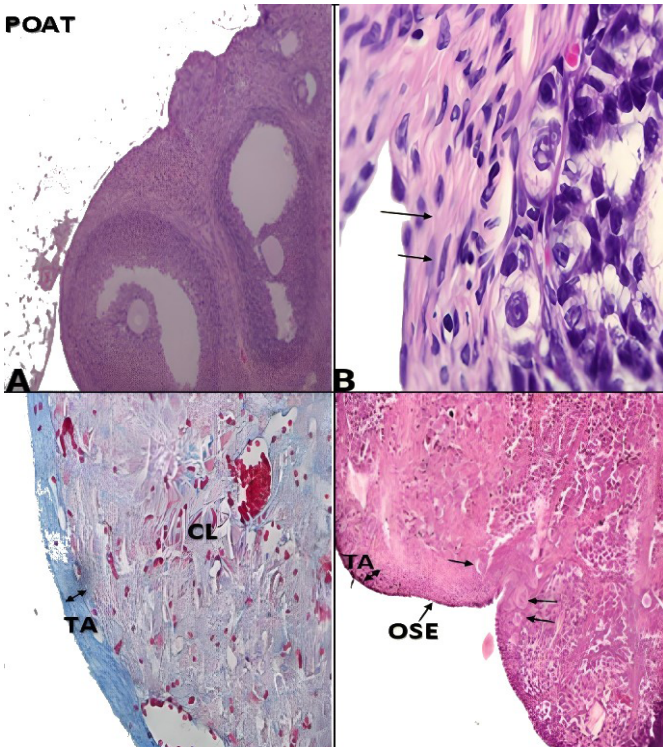

Figure 3. Ovarian surface epithelium (OSE) (X100)

A) Single-layered squamous epithelium B) Single-layered cuboidal epithelium, C and D) Multilayered epithelium

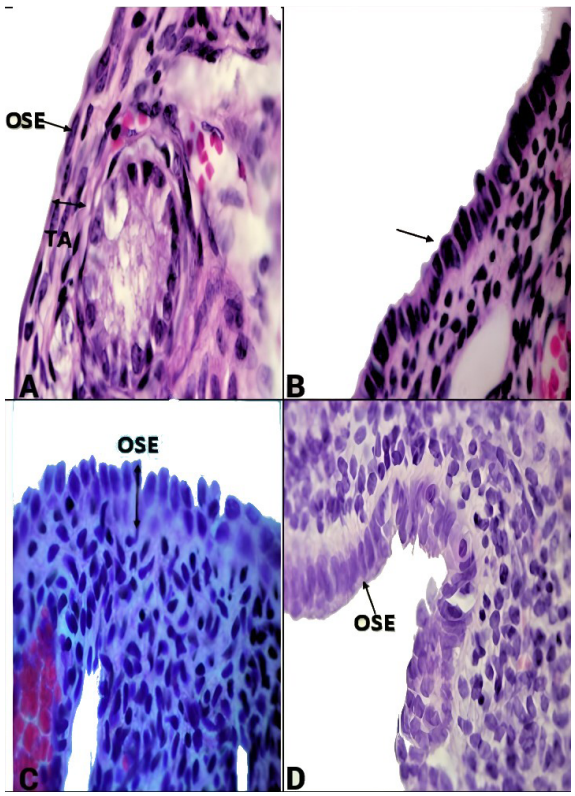

Figure 4. Localization of periovarian adipose tissue (POAT), tunica albuginea, and primordial follicles around the ovary

A) POAT (X10), B) Fibroblast-like mesenchyme cells and concentrically arranged collagen fibers in the tunica albuginea (X100), C) Tunica albuginea layer of corpus luteum (Masson's trichrome) (X20), D) Primordial follicles in the tunica albuginea layer (X20)

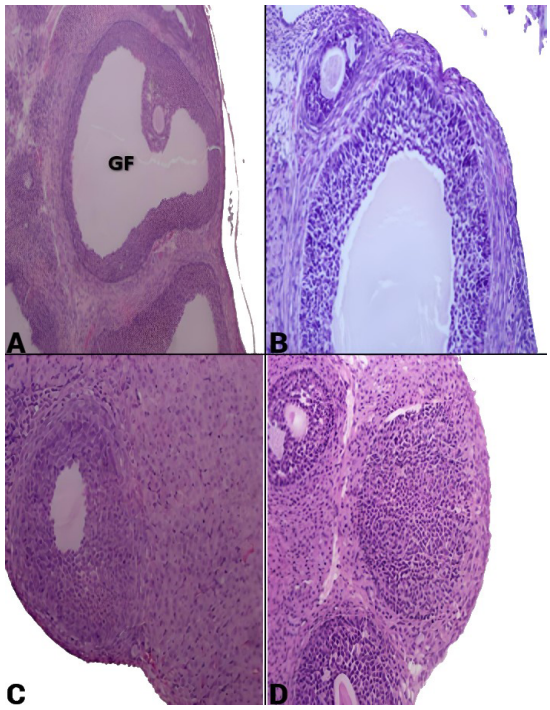

Figure 5. The process of Graafian follicle to corpus luteum formation

A) Graafian follicle (X10), B) Ovule graaf follicle (X100), C) Enlargement and proliferation of granulosa cells (X10), D) Granulosa cells proliferated and completely closed the lumen (X10)

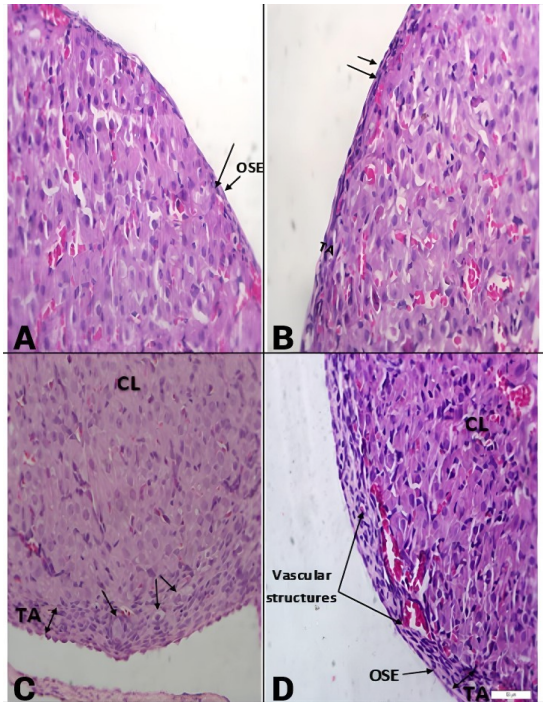

Figure 6. Appearance of the ovarian surface epithelium, corpus luteum, adjacent tunica albuginea, and follicles within the tunica albuginea (X20)

A and B) The appearance of the surface epithelium and tunica albuginea in the corpus luteum, the surface epithelium being of the squamous epithelial type, and the tunica albuginea composing of 1-2 rows of fibroblast-like mesenchyme cells; C) Follicles in the tunica albuginea beginning to thicken; D) Fibroblast-like mesenchyme cells in the tunica albuginea proliferate and thicken this layer, containing vascular structures

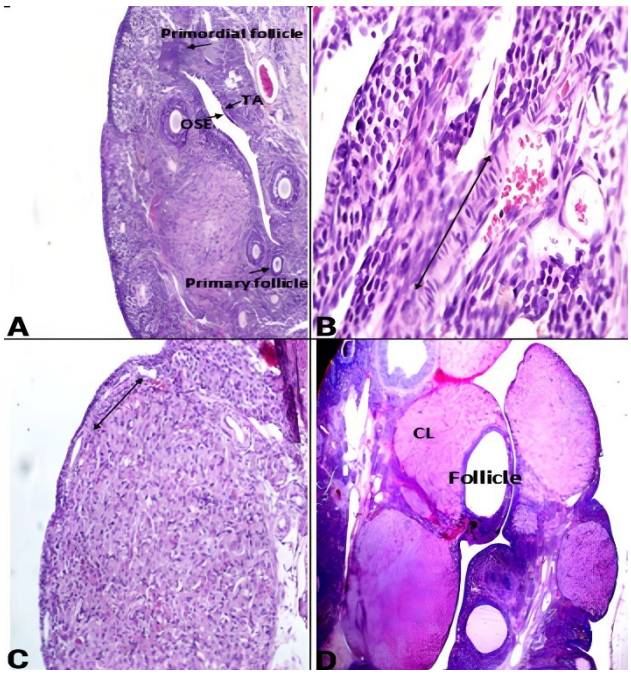

Figure 7. Appearance of inclusion cysts, spiral arteriole and corpus luteum cysts in the ovary

A) Invaginated surface epithelium forming inclusion cysts after ovulation and a part of the tunica albuginea (X10); B and C) Spiral arterioles (X40- X20), D) Follicle developing inside the corpus luteum and making cystic appearance (X4)

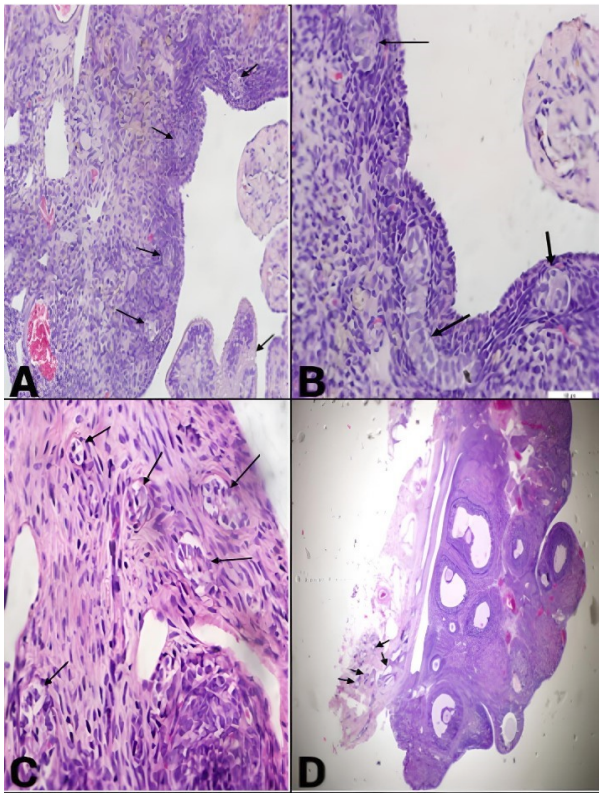

Figure 8. Appearance of the rete ovarii structures in the mesovarium and ovary

A and B) In the cortex layer of the ovary (X10- X20); C) In the medulla (X20), D) In the mesovarium rete ovarii and epoophoron structures (X4)

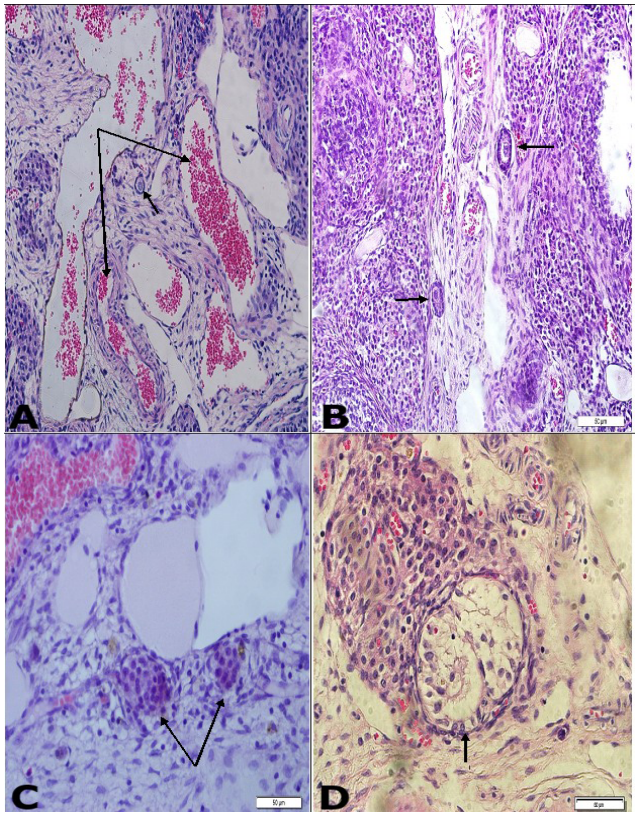

Figure 9. Appearance of vascular structures, follicles, atretic follicles and ganglion structures in the medulla layer of the ovary

A) Primordial follicle, adipose tissue, and vascular structures in the medulla layer (X20), B) Primary follicles in the medulla layer (X10), C) Atretic follicles in the medulla layer (X20), D) Ganglion structure in the medulla layer (X40)

sex cords degenerate and are replaced by the secondary sex cords surrounding the oogonium (Sadler, 2022).

Rete ovarii develops due to the differentiation of mesonephric cells that migrate to the developing gonad during the embryological period. Rete ovarii is the homolog of the male rete testis. Rete ovarii usually appear as groups of anastomotic tubules lined with cuboidal or columnar epithelium. Rete ovarii undertakes important functions in the control of meiosis in the ovary. Studies have shown that in the embryonic period, with the arrival of mesonephric-origin somatic cells that make up the rete ovarii, some germ cells enter meiosis. Observation of secretory substances in the lumen of the rete tubules may indicate that they have a secretory ability. Three different parts have been described in the rete ovarii: Extraovarian rete, connecting rete, and intraovarian rete. We have little information about the function and structure of the rete ovarii throughout adult life. Studies show that this structure is not a vestigial part but is a dynamic system that regulates the functions of the ovaries in the postnatal period (Wenzel & Odend'Hal, 1985; McNatty et al., 1995; Smith, 2012; Apperson et al., 2017; Mfoundou et al., 2021).

The epoophoron, another structure that develops in the embryological period, remains in the mesovarium between the ovary and the tuba uterine as mesonephric duct remnants. The epoophoron, which consists of several blind-ending tubules and ducts, is the equivalent of the ductuli efferentes and epididymis in males (Moore et al., 2008; Apperson et al., 2017).

Signaling pathways have activating and suppressive roles in transitioning from the primordial to the primary follicle. They protect the follicle pool so that it can operate independently and in a balanced way. In cases where the balance is disturbed, massive activation of the follicles and premature depletion of the follicle pool occur (Kabasakal, 2023). Primordial follicles, which are intensely located in the cortex and medulla of the ovary until the first four weeks of birth in rats, begin to appear mostly in the cortex region and in the tunica albuginea layer, which is more resistant to environmental factors during the reproductive period (Picut et al., 2015).

During ovulation, collagen fiber bundles in the theca externa and tunica albuginea layers continue the collagenolysis. The proteolytic activity in the ovarian surface

epithelium contributes to the restructuring and breakdown of the ovarian surface epithelium and the ovarian cortex during ovulation. Ovarian surface epithelium produces proteolytic enzymes such as urokinase plasminogen activator (uPA) and matrix metalloproteases 2 and 9 (Okamura et al., 1980; Ahmed et al., 2007). In addition, matrix metalloproteinases 1 and metalloproteinases 3 are proteolytic enzymes involved in the remodeling of the extracellular matrix of the ovary in the menstrual cycle (Bogusiewicz et al., 2000). In some cases, after ovulation, a part of the ovarian surface epithelium invaginates towards the cortex region and forms inclusion cysts by maintaining its presence together with parts of the tunica albuginea (Auersperg et al., 2001; Ahmed et al., 2007). The autonomic ovarian plexus innervates the ovaries and receives sympathetic and parasympathetic nerves. Parasympathetic ganglion cell groups in the ovary are distributed in the medulla (D'Albora & Barcia, 1996; Pastelín et al., 2017).

Gonadotropin-releasing hormone (GnRH) is released in a pulsatile way from the medial basal hypothalamus region and sent to the pituitary portal system. In this way, GnRH causes the release of FSH and luteinizing hormone (LH) from the anterior pituitary gland into the systemic circulation. While FSH and LH ensure follicle growth, ovulation, and corpus luteum formation in the ovary, they are also responsible for the coordinated release of hormones such as estradiol, progesterone, and inhibin from the follicles (Dadashpour Davachi et al., 2022).

There are periovarian and intraovarian adipose tissues in the ovary. Periovarian adipose tissue has been shown to play an important role in follicular development and whole-body lipid metabolism. Leptin and adiponectin are adipokines secreted from adipose tissue and play an important role in energy metabolism. Studies are investigating the potential of adiponectin, which is known to have effects on reproductive function, as a biomarker in some pregnancy-related disorders (such as gestational diabetes, preeclampsia, premature birth, and abnormal intrauterine growth) (Ketaby et al., 2023).

Polycystic ovary syndrome (PCOS) is a disease that causes infertility and leads to low oocyte and embryo quality. The incidence of ovarian hyperstimulation syndrome (OHSS) in patients with PCOS is considerably higher than in patients without PCOS. OHSS is a serious complication that poses a threat to patients undergoing ovulation stimulation. In PCOS, thickening of the tunica albuginea layer of the ovary is observed (Alhilali et al., 2022). In their experimental animal study, Omairi et al. (2022) investigated the histologic changes of commonly used drugs in ovarian tissue and the effects of these drugs on follicle structures. In another study, Elahinia et al. (2023) examined the changes in the effects of the drugs administered depending on the phases of the estrous cycle. As a result of the study, they found that the drugs showed different effects depending on the stages of the cycle. Fazlelahi et al. (2023) found the optimal dose of different drug doses depending on the ovary's estrous cycles. In our study, we tried to show the histologic appearance of the cells and their structures in the ovaries of early reproductive rats (8-10 weeks) and late reproductive rats (12-14 months) in a way that contributes to the literature. In addition, phase contrast microscopy was used to observe the interactions and proliferation of stromal cells and surface epithelium known to be present in the niche of follicles in mixed cell culture.

Conclusion

The growth of some follicles in the developing follicle pool and the ceasing of the development of other follicles are associated with hormones secreted from some of them. In addition, we think that antral follicles and corpus luteum, especially with their enlarged volumes, do not leave an area (niche) for primordial and primary follicles to develop in some cases and trigger their transformation into atretic follicles (which leads to apoptosis and autophagy). Adipose tissue around the ovary contributes to the development of follicles by secreting hormones and paracrine factors.

After ovulation, the ovule Graaf follicle underwent remodeling of the ovarian surface epithelium and tunica albuginea (surface epithelium and tunica albuginea adjacent to the future corpus luteum). This event occurs not only in the stigma, where ovulation occurs but also along the entire tunica albuginea and surface epithelium adjacent to the ovulated Graaf follicle. In addition, vascular structures (capillaries and venules) were also observed along the tunica albuginea adjacent to the corpus luteum.

The appearance of primordial follicles in the tunica albuginea layer, which is more resistant to environmental factors, suggests that their niches are fibroblast-like mesenchymal cells that support their development. The presence of primordial follicles in the tunica albuginea layer of the ovary provides them with significant advantages in terms of better protection. It is thought that the follicles in this layer differentiate from the oogonium, dispersed due to the breakdown of the ovarian cords and settle here.

Since the structure and functions of the tunica albuginea have not been investigated sufficiently so far, our knowledge about this layer is limited. In addition to all of these, the presence of tubules of the rete ovarii, which develop during the embryological period of the genitourinary system in the ovary and mesovarium during the reproductive period, indicates that these structures may also be important in terms of folliculogenesis.

Ethical Considerations

Compliance with ethical guidelines

Approval for this study was obtained from the Ethics Committee of Pamukkale University Animal Experiments, Denizli, Turkey (Code: E60758568-020-402580; dated 24.07.2023).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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