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Histomorphology of the ovaries of rabbits does during ovulation induced by the combined use of gonadotropins

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Abstract

Hormonal stimulation of folliculogenesis is a general method of increasing reproductive capacity in rabbits; however, studying the safety of the developed protocols remains an urgent scientific task. The use of hormonal agents is due to the physiological characteristics of rabbits, primarily coitus-dependent mechanisms of ovulation. Applying gonadotropin-releasing analogs depends on mature follicles ready for ovulation in the ovary. It, therefore, has limited effectiveness for rabbits of the "zero" cycle that have not yet given birth. Therefore, such females are recommended to use medications based on gonadotropins obtained from the serum of foal mares (eCG) or human chorionic gonadotropin (hCG). There is limited data on the combined use of gonadotropins, especially the long-term administration in different doses. Thus, the research aimed to conduct a morphological evaluation of rabbit's ovaries with combined serum and chorionic gonadotropins to stimulate folliculogenesis before artificial insemination to assess the safety of the proposed dosages and long-term administration. Stimulation of ovulation in rabbits of experimental groups was caused by the combined use of serum and chorionic gonadotropins (eCG – 400 IU; hCG – 200 IU) for experimental groups 1 and 2 at a dose of 40 IU and 24 IU, respectively. Females of the control group were induced to ovulate by subcutaneous injection of 0.2 ml of gonadotropin-releasing hormone analog. Ovaries were collected on the seventh day of gestation after the euthanasia of the rabbit with prior premedication. After dissection and macroscopic examination, the ovaries were fixed in a 10% aqueous solution of neutral formalin. Histological slides were made according to the general method by pouring in paraffin and subsequent staining with hematoxylin and eosin. The research has established that repeated (during five reproductive cycles) combined use of serum and chorionic gonadotropins has dose-dependent effectiveness and can cause dysfunction of ovaries and blood circulation. Pathological changes may acquire a systemic character with the manifestation of critical conditions for the health and life of rabbits. The stimulating effect of the combined use of eCG and hCG was detected at a dose of 40 IU, and the inhibition of folliculogenesis was observed at 24 IU. Since the premises for the hyperandrogenism were found microscopically, it was essential to investigate the level of androgens in the blood of rabbits after using a dose of 24 IU. At the same time, both doses of gonadotropins affect the reactivity of the endothelium, which is manifested by the presence of intravascular vesicles and changes in the rheological properties of blood.

Keywords: rabbit doe; reproduction; hormones; ovulation; ovary.

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1. Introduction

Ensuring effective management of rabbit breeding depends on a large number of factors, the leading one of which is reproductive ability (Naumenko & Koshevoy, 2017; Boiko et al., 2020; Koshevoy et al., 2022; Lesyk et al., 2022). Many factors affect the reproductive traits of rabbits, which are determined by physiological characteristics and keeping conditions (Myroshnychenko & Zhorina, 2021; Koshevoy & Naumenko, 2022).

The effectiveness of ovulation stimulation in rabbits during artificial insemination (using gonadotropin-releasing hormone) depends on the presence of mature follicles ready for ovulation in the ovary (Stevenson et al., 2007). The exploratory research showed that most often unsuccessful artificial insemination occurred in repair rabbits, which we attributed to the "zero" cycle (that were not yet pregnant. To solve this problem, induction of ovulation using gonadotropic hormones in different doses was used (Skibina et al., 2021).

The hormonal stimulating folliculogenesis is most developed in pig and cattle breeding (Cunha & Martins, 2022; Beasley et al., 2023). Most of those industries apply methods in rabbit breeding. The medications containing the serum of foal mares (eCG) and human chorionic gonadotropin (hCG) have been used for a long time. These are the most reproductive hormones necessary for the full-fledged course of pregnancy (Cho et al., 2021). These medications show a positive effect at certain levels, but later, with a few complications, they significantly increase the percentage of non-pregnant or culling female rabbits (Tverdokhlib, 2023).

Human chorionic gonadotropin was discovered after decades of research by many pioneers, and in 1920, Hirose induced ovulation in rabbits and guinea pigs by placental extract (Cole, 2009; Yoo et al., 2021). In 1929, it was discovered that the pituitary gland secretes two hormones that stimulate the gonads: plan A and plan B, which later became well-known as follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively. Fourteen years later, in 1943, Seagar-Jones and his colleagues demonstrated for the first time that a substance secreted from the urine of pregnant women was produced by the giant placental cells, not by the pituitary gland (Lunenfeld, 2004; Practice Committee of American Society for Reproductive Medicine, 2008). Purified hCG was first isolated from urine in the 1940s, and only much later, in 2000, a recombinant hCG became available (Gurin et al., 1940; Lunenfeld et al., 2019).

For a long time, the well-known role of hCG was to promote progesterone secretion by the corpus luteum in early pregnancy through the hCG/LH (luteinizing hormone) receptor. However, many other functions of hCG have recently been described in the Placenta, myometrium, uterus, and fetus (Cole, 2010; Heidegger & Jeschke, 2018). The hCG promotes angiogenesis in the uterine vessels during pregnancy and plays a vital role in fetal organ growth and differentiation (Berndt et al., 2009; de Medeiros & Norman, 2009). Another critical aspect of hCG treatment is its participation in the implantation as one of the crucial molecules (d'Hauterive et al., 2007). Also, hCG effectively modulates metabolic pathways, increasing the endometrium's receptivity (Makrigiannakis et al., 2017). The use of hCG is a promising therapy for secondary hypogonadism in males (Fink et al., 2021). Gonadotropins, widely used for ovulation induction and estrus stimulation in reproductive practice, have a similar impact and different features (Table 1).

Table 1
Properties, scope of production, and application of gonadotropins

Indicator	Equine chorionic gonadotropin (eCG)		Human chorionic gonadotropin (hCG)	
	Data	Source	Data	Source
Site of synthesis and source of production	The Placenta of pregnant mares: extracts from their blood	Byambaragchaa et al. (2021)	Human Placenta: transfer in maternal blood	Fournier (2016)
Chemical structure, producer cells	Glycoprotein hormone secreted by trophoblastic epithelial cells of fetal origin	Legardinier et al. (2005)	Heterodimeric glycoprotein consists of two subunits. The syncytiotrophoblast secretes it	Heidegger & Jeschke (2018)
Cost and quantity on the market, availability of analogs	Low cost and relatively high quantity on the market, no analogs	Gomes et al. (2020)	High cost and low quantity on the market, lack of analogs	Vilanova et al. (2019)
FSH/LH-like activity and half-life	Possesses FSH/LH-like activity, has a longer half-life	Thompson et al. (2023)	More pronounced FSH/LH-like activity	d'Hauterive et al. (2007); Cunha & Martins (2022)
Additional synthesis of progesterone	Increase in the number of corpora luteum and concentration of progesterone	Rigoglio et al. (2013)	After binding to LH receptors from auxiliary luteal cells	Spencer & Bazer (1996); Stevenson et al. (2007)
Side effects of high doses or long-term use	Ovarian hyperstimulation syndrome (OHSS) and increased long-term risk of ovarian cancer	Lin et al. (2021)	Excessive stimulation of folliculogenesis, which leads to the occurrence of HSS	Banker & Garcia-Velasco (2015)
Use in animal reproduction	Both gonadotropins induce follicular growth and ovulation in immature sows, stimulate ovulation in other animals, and in superovulation and embryo transfer			

The described properties of these hormones testify to their ability to provide effective induction of folliculogenesis, which increases the reproductive ability of rabbits and, therefore, the profitability of rabbit meat production. However, many sources indicate a high probability of side effects and morphological damage of the ovaries' parenchyma due to incorrect dosage and/or long-term use of gonadotropins (Herkert et al., 2022). The obtained negative consequences of the use of gonadotropins are probably related to the difference between the recombinant forms of gonadotropins and endogenous hormones circulating in the blood, which not only affects their pharmacodynamics but also changes their selectivity and, thereby, modifies the cellular response (Banker & Garcia-Velasco, 2015; Casarini & Simoni, 2021).

The work aimed to conduct a morphological assessment of rabbits' ovaries with combined serum and chorionic gonadotropins to stimulate folliculogenesis before artificial insemination to assess the safety of the proposed dosages and long-term administration.

2. Materials and methods

Organizing and conducting the experiment were performed according to the provisions of the "European Convention on the Protection of Vertebrate Animals Used for Experimental and Scientific Purposes" (Strasbourg, 1986), the 1st National Congress on Bioethics (Kyiv, 2001), and the Law of Ukraine "On the Protection of Animals from of cruel treatment" (2006).

The ovaries of Hyla in five reproductive cycles on the seventh day of gestation were studied. Artificial insemination was performed using the general method. Stimulation of ovulation in rabbits of experimental groups was caused by the combined use of serum and chorionic gonadotropins (eCG – 400 IU; hCG – 200 IU) at a dose of 40 IU and 24 IU for experimental groups 1 and 2, respectively. Induction of ovulation of rabbit does of the control group was carried out by subcutaneous injection of 0.2 ml of gonadotropin-releasing hormone analog. Ovaries were collected on the seventh day of gestation after the euthanasia of the rabbit with prior premedication.

After dissection and macroscopic examination, the ovaries were fixed in a 10 % aqueous solution of neutral formalin. Histological slides were made using the general paraffin embedding technique and subsequent staining with hematoxylin and eosin (Horalsky, 2015).

3. Results and discussion

During a histological examination of the ovaries of rabbits of the control group, it was established that each of them is surrounded by a protein shell made of dense connective tissue covered with a simple cuboidal epithelium. The cortex and medulla were determined on the median longitudinal section of the ovaries. The cortex outweighed the medulla significantly (Skibina et al., 2021).

The cortex comprises a connective tissue stroma and numerous ovarian follicles at various stages of development, as well as atretic bodies, corpora lutea, and interstitial cells. The functional reserve of the ovaries is formed by primordial and primary follicles, which are located under the protein shell in several rows. Secondary and tertiary follicles and atretic bodies are in the center of the cortex.

The analysis of various scientific and methodical sources showed that the study of the ovaries of various animals and the issue of folliculogenesis are pretty standard. However, despite this, there are still discrepancies in the classification of ovarian follicles, especially regarding determining the stages of secondary and tertiary follicles.

The classification of the ovarian follicles of rabbits was identified according to Edson et al. (2009). It was based on the morphology of the follicles, as well as their sensitivity to pituitary gonadotropins. The primordial, primary, secondary, and tertiary follicles were defined according to this classification. Primordial, primary, and secondary follicles are preantral and do not contain a cavity. Their development does not depend on the action of gonadotropins; it is controlled only by ovarian factors. Tertiary follicles contain a few small cavities (or one large) filled with follicular fluid. The development of tertiary follicles depends on pituitary gonadotropins.

As Figure 1 illustrates, the primordial ovarian follicles were the smallest and were formed by oocytes and only one layer of squamous cells of the follicular epithelium. Primary follicles contain oocytes in the initial stages of vitellogenesis. From 1 to 2, layers of cuboidal follicular cells were determined in the wall of primary follicles. In the presence of 2 layers of epithelium, the initial formation of the follicle theca (connective tissue membrane) was detected. Concentrically layered fibrocytes and collagen fibers formed the theca. In such primary follicles, oocytes were surrounded by a shiny zone (transparent shell) in the form of an oxyphilic strip.

Secondary follicles were classified as follicles with a multilayered epithelium and a theca differentiated into "theca interna" (which contains thecal endocrinocytes and blood vessels) and "theca externa" (formed by fibrous connective tissue). Tertiary (antral) follicles of the ovary of pregnant rabbits had one large cavity or several small ones among the follicular cells. Such cavities are formed by fluid accumulation during the follicular cells' secretion. The follicular epithelium in such follicles was differentiated into three cell types: granulosa cells, cumulus cells (egg tubercle cells), and cells of radiating crown. Granulosa cells line the surface of the follicle wall. Cumulus cells attach the oocyte to the follicle wall. The cells of the radiating crown surround the oocyte.

The ovaries from three rabbits contained 2–4 tertiary follicles with hemorrhages or follicular fluid with impurities of blood cells, mainly erythrocytes. These follicles were characterized by a complete absence of granulosis, thickening of the theca, and absence of signs of luteinization of thecal endocrinocytes. Such follicles were considered cystic atresia of tertiary follicles that have not reached ovulation.

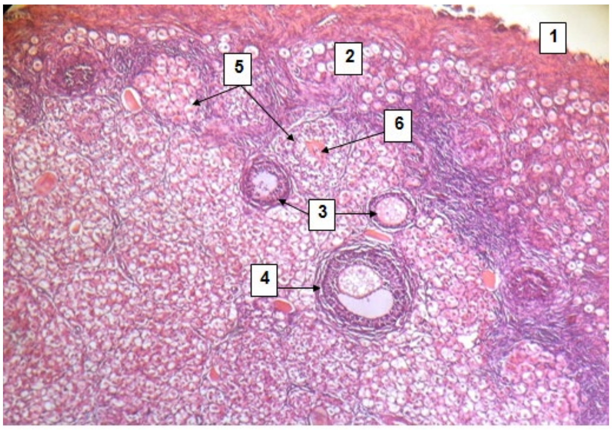

Fig. 1. Histology slide of the ovary of rabbit doe (control group). Staining with hematoxylin and eosin, ×100:
1 – protein shell; 2 – primordial follicles; 3 – secondary follicles; 4 – tertiary (antral) follicle; 5 – atretic bodies; 6 – the remnants of shiny shell

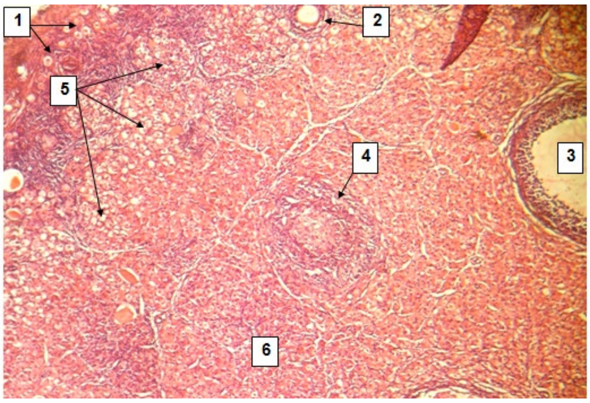

Fig. 2. Histology slide of the ovary of rabbit doe (control group). Staining with hematoxylin and eosin, ×100:
1 – primordial and primary follicles; 2 – secondary follicle; 3 – tertiary (antral) follicle; 4 – atresia of the follicle according to the obliterating type; 5 – atretic bodies, 6 – interstitial glandular tissue

Regardless of the use of hormones, a feature of the ovaries of rabbits after several cycles is a high level of luteinization due to the formation of corpora lutea during pregnancy, atretic bodies, and interstitial glandular tissue (Fig. 2). Atretic bodies are formed because of luteinization of cells of the "theca interna" and sometimes granulosa cells of atretic follicles. Atresia covered all generations of follicles in the rabbit experimental groups except for primordial ones. In the primary and secondary follicles, atresia occurred according to the obliteration type. It was mainly accompanied by the proliferation and luteinization of endocrinocytes of "theca interna". Granulosis cells were luteinized less often, mainly in the early stages of forming tertiary follicles with several small cavities. The cell apoptosis was observed more often. Luteinized primary and secondary follicles formed round atretic bodies. They looked like accumulations of large cells with unstained vacuolated cytoplasm.

The atretic bodies in the central part sometimes contained the remains of a shiny membrane and were externally surrounded by "theca externa". Such atretic bodies were densely placed in the cortex and formed a light zone that separated the primordial and primary follicles from the interstitial glandular tissue.

Interstitial glandular tissue was detected in the cortex and medulla of the ovary. Interstitial endocrinocytes had optically dense oxyphilic cytoplasm and formed cell arrays of arbitrary shape, separated by thin layers of loose connective tissue. As part of the interstitial glandular tissue, the capillary bed was visualized as an empty slit space. Spaces were bounded by endothelium and sometimes contained erythrocytes.

Corpora lutea of pregnancy were large and round and had a well-developed capsule and vascularization. Histology slides were often observed corpora lutea of pregnancy from previous cycles, which may indicate their activity during several cycles (Fig. 3). Such corpora lutea differed in size and light color. Their luteocytes had an optically transparent and weakly oxyphilic cytoplasm with large vacuoles. The nuclei of such cells were mainly hyperchromic or pyknotic, and the vessels of the capillary bed were dilated but bloodless. Such morphological signs testify to the gradual regression of these corpora lutea.

The vascular reaction with signs of hyperemia and venous stasis was less pronounced with the combined use of eCG and hCG in a dose of 40 IU (Fig. 4). Most of the vessels of the cortex and medulla of the ovaries were dilated. They appeared empty, but there were areas of the medulla with signs of venous hyperemia. The lumen of some vessels was filled with blood plasma in the absence of blood cells. The unstained vacuoles, which often had parietal location, were well defined in such vessels, mainly veins, in the homogenous oxyphilic mass of the vascular content. It may be evidence of the reactivity of endotheliocytes. Lymphatic vessels of the medulla were not visualized.

The follicles of all generations and the state of atresia were determined in the cortex of ovaries (Fig. 5). The pool of primordial follicles was more preserved compared to the ovaries of rabbits of the first and second experimental groups. However, it was noticeably smaller compared to the control group. The number of atretic bodies was significantly higher than the ovaries of rabbits, the ovulation of which was inducted by only eCG (Tverdokhlib, 2023). Despite this, the atresia of most primary and secondary follicles was characterized by degenerative changes in oocytes and, mostly, was not accompanied by luteinization of follicular epithelial cells and thecal cells. Their theca was thickening, sometimes very significant, due to connective tissue.

Among the tertiary follicles, the individual follicles with a multilayered follicular epithelium that showed signs of secretory activity were identified (Fig. 6). Their number was significantly smaller compared to the ovaries of a rabbit treated only by eCG (Tverdokhlib, 2023). Such follicles mostly did not reach the size of preovulatory follicles and contained luteinized thecocytes. Atretic follicles reaching the size of preovulatory follicles did not have granulosa.

The present atretic bodies were formed by rounded or polygonal cells, which contained large transparent vacuoles in the cytoplasm. So, the area where such cells were located generally looked lighter. Cell nuclei of atretic bodies were mostly hyperchromic and pyknotic; it indicated a decrease in their secretory activity. Such atretic bodies, as well as their perifollicular connective tissue, had cells of the lymphoid-macrophage series. Sometimes, they formed cellular infiltrates. Such a picture may indicate that the existing atretic bodies are in a state of restructuring. Those formed in previous reproductive cycles are in a state of regression; their functional activity is suppressed.

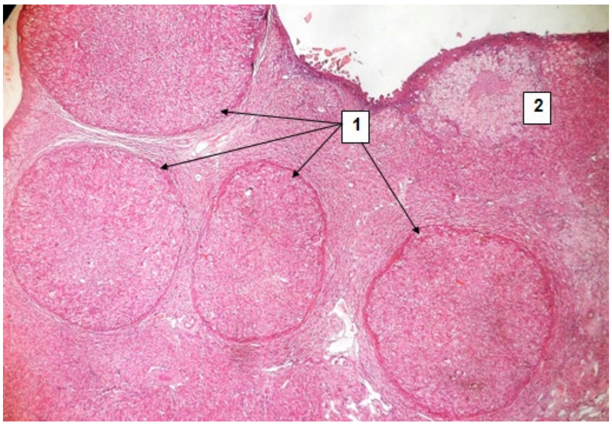

Fig. 3. Histology slide of the left ovary of rabbit doe (control group). Staining with hematoxylin and eosin, ×32:
1 – corpora lutea of pregnancy of the current cycle; 2 – corpus luteum of pregnancy of the previous cycle

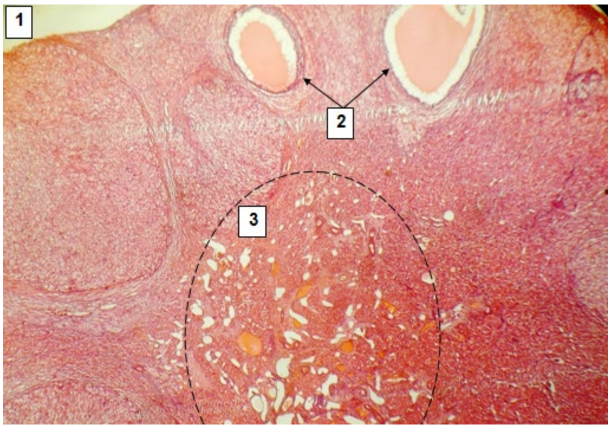

Fig. 4. Histology slide of the ovary of rabbit doe (experimental group 1). Staining with hematoxylin and eosin, ×100:
1 – corpus luteum of pregnancy; 2 – tertiary follicles with hypersecretory activity of granulosa cells; 3 – medulla

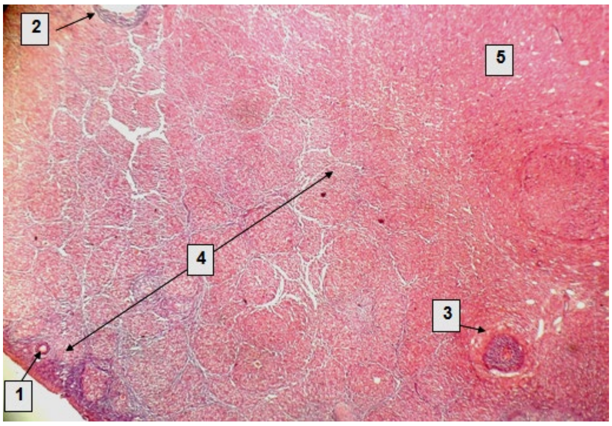

Fig. 5. Histology slide of the ovary of rabbit doe (experimental group 2). Staining with hematoxylin and eosin, ×32:
1 – secondary follicle at the stage of atresia; 2 – a fragment of a tertiary follicle; atretic follicle with a thickened theca, obliterating type atresia; 4 – the area of atretic bodies

The interstitial glandular tissue, contained in the deep layers of the cortex and medulla, was characterized by an oxyphilic color. Large glandular cells, polygonal or close to a rounded shape, were closely adjacent. Between them, the vessels of the capillary bed were determined in the form of transparent, endothelium-bounded slits. In the cytoplasm of glandular cells, eosinophilic granules and small, single, medium-sized transparent vacuoles were determined. They have a peripheral location. The nuclei of such cells had a rounded or oval shape and contained a moderate amount of finely dispersed heterochromatin and 1–2 nucleoli.

After the combined use of eCG and hCG at a dose of 24 IU, the microstructural features in the ovaries of rabbit does were established (Fig. 7). These features indicate a higher secretory activity of interstitial glandulocytes, compared with the ovaries of rabbit does of all previous groups. The cells of the interstitial glandular tissue were polygonal in shape with pronounced pointed corners, had noticeably smaller sizes, and optically dense oxyphilic cytoplasm. Between them, the slit spaces with expanded vessels of the capillary bed were defined clearly. Nuclei of interstitial endocrinocytes were mostly round with finely dispersed heterochromatin and 1–2 nucleoli.

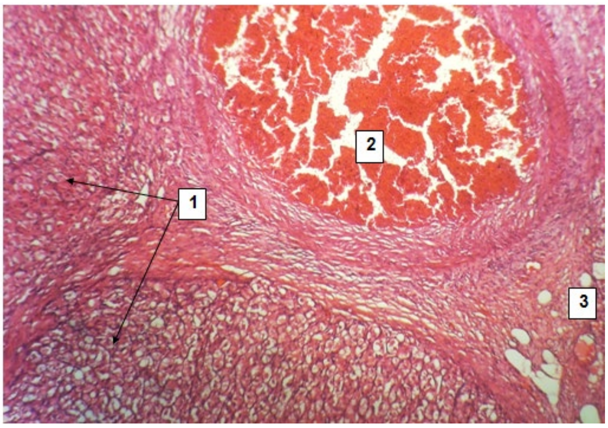

Fig. 6. Histology slide of the ovary of rabbit doe (experimental group 1). Staining with hematoxylin and eosin, ×100: 1 – corpora lutea of pregnancy; 2 – cystic atresia of a tertiary follicle with hemorrhagic content. The capsules of the corpora lutea and the follicle are thickened due to connective tissue of the external theca. 3 – a fragment of medulla

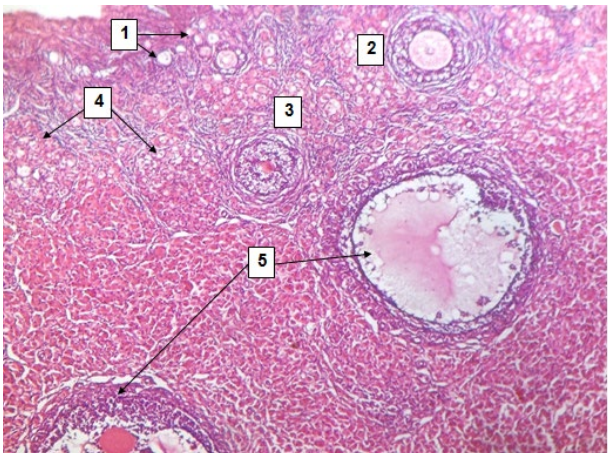

Fig. 7. Histology slide of the ovary of rabbit doe (experimental group 2). Staining with hematoxylin and eosin, ×100: 1 – primordial and primary follicles; 2 – secondary follicle with luteinization of thecocytes and granulosa; 3 – atresia of the secondary follicle according to the obliterating type; 4 – atretic bodies; 5 – cystic atresia and luteinization of thecocytes and granulosa cells

The size and shape of the cells and the nuclei indicate increased synthetic and secretory activities of the cells. Due to active secretion, secretory products are constantly released into the blood, which can cause an increase in the level of the corresponding hormones in the blood with the manifestation of specific effects. There is an opinion that interstitial endocrinocytes are the source of ovarian testosterone.

Indirect morphological signs of hyperandrogenism in some rabbits of this group were determined on histology slides of the ovaries. The inhibition of folliculogenesis at the stage of early antral follicles with their cystic degeneration was accompanied by necrosis and exfoliation of granulosa without luteinization. At the same time, atresia of primary and secondary follicles occurs according to the obliterating type with luteinization of granulosa cells and thecocytes. Such a morphological pattern is a leading feature of polycystic ovary syndrome.

4. Conclusions

1. Multiple (within five reproductive cycles) combined use of serum and chorionic gonadotropins is dose-dependent and can cause dysfunctional disorders of ovaries and blood circulation. Blood circulation dysfunction can become systemic, resulting in critical conditions for the health and life of rabbits.

2. The stimulating effect of the combined use of eCG and hCG was detected at a dose of 40 IU, while the inhibition of folliculogenesis was observed at 24 IU. It is essential to investigate the level of androgens in rabbit blood after using a dose of 24 IU since the prerequisites for developing hyperandrogenism were found in the microstructure of the ovary.

3. Both doses of gonadotropins that were used in the studies affected the endothelium reactivity. The presence of intravascular vesicles and changes in the rheological properties of blood manifested it.

Conflict of interest

The authors declare no conflict of interest.

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