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ULTRASTRUCTURE OF GRANULOSA CELLS OF BOVINE OVARIAN ANTRAL FOLLICLES IN RELATION TO ATRESIA

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ABSTRACT

The aim of this study was to describe the most common forms of ovarian follicle atresia in large antral follicles of cows and quantify the occurrence of different cell structures in each form. Atresia of antral follicles in ovaries was determined on the basis of ultrastructural images acquired by electron microscopy of ultrathin sections contrasted with uranyl acetate and lead citrate to visualize cell organelles. All forms of atresia in large follicles are accompanied by regressive changes of the granulosa cells. The initial form of atresia is characterized by enlarged intercellular spaces after the disruption of the gap junctions and desmosomes. Small collapsed cells with pyknotic nuclei, substantially reduced the cytoplasm and a higher incidence of lysosomes are located on the surface of the granulosa layer. The *stratum granulosum* wall collapses and the basal membrane is swollen with a rupture of the *lamina basalis*. Obliterative atresia is characterized by a multiplied loose connective tissue consisting of collagen fibers, fibroblasts, histiocytes, blood capillaries and sporadically granulocytes. The cystic form of atresia is characterized by small collapsed, pyknotic granulosa cells settled in one or two layers. In luteinization-associated atresia, granulosa cells are hypertrophied, their cytoplasm contains smooth endoplasmic reticulum and mitochondria with tubules. In conclusion, the initial atresia of large antral ovarian follicles is associated with processes of cell death, followed by multiplication of the loose connective tissue cells, its dystrophy and hyalinization of the collagen fibers. Ultrastructural examination could be used as a complementary method to improve histopathological diagnostics of cow reproductive organs in veterinary practice.

Key words: atresia; granulosa; ovarian follicle; ultrastructure

INTRODUCTION

In mammalian ovaries, more than 90 % of the follicles undergo a degenerative process known as atresia [9, 13]. Follicular atresia in mammals is a process that destroys ovarian follicles and significantly eliminates from the ovary the oocytes prepared for selection, ovulation and potential fertility [19].

Using morphological assessments, Byskov [2] and Blondin et al. [1] described the occurrence of pyknotic nuclei in the *stratum granulosum*, or in the *antrum* of the atretic ovarian follicles of cows. Later on, the antral and basal forms of atresia were described as two basic patterns of bovine antral follicle atresia [6]. Basal atresia occurs in small follicles (≤ 5 mm) and antral layer atresia occurs in large antral follicles (≥ 5 mm), including dominant, which can be identified histologically and biochemically [17]. It has also been found that the occurrence of morphological, phenotypic changes in *granulosa basal lamina* in the form of loops and openings is related to the functional competence of bovine oocytes [7].

The concentrations of follicular fluid steroid hormones can be used to classify atresia and distinguish some of the different types of atresia. However, this method is unlikely to identify follicles early in atresia, and hence misclassify them as healthy. Other biochemical and histological methods can be used, but since cell death is a part of normal homeostasis, deciding when a follicle has entered atresia remains somewhat subjective [17].

The aim of this study was to describe the ultrastructural changes in the granulosa cells of non-ovulated antral follicles (≥ 5 mm) of cows. In addition to the morphological description, we attempted to quantify the incidence of different cell structures related to each status of follicles: normal, initial atresia, atresia with luteinization, cystic and obliterative atresia.

MATERIALS AND METHODS

The non-pregnant cows of Slovak spotted breed and Holstein cross-breed (n = 30), slaughtered at a local abattoir at the age of 4—8 years, were used as a source of ovaries without previous ovary examination. Ovaries were isolated following 20 min after the slaughter of cows and transported to the laboratory in thermos at a temperature of 26 °C. Antral follicles (n = 98) of 5—10 mm (1—5 follicles per ovary) were picked out of the ovary and the follicular wall was cut off in a size of 1.1—2.0 mm and processed for transmission electron microscopy.

The ovarian tissue samples containing antral follicles were fixed in the aldehyde mixture (2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M sodium cacodylate; all from Fluka, Buchs, Switzerland) at 4 °C for 1 hour and then post-fixed in 1 % OsO_4 (Fluka, Buchs, Switzerland) in 0.1 M sodium cacodylate for 1 hour. Samples were dehydrated in acetone for 30 min and then embedded into Durcupan ACM (Fluka, Buchs, Switzerland). Ultrathin sections (70 nm) were contrasted with uranyl acetate and lead citrate to visualize cell organelles. The contrasted sections were analysed under a JEM100 CXII transmission electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

The follicles were classified as normal (N) without pyknotic nuclei or atretic, which had pyknotic nuclei and ≤ 4 granulosa cell layers. Atresia was determined on the

Table 1. Ultrastructure of granulosa cells from normal or atretic bovine ovarian follicles

Granulosa cell structures	Forms of ovarian follicle atresia				
	N	IA	LA	CA	OA
Cell contacts	+++	+	+	++	+
Intracellular spaces	small	large	large	small	large
Pyknotic nuclei	-	+++	++	+	+
Endoplasmic reticulum					
rough	+++	+	+	+++	++
smooth	+	+	+++	+	-
Mitochondria					
with cristaes	+++	+	+	+++	++
with tubules	+	+	+++	+	-
Lipid droplets	+	++	++	+	+
Lysosomes	+	+++	+	+	++
Autophagic vacuoles	+	+++	+	+	++

N—normal follicle; IA—initial atresia; LA—atresia with luteinization; CA—cystic atresia; OA—obliterative atresia (initial phase)
Occurrence: „-"—none; „+"—small, less than 30 %; „++"—moderate, 30—50 %; „+++"—abundant, more than 50 %

basis of ultrastructural features and assigned to the following forms: initial (IA), atresia with luteinization (LA), cystic (CA) and obliterative (OA). The presence of important cell structures (in %) is shown in Table 1, as a small (less than 30 %), moderate (30—50 %) or abundant (more than 50 %) occurrence.

RESULTS

In the wall of normal (N) antral follicles—*stratum granulosum*, we observed granulosa cells as polyhedral cells with heterochromatic nuclei; their cytoplasm contained rough endoplasmic reticulum in the form of short seg-

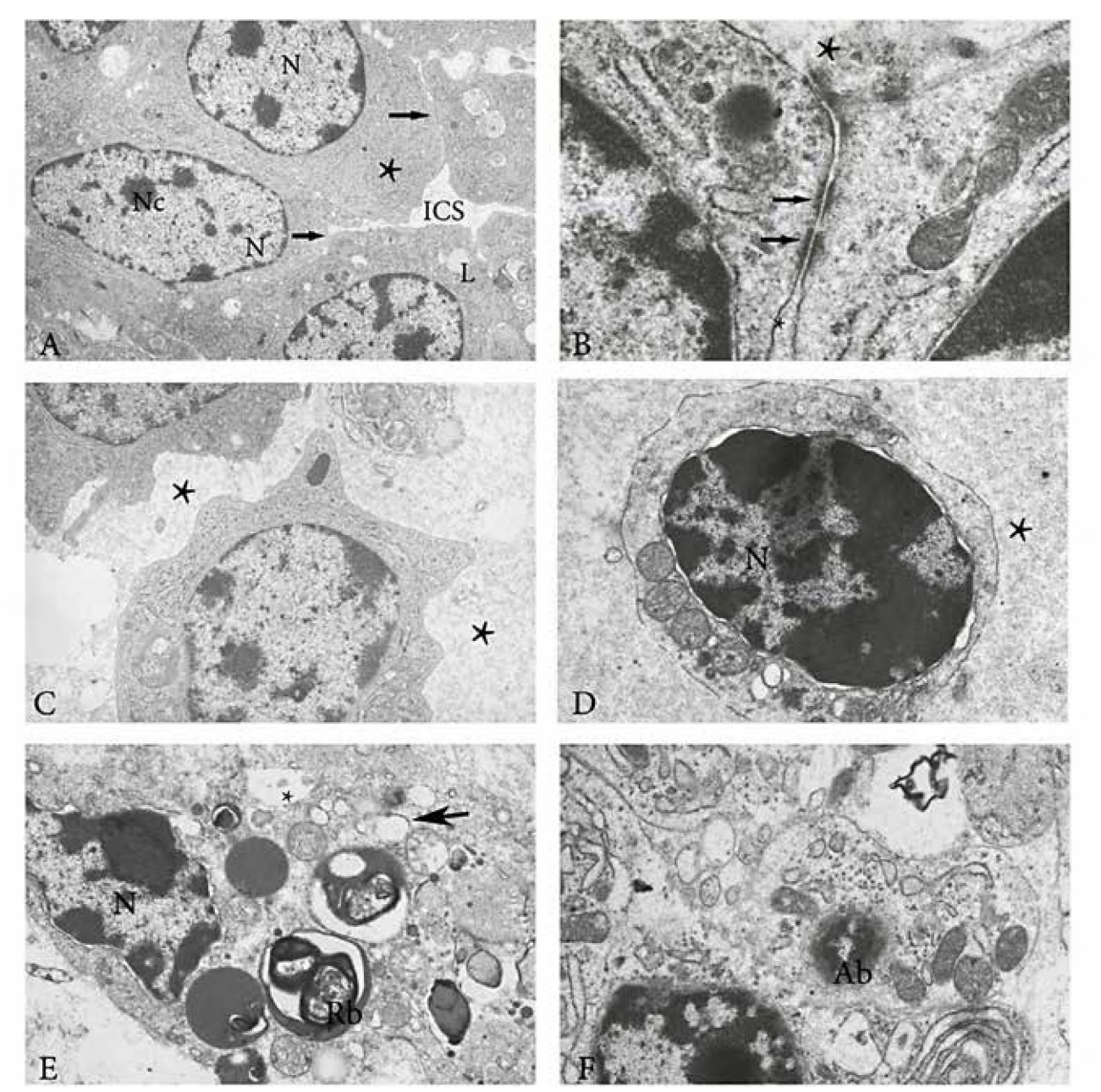

Fig. 1. Ultrastructure of granulosa cells of normal and atretic ovarian follicles of cows

A—Granulosa cells with hyperchromatic nuclei (N) and nucleoli (Nc) in close contact coupled by gap junctions (arrows), rough endoplasmic reticulum (asterisk) and lipids (L). Intercellular spaces (ICS) are small. Magn. ×4800; B—Intercellular junctions of the desmosomal type (*macula adherentes*; arrows), connecting two adjacent granulosa cells, broken gap junctions and enlarging intercellular space (asterisks). Magn. ×29 000; C—Granulosa cell after gap junction loosening. The large intercellular space is filled with finely granulated extracellular matter (asterisks). Magn. ×7200; D—Pyknotic cells and enlarged intercellular spaces in *stratum granulosum*. Pyknotic nucleus (N) in the antral cavity of the atretic follicle and enlarged intercellular spaces (asterisks). Magn. ×14 000; E—Necrotic granulosa cells with pyknotic nucleus (N), cytoplasmic breakdown and cytoplasmic membrane rupture. Dilated tubules of rough endoplasmic reticulum (arrow), vesicles (asterisk) and residual bodies (Rb). Magn. ×10 000; F—Apoptotic bodies (Ab) in the cytoplasm of granulosa cell. Magn. ×10 000

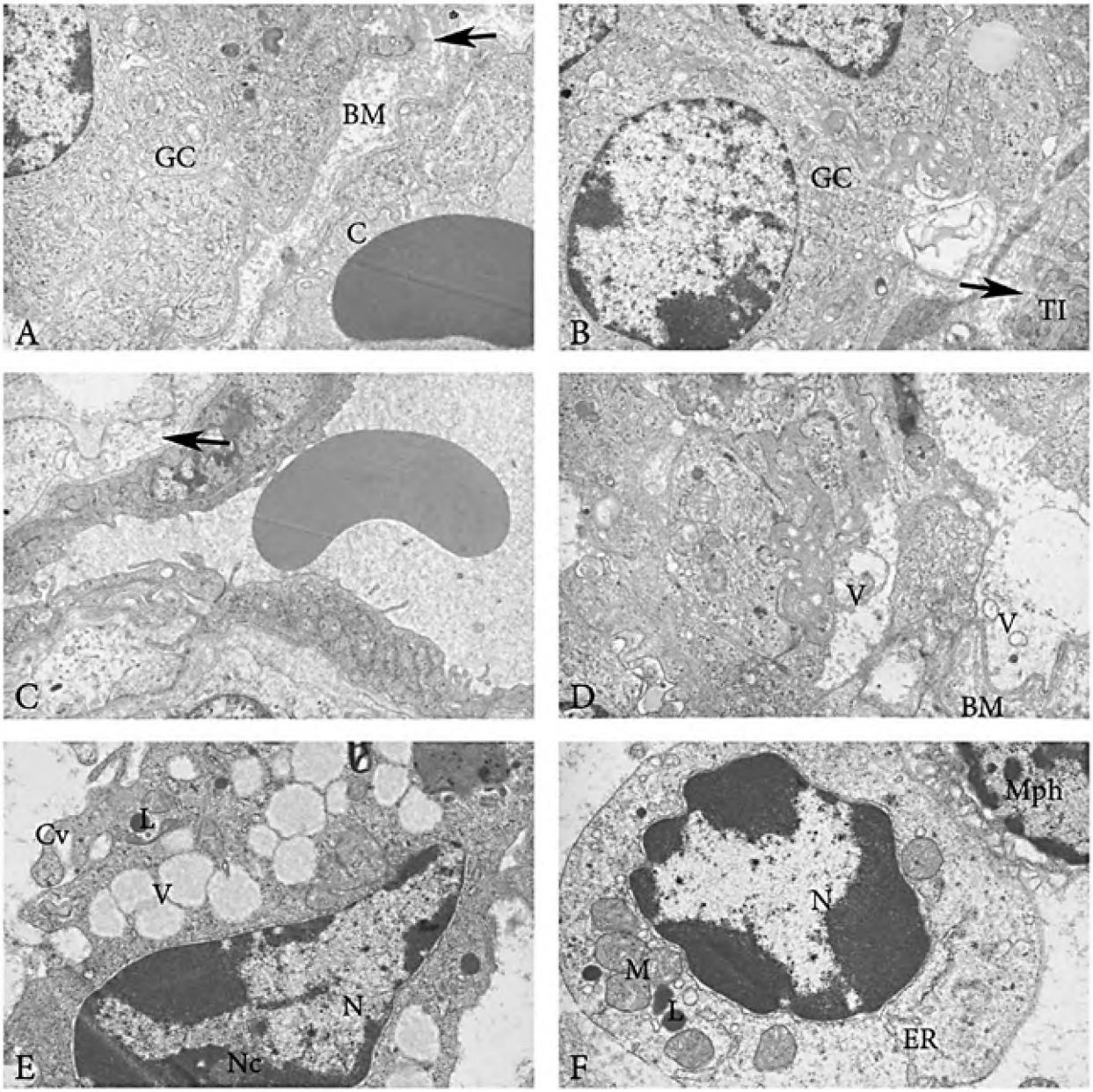

Fig. 2. Ultrastructure of the basal lamina of cow ovarian follicles at the initial form of atresia

A—The basal layer of granulosa cells (GC) adheres to the waved and swollen basal membrane (BM). Disrupted *lamina basalis* from the side of *stratum granulosum* cells (arrow); capillary (C). Magn. ×7200; B—Disrupted *lamina basalis* (arrow) from the side of the *theca interna* (TI) cells. Granulosa cells (GC) are present on the left side. Magn. ×7200; C—Disrupted *lamina basalis* in contact with the sub-endothelial space (arrow). Magn. ×7200; D—Vesicles (V) between granulosa cells and disintegrated plasma membranes. Basal membrane (BM) is located on the right lower part. Magn. ×7200; E—Macrophage in the *stratum granulosum* of the antral follicle, heterochromatic nucleus (N) and nucleolus (Nc). Numerous occurrence of large vacuoles after endocytosis (V), lysosomes (L), cytoplasmic projections (Cv). Magn. ×10 000; F—Luteinized granulosa cell (*stratum granulosum*) of the cow antral follicle in contact with macrophages (Mph). The heterochromatic nucleus (N) of a cell. Cytoplasm containing mitochondria with tubules (M), small amounts of rough endoplasmic reticulum (ER) and several lysosomes (L). Magn. ×10 000

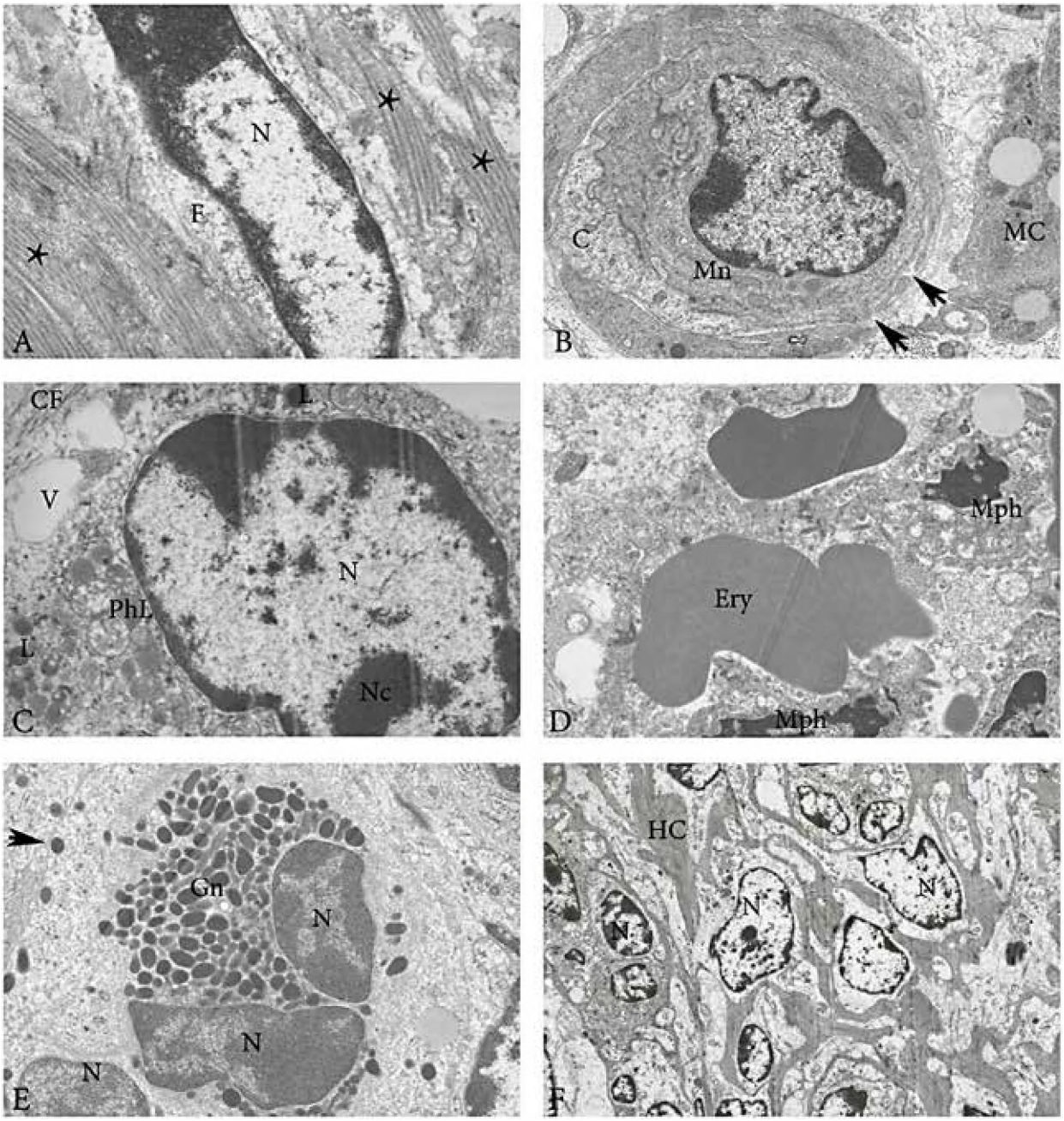

Fig. 3. Ultrastructure of cells from the antral ovarian follicles of cows at obliterative form of atresia

A—Initial stage of obliterative antral follicle atresia. In close proximity to the fibroblast (F) with oval-shaped nucleus (N) there are numerous fibrils with periodic transverse striations forming bundles of normal collagen (asterisk). Magn. ×14 000; B—Capillary (C) of a loose connective tissue with a monocyte (Mn) at the process of diapedesis (arrow). On the right side - part of the mast cell (MC) in close proximity to the capillary wall. Magn. ×7200; C—Histiocyte-macrophage in loose connective tissue with hyperchromatic nucleus (N) and compact nucleolus (Nc). The cytoplasm contains smaller amounts of rough endoplasmic reticulum, mitochondria and a number of vacuoles (V), lysosomes (L) and phagolysosomes (PhL), with larger vacuoles on the surface of the cytoplasm in contact with collagen fibrils (CF); (top left). Magn. ×1000; D—Phagocytosis of degraded connective cell elements, including erythrocytes (Ery), by macrophages (Mph). Magn. ×7200; E—Basophilic granulocyte in the loose connective tissue of the antral follicle. Large three-lobed nucleus (N). Numerous round granules (Gn); some of them with fine granulation and many of them in close contact with the cytoplasmic membrane (arrow). Magn. ×10 000; F—Advanced collagen hyalinization (HC) at obliterative follicular atresia with numerous necrotic connective tissue cells in hyalinized follicle. Hyperchromatic nuclei (N) of necrotic cells. Magn. ×1900

ments. Small rod-shaped mitochondria were mainly with cristae and to a lesser extent with tubules. The Golgi complex was located near the nucleus. Small amounts of lipid droplets, multi-vesicular bodies and autophagic vacuoles were observed. The cell-to-cell contacts were provided by frequently occurred gap junctions and desmosomes (*maculae adherentes*). Intercellular spaces were small (Table 1; Fig. 1A). The granulosa cells by their basal layer were settled on a thin intact extracellular *lamina basalis* layer.

In antral follicles with initial atresia (IA), enlarged cell spaces filled with intercellular mass (Fig. 1C) were observed due to the broken intercellular junctions of the desmosomal and gap junction types (Fig. 1B). On the surface of the *stratum granulosum*, we observed the frequent occurrence of collapsed granulosa cells with pyknotic nuclei (Fig. 1D). In the cytoplasm of granulosa cells of atretic follicles we found frequent dilation of tubules of the rough endoplasmic reticulum and formation of vesicles, autophagic and residual bodies (Fig. 1E). The mitochondria number decreased, they were diminished in size and often dark-pyknotic; the numbers of autophagic vacuoles and lysosomes were increased (Table 1). In some granulosa cells of antral follicles the apoptotic bodies had been found (Fig. 1F). The *lamina basalis* underwent disintegration and, to a greater extent, created openings into the intercellular area of *stratum granulosum* (Fig. 2A) and into the *theca interna* area (Fig. 2B). We also observed a break in the integrity of the capillary sub-endothelial basal lamina (Fig. 2C) and the formation of vesicles between the granulosa cells following the plasma membrane disintegration (Fig. 2D). Among granulosa cells of the basal part of the atretic follicles the macrophages were frequently evident (Fig. 2E).

At the atresia with luteinization (LA), we observed the hypertrophied polygonal cells among the granulosa cells, which contained a nucleus with 1—3 nucleoli. The cytoplasm contained smooth endoplasmic reticulum, overspread tubules and vesicles of Golgi complex, numerous mitochondria with tubules and the lysosomes (Fig. 2F).

The cystic form of atresia (CA) was characterized by several pyknotic granulosa cells grouped in one or two layers, similar as at the initial form of atresia (data not shown). There were intercellular connections; intercellular spaces were small, endoplasmic reticulum was rough and the mitochondria had cristaes (data not shown).

Granulosa cells in the initial phase of the obliterative form of atresia (OA) substantially lost intercellular connections, intercellular spaces were large, endoplasmic reticulum was rough and mitochondria had cristaes (Fig. 3A—F). In older atretic follicles, at an advanced phase of an obliterative form of atresia we observed an increased incidence of fibroblasts (Fig. 3A). At this stage, a diapedesis of monocytes that penetrate the capillary wall into the loose connective tissue was present (Fig. 3B). The macrophages in the loose connective tissue of atretic follicles had a typical hyperchromatic nucleus and contained smaller amounts of rough endoplasmic reticulum, mitochondria, a prominent Golgi complex, a number of vacuoles, lysosomes and phagolysosomes (Fig. 3C). The macrophage activity gradually increased, and phagocytosis of all degraded elements of connective cells including erythrocytes occurred (Fig. 3D). The polymorphonuclear neutrophil granulocytes with numerous typical granules in the cytoplasm were also observed in the loose connective tissue (Fig. 3E). We have observed that the loose connective tissue gradually became dense, and its fine-fiber tangle of collagen fibrils underwent hyaline dystrophy converting to a strongly hyalinized connective tissue, manifested as an unstructured homogeneous dense mass (Fig. 3F).

DISCUSSION

In mammals during the development of ovarian follicles most of the antral follicles undergo atresia at different stages of their development [17], however, the mechanisms controlling this selective process are not known. T e e r d s and D o r r i n g t o n [18] reported histological differences in atresia of preantral and antral follicles: oocyte fragmentation, disordered granulosa layer and hypertrophied theca layer were characteristic for atresia in preantral follicles, whilst massive apoptosis of granulosa cells in the presence of a more or less intact oocyte being characteristic of atresia in antral follicles.

The basic physical mechanism of follicular atresia is granulosa cell apoptosis [22]. Atretic changes may affect cells of both antral and non-antral follicles but apoptotic changes only occur in the antral follicles [5, 21]. The degeneration of cattle oocytes, however, may occur at any stage of atresia. Several studies have shown that apoptosis in granulosa cells may occur much earlier than the morphological changes in follicular atresia [11, 12]. M e n g et al. [13] studied the ovarian atresia, besides immuno-

histochemistry, also from the functional viewpoint using certain molecular markers of autophagy and apoptosis: microtubule-associated light-chain protein 3 (LC3), sequestosome 1 (SQSTM1/P62), Beclin1, autophagy-related protein 7 (ATG7) and cleaved caspase 3 (cCASP3). These authors had found that preantral and antral follicular atresia was the result of the activation of different cell-death pathways. Antral follicular degeneration was initiated by massive granulosa cell apoptosis, while preantral follicular atresia occurred mainly via enhanced granulosa cell autophagy [13].

The granulosa cells of the antral ovarian follicles are interconnected by the gap junctions and desmosomes—*maculae adherentes*, and the whole ovarian follicle is surrounded by the *lamina basalis* [16]. At the initial form of follicular atresia, some extracellular changes lead to the breakdown of the intercellular junctions and the enlargement of the extracellular space, which is filled with the granularly structured extracellular matrix.

One form of cow ovarian atresia is also cystic atresia of ovarian follicles. This form of cysts affects the antral follicles of 3—5 mm in size and it must not be misinterpreted with the cystic ovarian disease of the antral follicles 15—20 mm in size. Cystic atresia does not affect the estrous cycle of cows, while cystic-diseased cows have an abnormal estrous cycle. At cystic atresia, the *stratum granulosum* is absent, and the internal part of the cyst is lined with one or two layers of small cubic, partially pyknotic granulosa cells.

Atresia with luteinization may be caused by the action of serum luteinization-controlling factors, which, after changing the permeability of the basal membranes of atretic follicles, act untimely even with the involvement of capillaries penetrating between granulosa cells [14]. The fact, that even non-ovulated follicles can luteinize, had been confirmed by W e s t f a h l [23], when he found that ovulation was not necessary for the follicle luteinization process. According to E l f o n t et al. [4], also the lysosomal cell system may participate in ovarian steroidogenesis. Similar results were observed in our study, when there were more lysosomes in the cytoplasm of atretic and luteinizing granulosa cells.

We also observed that when the process of antral follicle atresia progressed over time, fibroblasts began to appear in the follicles; the follicle became occupied with the loose connective tissue and the process of obliterative atresia was initiated. This process was accelerated especially by the formation of cracks of the granulosa epithelium, its separation from the theca cells, as well as with the increased incidence of pyknotic nuclei of the granulosa cells associated with the oocyte degeneration.

In addition to necrotic and apoptotic processes, also macrophages were involved in the regression of the antral follicle cells [8]. Macrophages penetrated the wall of the atretic follicles between the granulosa and theca cells, infiltrated into the loose connective tissue of obliterated follicles, phagocytosed necrotic cells and gradually disappeared. The abundance of rough endoplasmic reticulum in their cytoplasm suggests an increased synthesis of proteins, e.g. cytokines required for the apoptotic process. We assume that macrophages can play an important role in fibrosis and, by secreting fibroblast activation factor [20], accelerate the process of obliterative atresia. Various mediators, in particular histamine, released by the mast cells, may be greatly involved in the onset of the local immunopathological response by increasing the permeability of the *theca interna* capillaries, thus creating the conditions for the formation of edema and diapedesis of neutrophils, that we observed in the loose connective tissue of atretic follicles in our study.

In general, atresia is considered a functional ovarian disorder in which maturing follicles do not ovulate, undergo various forms of regression and luteinization and gradually vanish. The development of atresia is influenced by various factors, the most common being internal factors, especially hormonal misbalance of gonadotropins. For example, B i t e c o u r t et al. [3] found that endogenous gonadotropins in rats promoted ovulation and protected pre-ovulatory follicles from atresia.

Among the external factors, this may be, for example, the body condition of cows and level of milk productivity. In our previous study [15] the emaciated cows with BCS (body condition score) 1 or BCS 2 had a higher incidence of late (cystic) and lutenization-associated atresia in the antral ovarian follicles compared to the normal (BCS 3) body conditioned cows. Moreover, cows from the higher milk lactation group showed a higher incidence of non-ovulated antral ovarian follicles and increased incidence of cystic or luteinization-associated atresia, than cows with low milk yield [10]. These factors affect the ovarian status and ultimately lead to an increased incidence of various forms of atresia, which may affect the regularity of the estrous cycle and adversely influence the fertility of dairy cows.

Currently, miRNA-mediated regulation is widely valued in ovarian function studies. In particular, Zhang et al. [24] reported about bioinformatic prediction of key miRNA-mediated pathways in the follicular atresia, which suggests a causal link between the levels of certain miRNAs and the fate of granulosa cells or the whole ovarian follicles. A deep understanding of the roles of miRNA networks will not only help elucidate the mechanisms of granulosa cell apoptosis, follicular development, atresia and their disorders, but also offer new diagnostic and treatment strategies for infertility and other ovarian dysfunctions.

CONCLUSIONS

On the basis of our electron microscopy examination, we can conclude that the atresia of the ovarian follicles in cows appears in several forms. It is manifested by varying incidence of initial, obliterative and cystic forms of atresia, as well as luteinization-associated atresia of granulosa cells. Granulosa cells degenerate along with the oocyte, and the *stratum granulosum* of the antral follicles collapses after swelling of the basal membrane and disintegration of the *lamina basalis*. A loose connective tissue with macrophages grows among the degenerated granulosa cells, and the macrophages phagocytose them. Subsequently, the loose connective tissue of the vanishing atretic follicle hyalinizes and turns into a dense connective tissue that persists for a time in the form of a small white body—*corpus albicans*. Using the ultrastructural analysis, we obtained a morphological image as well as a sequence of regression processes characterizing some forms of atresia that take place in the atretic and luteinized antral follicles of cows. Ultrastructural examination could be used as a complementary method to improve histopathological diagnostics of cow reproductive organs in veterinary practice.

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