

Histomorphological and histochemical features of atresia of antral follicles of water buffaloes

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ABSTRACT

The present study aimed to characterize the atresia of antral follicles of buffalo. Two types of atresia, cystic and obliterative, were observed in the buffalo ovary. The cystic atresia was characterized by complete degeneration of the granulosa cells with the antrum filled with fluid. In obliterative atresia, the granulosa cells degenerated, and the surrounding theca cells started invaginating into the antrum. The ingrowth of these connective tissues filled the antrum. The histological picture of tertiary follicles showed that the atresia occurred in successive stages. The atretic changes observed were pyknosis of granulosa cells, the creation of small spaces between the cells, and the loosening of the cumulus oophorus cells. Two patterns were observed based on the start of atresia, i.e. antral atresia and basal atresia. At places, call-exner bodies were observed in the granulosa cell layer. The collagen fiber-containing basal lamina started separating from the theca layer at locations and later became hypertrophied in the cystic atresia type. In contrast, in the obliterative atresia, the collagen fibers were seen as abundant in growth towards the lumen and later, it filled the whole antral cavity. Disruptions of reticular fibers as stained with Gridley's stain were observed in the cystic type of atresia, and ingrowth of reticular fibers in the antrum and formation of corpus atreticum were contributed by reticular fibers also.

Keywords: Atresia, Antral Follicle, Buffalo, Histochemical, Histomorphological

In Asian nations, water buffaloes are raised mainly for milk and meat production. According to the 2019 Livestock Census, buffaloes, of number 109.85 million, make up around 20.45% of all livestock in India and produce 49.2% of all milk produced there, making it extremely important to the Indian economy (DAHD 2017-18).

The primary dynamic reproductive organ, the ovary, does both gametogenesis and steroidogenesis simultaneously. Folliculogenesis is a physiological process that ultimately results in either atresia or ovulation. The interaction between cell death and cell survival signals regulates the growth of ovarian follicles and atresia. Apoptosis is the primary cause of follicular atresia in the mammalian ovary (Hussein 2005, Liang *et al.* 2017). One of the critical causes of buffalo reproductive failure has been linked to an increased risk of follicular atresia (Rajesh *et al.* 2001). All stages of follicle development in ovaries are affected by follicular atresia, which is characterized by a stop in development and follicle degradation. The basal membrane, ovum, theca, membrane granulosa, and blood capillaries are all affected by degenerative changes (Irving-Rogers *et al.* 2009). Antral follicles may become

luteal cysts or succumb to premature luteinization. Under poor life circumstances, especially during starvation, such atresia in the follicles containing the oocytes can increase. Cystic atresia and atresia with luteinization are the most prevalent types of follicle degeneration in malnourished dairy cows (Makarevich *et al.* 2011). Pivko *et al.* (2012) observed an increase in the incidence of atresia in emaciated dairy cows, thus decreasing fertility. Singh *et al.* (2019) recorded follicular atresia in preantral follicles of buffalo ovaries. The higher prevalence of atresia, particularly cystic atresia in highly lactating cows, may negatively impact dairy cow fertility (Makarevich *et al.* 2018).

This work aimed to characterize the atretic process of antral follicles in bovine ovaries from a histological and histochemical standpoint.

MATERIALS AND METHODS

Sample collection and processing: The ovaries of buffaloes aged 2-7 years of age (n=48) were collected from the local abattoirs and Teaching Veterinary Clinical Complex. These samples were utilized for histomorphological and histochemical studies. The samples were fixed in 10% neutral buffered formalin for 48 h. Dehydrating in increasing grades of alcohols and acetone, clearing in benzene, and infiltrating and embedding in paraffin were the processes for paraffin sectioning.

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Sections were cut to 4-5 μm thickness for histological and histochemical studies (Pathak and Bansal 2012).

Histological staining: Paraffin sections were stained with haematoxylin and eosin for morphological studies. For the demonstration of collagen fibers and reticular fibers, cells were stained with Masson's trichrome and Griedley's stains, respectively.

Picrosirius red staining for collagen fibres: Paraffin sections were stained with Picrosirius red stain for collagen fiber, as described earlier by Choudhary *et al.* (2017). The sections were observed in a light and fluorescent microscope (in a TRITC filter). PSR-stained collagen appeared red in light microscopy and showed a red fluorescence in the TRITC filter.

Histochemical studies: For a histochemical demonstration of neutral mucopolysaccharides, acid

mucopolysaccharides, and both neutral and acid mucopolysaccharides, paraffin sections were stained with Periodic acid Schiff (PAS), Alcian blue (AB), and PAS-AB stains, respectively. The bromophenol blue staining method demonstrated essential proteins in the paraffin sections (Singh *et al.* 2019).

RESULTS AND DISCUSSION

Histological findings: The atresia present in this study's antral follicles of buffalo ovaries was of two types. They were cystic (Fig. 1A) and obliterate atresia (Fig. 1B), as described earlier by Sassan *et al.* (2016) in buffaloes and Makarevich *et al.* (2011) in dairy cows. In the cystic type of atresia, there was complete degeneration of the granulosa cells, and the antrum remained filled with fluid, thus giving the appearance of a cyst. In the second type, obliterate

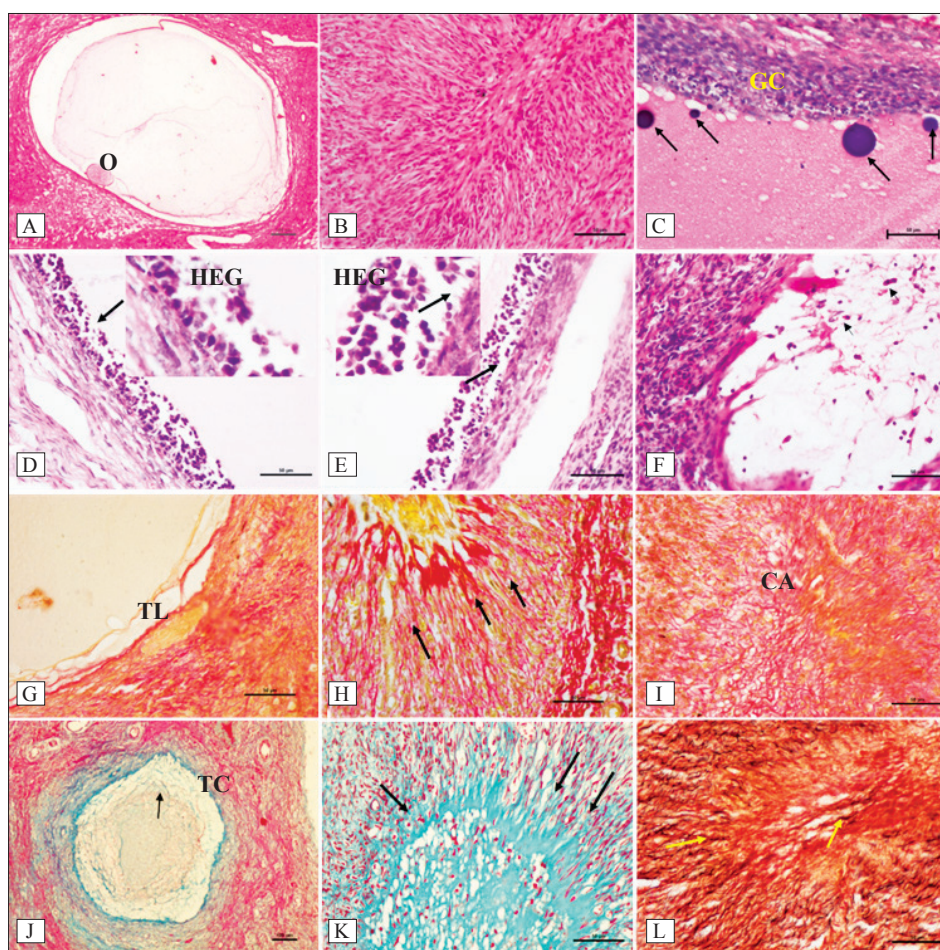


Fig. 1. Paraffin Section of buffalo ovary showing: A. Cystic atresia with oocyte (O) having no effect; B. Advanced stage of obliterate atresia showing collapsed follicle after invagination of proliferating cells; C. Tertiary follicle with the initiation of atresia with macrophages and pyknotic nuclei (arrows) in the granulosa cell layer and within the cystic fluid; D. initiation of atresia starting granulosa cells at the periphery; i.e. Antral atresia. Inset: vacuolation and somewhere healthy granulosa cells (HGC); E. The initiation of atresia started in the granulosa cells near the theca layer and basement membrane. Inset: disruption of granulosa cells towards thecal layer (arrow) and healthy granulosa cells (HGC) at the periphery; F. Obliterate atresia with proliferating cells (arrows) invigorating H and E stain. Original magnification A. $\times 100$; B-F. $\times 400$. G. Tertiary follicle with discontinuity in the theca layer (TL) also shows the cystic atresia type; H. obliterate atresia with invagination of proliferating cells (arrows); I. Advanced stage of obliterate atresia with corpus atreticum (CA) formation. Picro Sirius red stain. Original magnification G., H. and I. $\times 400$; J. Cystic type of atresia with intact theca cells (TC). Masson's trichrome stain $\times 400$; K. Advanced stage of obliterate atresia with collagen fibers invaginating inwards (arrows). Masson's trichrome stain $\times 400$; L. Follicle gone through the obliterate type of atresia showing invagination of proliferating cells (arrows). Gridley's reticular stain $\times 400$.

atresia, the surrounding theca cells started invading the antrum as the granulosa cells degenerated. Sassan *et al.* (2016) have given a similar description of buffaloes' ovaries. The ingrowth of these connective tissues filled the antrum. Similar observations have been recorded by Irving-Rodgers *et al.* (2001), Makarevich *et al.* (2018) and Pivko *et al.* (2020) in cows.

The histological picture of tertiary follicles showed that the atresia occurred in successive stages. Early atretic changes were noted as pyknosis of granulosa cells and the creation of small spaces between the cells. The initial symptom of atresia, pyknosis of granulosa cells, was suggestive of the overall strategy and course of atretogenic alterations in mammals. There was also a loosening of the cumulus oophorus cells. Theca layer appeared normal. The necrotic changes in the granulosa cells led to vacuoles in the membrana granulosa cells. Atretic changes were observed only in the granulosa. At an earlier stage of atresia, pyknosis of granulosa cells was observed in regions and not in general, and pyknotic nuclei were present only in the granulosa layer (Fig. 1C). In contrast, in tertiary follicles, the process begins at the level of the granulosa cells (Sreejalekshmi *et al.* 2011). Pivko *et al.* (2012) also observed degenerated cells with pyknotic nuclei on the surface, within each layer of membrana granulosa, and on the basal membrane in dairy cows. Celestino *et al.* (2009) reviewed the mechanisms of follicular atresia and described various morphological alterations in the apoptotic cells.

In tertiary follicles, certain nuclear particles resulting from pyknosis were scattered at the periphery of the antrum. Corona radiata of the oocyte was seen at the second stage of atresia. The oocyte was last to degenerate and was seen naked in the antrum, even at the third stage of atresia (Fig. 1A). The start of atresia was the presence of degenerative changes in the granulosa cells, which occurred in two patterns. In some follicles under degeneration, granulosa cells towards the antrum showed degenerative signs, while the basal cells were healthy (Fig. 1D and inset). This pattern of granulosa cell degeneration was called antral atresia.

In some other follicles under regression, granulosa cells towards the basement membrane and theca cells showed signs of deterioration, while the cells towards the antrum were healthy (Fig. 1E and inset). This pattern of granulosa cell degeneration was called basal atresia. These findings are in accordance with the classification suggested by Irving-Rodgers *et al.* (2001). They suggested that the initial elimination of granulosa cells proximal to the antrum characterized the antral atresia. Numerous pyknotic nuclei are evident in these antral layers of the membrana granulosa and sometimes within the antrum itself. Conversely, the basal granulosa cells, i.e. those aligning the basal lamina remained intact. They showed many ultra-structural characteristics of healthy cells, e.g. moderate numbers of mitochondria, lipid droplets, and moderate amounts of the endoplasmic reticulum. Antral atresia was viewed as the classic and most widely observed form of follicular atresia

because it occurs at all stages of follicle development in most species, and it is universally seen in large follicles (>5 mm in diameter), including the dominant follicle, of mono-ovulatory species (Irving-Rodgers *et al.* 2001). Basal atresia entails the destruction of the basal layer of the follicle, whereas most antral layers remain intact and healthy (Irving-Rodgers *et al.* 2001).

The basal lamina is often penetrated by macrophages and invading capillaries, and the theca layer of the follicle has an additional collagen deposition. The middle layers of the membrana granulosa exhibit a progression of cellular morphology and ultrastructure from the fragmented, pyknotic cells typical of the basal layers to the healthy, intact cells found in the antral layers. In the cow/heifer, this form of atresia occurs only in small follicles (<5 mm in diameter) (Irving-Rodgers *et al.* 2001).

At a later stage of follicular atresia, the cumulus oophorus cells and corona radiata cells also underwent degenerative changes, and at places, the denuded oocyte was seen. At this stage, at locations in the follicle, granulosa cells were detached from the theca layer. At places, call-exner bodies were observed in the granulosa cell layer. This structure comprised a central fluid-filled cavity surrounded by one layer of granulosa cells. Similar observations have been made by Sasan *et al.* (2016) in buffalo ovaries. Makarevich *et al.* (2011) recorded the histopathological alterations characterizing atresia of antral follicles in dairy cows. They observed the successive changes as degenerative changes in the nuclei of granulosa cells (pyknosis) and separation of the basement membrane from the basal layer of granulosa cells followed by the destruction of granulosa and theca cells. According to Pivko *et al.* (2020), there are several manifestations of ovarian follicular atresia in cows. It shows different incidences of luteinization-associated atresia of granulosa cells and initial, obliterative, and cystic forms of atresia. Granulosa cells degenerate along with the oocyte, and the antral follicles' stratum granulosum collapses after swelling the basal membrane and disintegrating the lamina basalis.

In the second and third stages of follicular atresia, phagocytic cells were observed in the membrana granulosa layer and the antrum. These phagocytic cells included macrophages, lymphocytes, and neutrophils (Fig. 1F). Pivko *et al.* (2020) concluded that the degenerating granulosa cells were surrounded by loose connective tissue filled with macrophages that phagocytose them.

In the cystic type of atresia, most granulosa cells degenerated and resorbed by these phagocytic cells and left behind a cavity filled with fluid, and in the obliterative type, proliferating theca cells invaded the cavity and filled the lumen with connective tissue (Fig. 1B). Studies suggested that follicular atresia is a complex physiological process regulated by endocrine and paracrine changes. Granulosa cell apoptosis is considered the primary potential mechanism for follicular atresia (Hussein 2005, Liang *et al.* 2017). As atresia progressed, the granulosa layer gradually became thinner, and theca cells hypertrophied

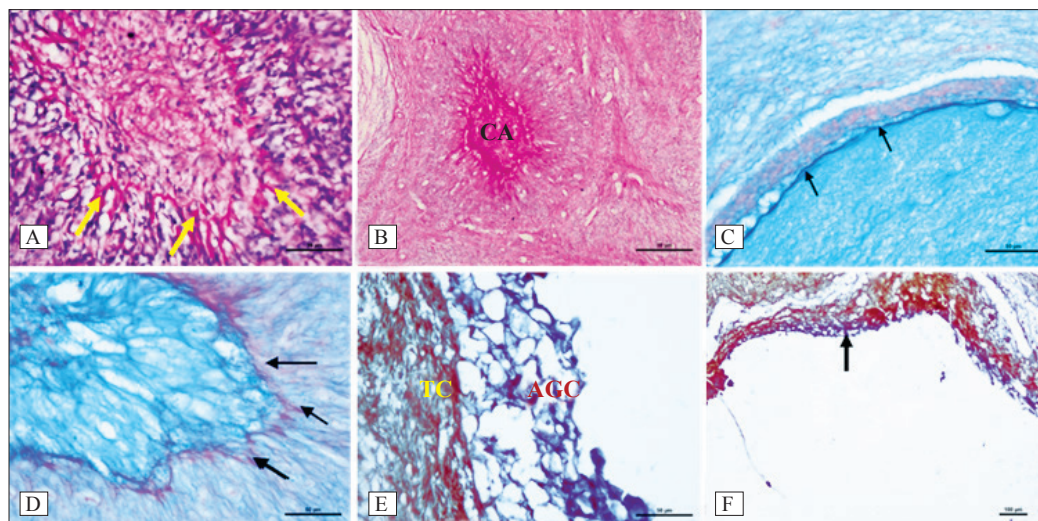


Fig. 2. Paraffin section of buffalo ovary showing: A. Obliterative type of atresia, cells invaginating along with basal lamina (arrows). PAS stain $\times 400$; B. Obliterative type of atresia (advanced stage) corpus atreticum (CA). PAS stain $\times 400$; C. Cystic atresia with PAS-positive reaction in the granulosa cells (arrows). PAS-AB stain $\times 400$; D. Obliterative atresia type with basal lamina invading inwards (arrows). PAS-AB stain $\times 400$; E. Apoptotic granulosa cells (AGC) stained blue, showing apoptosis and breakage in the theca cell layer (TC). Bromophenol blue stain. Original magnification $\times 400$; F. Paraffin section of buffalo ovary showing apoptosis in granulosa cells (arrow) stained blue in tertiary follicle showing cystic atresia. Bromophenol blue stain. Original magnification $\times 400$.

to form theca-type interstitial cells in some follicles; with the advancement of atresia, the theca cells hypertrophied to form theca-type interstitial gland cells, which, by losing cytoplasm and lipids, ultimately revert to the stromal element lying in the scars left behind after the degeneration of the follicle. Similarly, Pastor *et al.* (2001) recorded many apoptotic cells in the granulosa and in the theca during atresia of antral follicles in pigs.

The picosirius red-stained sections showed that the basal lamina was distinctly stained and separated the granulosa layer and theca interna layer and was visible when viewed in the fluorescent microscope. The collagen fiber containing basal lamina started separating from the theca layer at places, and later stage became hypertrophied in the cystic type of atresia (Fig. 1G). In the obliterative type of atresia, the collagen fibers were abundant in amount towards the lumen and later, it filled the whole antral cavity (Fig. 1H and 1I). The formed structure is called corpus atreticum. Sasan *et al.* (2016) also described in the buffalo ovary that the antrum was filled with connective tissue, and the degenerated follicle was termed as corpus atreticum.

The observations recorded by picosirius red-stained sections were also supported by the observations made on the Masson's trichrome-stained sections. During the early stage, a few collagen fibers penetrated the antrum (Fig. 1J) and filled the lumen (Fig. 1K). The reticular fibers stained with Gridley's stain were seen as black wavy fibers in the theca layers and basal lamina of a healthy tertiary follicle. Disruptions of reticular fibers were observed in the cystic type of atresia (Fig. 1L) and ingrowth of reticular fibers in the antrum and formation of corpus atreticum was contributed by reticular fibers also. The variable distribution of these fibres suggests atresia being a dynamic process involving cellular reorganization.

According to Pivko *et al.* (2020), the fading atretic follicle of dairy cows features a loose connective tissue that hyalinizes and transforms into thick connective tissue, which survives for a while in the form of a little white body, the corpus albicans.

Histochemistry: In the obliterative type of atresia, strong PAS-positive reactions were observed in the invading cells and the margin of the atretic follicle (Fig. 2A). The corpus atreticum was intensely positive for neutral mucopolysaccharides (Fig. 2B). In the cystic type of atresia, fluid inside atretic follicle showed PAS-positive solid reaction. A distinct line of PAS-positive reaction was observed on the wall of the cystic follicle. Alcian blue-positive mucopolysaccharides and PAS-positive neutral mucopolysaccharides were distinctly localized in the wall of the cystic atretic follicle (Fig. 2C), as revealed by PAS-AB staining. Fluid in the cystic atretic follicle was Alcian blue positive. The early stage of obliterative atresia showed both acidic and neutral mucopolysaccharides. In the advanced stage of obliterative atresia, the reaction was stronger for Alcian blue and PAS (Fig. 2D). Singh *et al.* (2019) observed the location of neutral and acid mucopolysaccharides in atretic preantral follicles of buffaloes. Based on a PAS-positive reaction, they recorded the hyalinization and folding of basal lamina in the atretic follicle. The presence of a strong accumulation of carbohydrates in both types of atresia suggests a secretory process by degenerating cells and cells undergoing remodelling during the process of follicular atresia.

Sasan *et al.* (2009) recorded strong to intense reaction for PAS staining in membrana granulosa of atretic follicles showed whereas it was low in oocytes of buffaloes. It has been reported that surface carbohydrates of granulosa cells contain hyaluronic acid, sulphated glycosaminoglycans and

chondroitin sulphate. These carbohydrates may be involved in adhesion of granulosa cells, thus playing an important role in follicular growth, atresia and luteinization.

Bromophenol blue-positive protein was strongly localized in the membrane granulosa layer in a healthy tertiary follicle. At the same time, a moderate reaction was observed in the theca layer. In the early-stage atresia, the antral granulosa cells were positive for bromophenol blue, while the basal cells were negative. This might be an antral form of the atretic follicle. In the advanced stage of atresia, a bromophenol blue positive reaction was observed in the membrana granulosa layer. However, a disrupted appearance was observed, significantly reducing at the third degree of atresia (Fig. 2E and 2F). In the obliterative atresia type, bromophenol blue-positive proteins were present in the invading proliferating cells. The abrupt protein localization of the atretic cells reflected the process of cell death, leading to a differential chemical secretory process as compared to the healthy follicles.

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