Ultrastructural features of atretic ovarian follicles in buffalo (*Bubalus bubalis*)


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ABSTRACT

The process of follicular development and features of morphological changes are essential to understand the factors involved during folliculogenesis and to improve fertility in buffalo. Hence, the objective of the present study was to report ultrastructural features of atretic ovarian follicles in buffalo. In the early stage of atresia, the involvement of mitochondria was observed and whereas in the later stages of atresia, involvement of macrophages were observed. The granulosa cells of buffalo atretic tertiary follicles were observed to contain undulation of the nuclear membrane, crescent formation of the pyknotic nuclei and loopiness and undulations of the basal lamina. These features are similar to the reports in other species. The involvement of mitochondria in the early stage of atresia suggested the probable role of a reactive oxygen free radical induced damage.

Key words: Atresia, Buffalo, Electron microscopic features, Follicle.

INTRODUCTION

The ovarian follicular development occurs in waves and only a very few number of follicles (<1%) that begin to grow successfully ovulate (Hughes and Gorospe, 1991). The remaining follicles demise by apoptosis, a physiologically active process in which cell death occurs in a controlled fashion. The atresia limits the number of oocytes available for fertilization and embryonic development. The morphological criteria used to classify the follicles as atretic were the presence of pyknotic nuclei (shrunken nuclei staining strongly with hematoxylin) in the membrana granulosa or the antrum (Blondin *et al.*, 1996), nuclei with marginated chromatin, a single condensed nucleus, multiple nuclear fragments, and/or membrane bound structures containing variable amount of chromatin and or apoptotic bodies (Arends *et al.*, 1990; Hsueh *et al.*, 1994).

The atretic process is reported to be different in buffaloes as compared to cattle (Feranil *et al.*, 2004). In buffalo, low primordial follicular pool along with higher rate of atresia resulted in poor reproductive efficiency. Hence studies on follicular atretic features have considerable implication for fertility control and treatment of infertility (Aboul-Ela 2000; Sreejalekshmi *et al.*, 2011). Identification of mechanism involved in the process of follicular atresia will provide an insight into the intricate factors involved in follicular development and methods of manipulation for improving reproductive efficiency. So far in buffalo, changes in ultrastructural features during atretic process are not described in detail. Hence, the present study was undertaken to describe the ultrastructural features associated with atresia in buffalo ovarian follicles.

MATERIALS AND METHODS

Ovaries (n=20) were procured from visually non-pregnant buffaloes from the Corporation Slaughterhouse, Bangalore. The ovaries, collected immediately after slaughter, were transported in ice cold buffer to the laboratory. The ovaries were then washed in normal saline to remove the blood clots adhering to it. The stages of estrous cycle were assessed by the appearance of follicle and corpus luteum (Ireland *et al.*, 1980 and Selvaraju *et al.*, 2010). The ovaries were longitudinally sliced at approximately 4-5 mm thick for further studies.

The dissected ovaries were fixed by immersing in 2.5% glutaraldehyde in 0.1M phosphate buffer for 24 hours at 4°C. The specimens were subsequently washed in 5% sucrose in 0.1M phosphate buffer, post fixed in aqueous 2% osmium tetroxide for 60 minutes at 4°C and rinsed with distilled water three times each for five minutes. The tissue was dehydrated using ascending concentration of acetone, infiltrated with epoxy resin at room temperature overnight and cured with fresh resin overnight at 60°C. The sections of 0.5µm were cut, stained with 1% methylene blue prepared in 1% sodium tetraborate and examined by light microscope. Then the sections of 50 nm were made, stained with uranyl acetate and Reynolds lead citrate (Bancroft and Stevens, 1996) and observed under transmission electron microscope (Hitachi H-7500, work done at College of Veterinary Science, Rajendranagar, Hyderabad).

RESULTS AND DISCUSSION

The atretic Graafian follicles had highly condensed pyknotic nuclei in the apoptotic granulosa cell layer (Plate1).
Further, the presence of deformed nuclei and intracytoplasmic vesicles were also observed. The nuclear envelope was observed to be undulated and the condensed chromatin adhered to the nuclear envelope resulted a crescent shape appearance. The cytoplasmic vacuolations characterized the alterations in the integrity of the cytoplasmic organelles (Plate 1). The multivesicular bodies were prominent in the cytoplasm and the remnants of nuclear material, lysosomes formed apoptotic bodies (Plate 2). The intercellular space contained abundant electron dense material representing the blebbing and budding of the cells to form apoptotic bodies.

In some of the granulosa cells, cytoplasmic vacuolation was wide spread without defining the membrane of functional organelles. In a very high magnification, there was disruption of the architecture of the mitochondria characterized by damage of the mitochondrial membrane, condensation and loss of cristae. The loss of organelles was also detected. In focal areas of the granulosa cell layer, the alteration in the shape of the granulosa cells were observed indicated by blebbing of the cytoplasm capped by the plasma membrane. In addition, a large number of highly condensed electron dense cytoplasm with reduced organelles and ribbon-like appearance of electron dense structures were observed.

The basal lamina aligning the basal surface of the basal granulosa cell layer exhibited loopiness and the undulation of the basal lamina was directed inwards into the granulosa cell layer. In some areas, the thecal cells were observed to project into the granulosa cell layer. Healthy granulosa cell phagocytosing an apoptotic body was observed. Macrophages were not detected in the present study. The cell debris without distinct membranes but with condensed chromatin was observed. The cell debris was observed to be surrounded by neighbouring granulosa cells. A normal oocyte with a well defined nucleus and nucleolus was observed in the granulosa cell.

In the present study, it was observed that, during initial stages of atresia, there were indented mitochondria with irregular shape, reduction in cristae and changes in the matrix density (Plate 1c). In the terminal stages of atresia, the mitochondrial membrane was ruptured leaving empty remnants of the mitochondrial structure. In the last stages, the nuclear membrane showed large number of indentations or rupture and cytoplasm contained small to very large vacuoles.

The molecular mechanism involved in the ultrastructural features of atresia involves a cascade of events. Highly condensed pyknotic nuclei observed in the granulosa cell layer was in conformity with the finding in humans (De Pol et al. 1997) in bovines (VanWezel et al. 1999) and pigs (Park et al. 2004). The condensed chromatin in the pyknotic nuclei was uniformly electron dense as reported in bovine (VanWezel et al. 1999). The present study confirms that the granulosa cell degeneration during follicular atresia in buffaloes occurs by apoptosis as reported in other species (Kaipia and Hsueh, 1997; Yang and Rajamahendran, 2000). The undulations of the nuclear envelope and vacuolation of the nucleus was observed in the granulosa cell layer indicated characteristic features of apoptosis. The alterations in the integrity of the cytoplasmic organelles such as large number of intracytoplasmic vacuoles mitochondrial membrane damage, and loss of cristae observed in the study were similar to the report in goats (Silva et al. 2001).

The blebbing of the cytoplasm, loopiness and undulation of the basal lamina was also observed in atretic ovarian follicles as reported earlier by Irving-Rodgers et al. (2000 and 2001). In addition, a large number of highly electron condensed cytoplasm with reduced organelles and ribbon-like appearance of electron dense structures were observed in the cytoplasm as reported in rats by Ortiz et al. (2006). The vacuolation of the cytoplasm leading to formation of apoptotic bodies is in agreement with the findings of Devine et al. (2000) and Silva et al. (2001). These apoptotic bodies will be phagocytosed by the neighbouring granulosa cells and during phagocytosis, the lysosomes of the granulosa cells fuses with the apoptotic bodies to form secondary lysosomes (Peluso et al. 1980; Devine et al. 2000).

The study revealed the early involvement of mitochondria in the apoptotic process and suggested the role of a reactive oxygen free radical induced damage. Since, mitochondria are the sites of the oxygen free radical production, mitochondrion is the first organelle to show degeneration. Through the permeability transition pores, the oxygen radical come out and induce cellular changes (Kitagawa et al. 1993; Keefe et al. 1995). The absence of macrophages observed during early part of apoptosis whereas, phagocytosis of the apoptotic bodies and cell debris by macrophages were detected only during later stages of atresia (Sugimoto et al. 1998).

For apoptosis to occur, signals either in the form of positive inducers included Fas ligand and Bcl-2 family of ligands maintained ion channels to promote mitochondrial homeostasis and integrity (Kalekar and Thompson, 1998). The apoptosis inducer proteins dimerized with anti-apoptotic proteins leading to creation of nonfunctional or dysfunctional pores and compromised mitochondrial integrity. The cell death might be due to the translocation of cytochrome-C from the inner mitochondrial membrane to the cytosol (Kalekar and Thompson, 1998). The activated cytochrome-C released from mitochondria is caused by the mitochondrial swelling and rupture of outer mitochondrial membrane. The binding of cytochrome-C with apoptosis activating factor (Apa-1) activated Caspase-3 (Susin et al. 1996). Then the caspases entered into the nucleus and fragmented the chromatin after activating the endonucleases (Arends et al. 2004).
Plate 1: The electron microscope showing (a): atretic changes in granulosa cells (X7000), (b): apoptotic body being phagocytosed by neighbouring normal granulosa cell (X3500) and (c): loss of cristae in the mitochondria of granulosa cells undergoing atresia (X12000). (Arrow: undulation; VES: vesicles; AP: Apoptotic bodies; Cr: Crescent formation; M: Mitochondria; N: Nucleus; L: Lysosomes; V: Vacuole; cd: cytoplasmic condensation; MB: Multi-vesicular body)
1990). The externalization of the phosphatidylserine in the plasma membrane makes these cells to be bound to the receptors on the macrophages culminating in their phagocytosis. The observed ultrastructural changes in the buffalo ovarian granulosa cells followed similar patterns of these molecular mechanisms.

REFERENCES