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ULTRASTRUCTURE OF OVARIAN FOLLICLES OF BUFFALO (*Bubalus bubalis*) IN CAUVERY DELTA DISTRICTS

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Abstract: The ovaries from non-pregnant buffaloes were post fixed in aqueous 1% Osmium tetroxide and processed for transmission electron microscopic study. Highly condensed pyknotic nuclei were observed in the apoptotic granulosa cell layer and prominent multivesicular bodies detected in the cytoplasm. The intercellular space contained abundant electron dense material representing the blebbing and budding of the cells to form apoptotic bodies. There was disruption of the architecture of the mitochondria characterized by damage of the mitochondrial membrane, condensation and loss of cristae. The granulosa cell layer showed alteration in the shape due to blebbing of the cytoplasm capped by the plasma membrane. The basal lamina aligning the basal surface of the basal granulosa cell layer exhibited loopiness and undulation of the basal lamina directed inwards into the granulosa cell layer. Undulations of the nuclear envelope and vacuolation of the nucleus was observed in the granulosa cell layer. Atretic changes of granulose cells viz. indented mitochondria with irregular shape, reduction in cristae and changes in the matrix density, mitochondrial membrane rupture leaving the empty remnants, appearance of small to very large vacuoles were recorded in the present study. The apoptotic bodies phagocytosed by the neighbouring granulosa cells by release of lysosomes leading to the formation of multiple small vacuoles was noticed.

Keywords: Ultrastructure, buffalo, ovary, follicles and atresia.

Introduction

Buffalo is the major contributor for the white revolution and ovary is the root organ for such progressive development. Buffalo (*Bubalus bubalis*) farming is an important source of income to rural population and plays a significant role in livestock production and agricultural economy of India. The reproductive efficacy is hampered in buffaloes due to the factors viz. inherent late maturity, poor estrous expression with seasonal reproductive patterns and prolonged calving intervals due to postpartum delayed ovarian activity.

Studies on ovarian follicle have considerable implication for fertility control and treatment of infertility. In buffalo, less than one percent of the growing follicles ovulate successfully while ninety nine percent undergo atresia at various stages of follicular development (Mariana *et al.,* 1991). Atresia limits the number of oocytes available for fertilization and embryonic

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development. Recent studies have demonstrated that the death of the granulosa cell during follicular atresia in the ovaries of domestic animals occurs by apoptosis- a physiologically active and governed process whereby death of cell occurs in a controlled fashion triggered by changes in the level of specific physiological stimuli (Hughes and Gorospe, 1991). According to Hsueh *et al.,* (1994) a reduction in number of mitotic nuclei were found to be an indication of early stage of atresia, followed by the destruction of the whole membrana granulosa and cumulus. Identification of mechanism involved in the process of follicular atresia will provide an insight into the intricate factors so as to reduce the higher infertility rate.

Materials and Methods

Ovaries from visually non-pregnant buffaloes were procured from the slaughterhouse, immediately after slaughter and transported in icebox to the laboratory. The ovaries were then washed in normal saline to remove the blood clots. Stages of estrous cycle were assessed by the appearance of follicle and corpus luteum. Four or five longitudinal slices of each approximately 4-5 mm thickness were made.

The dissected ovaries were fixed in 2% gluteraldehyde in Phosphate Buffer for 6 hours at 4^0C. Subsequently the specimens were washed in Phosphate Buffer, post fixed in aqueous 1% Osmium tetroxide for 15 minutes at 4^0C, rinsed thrice in distilled water for five minutes each. The tissue was dehydrated using ascending concentration of acetone, infiltrated with epoxy resin at room temperature overnight, cured with fresh resin overnight at 60^0 C. Sections of 0.5µm were cut, stained with 1% toludine blue. Sections of 100 nm were made and stained with uranyl acetate and Reynolds lead citrate (Bancroft and Stevens, 1996).

Result and discussion

The Graafian follicles were subjected to transmission electron microscopy. Highly condensed pyknotic nuclei were observed in the apoptotic granulosa cell layer (Figure 1). Further, the presence of deformed nuclei and intracytoplasmic vesicles were also observed (Figure 3). Undulations of the nuclear envelope and the condensed chromatin adhering to the nuclear envelope presented a crescent shaped appearance (Figure 1). Alterations in the integrity of the cytoplasmic organelles characterized by cytoplasmic vacuolations were observed (Figure 1). Prominent multivesicular bodies detected in the cytoplasm as containing the remnants of nuclear material, lysosomes formed apoptotic bodies (Figure 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. Loss of organelles was also detected (Figure 3). 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 (Figure 4). In addition a large number of highly condensed electron dense cytoplasm with reduced organelles and ribbon-like appearance of electron dense structures were observed in the cytoplasm (Figure 5).

The basal lamina aligning the basal surface of the basal granulosa cell layer exhibited loopiness and undulation of the basal lamina directed inwards into the granulosa cell layer (figure6). In some areas the thecal cells were observed to project into the granulosa cell layer.

Highly condensed pyknotic nuclei observed in the granulosa cell layer is in confirmity with the finding of Depol *et al*. (1997) in human, VanWezel *et al*. (1999) in bovines and Sugimoto *et al*. (1998) and Park *et al*. (2004) in pigs. The condensed chromatin in the pyknotic nuclei was uniformly electron dense as reported by VanWezel *et al*. (1999). The deformed nuclei and the crescent shaped nuclei observed in the present study agreed with the reports of VanWezel *et al*. (1999) in bovines. Hence, the present study confirms that the granulosa cell degeneration during follicular atresia in buffaloes occurs by apoptosis as described by Hughes and Gorospe (1991) and Kaipia and Hsueh (1997) in rats and Yang and Rajamahendran (2000) in bovines.

Undulations of the nuclear envelope and vacuolation of the nucleus was observed in the granulosa cell layer as observed by Assey *et al*. (1994) in cattle. Alterations in the integrity of the cytoplasmic organelles such as mitochondria and large number of intracytoplasmic vacuoles were observed in the present study. The changes detected in the mitochondria were damage of the mitochondrial membrane, mitochondrial condensation and loss of cristae. These finding get support from Devine *et al*. (2000) in rats, Silva *et al*. (2001) in goats and deBruin *et al*. (2002) in human. As reported by deBruin *et al*. (2002) during initial stages of atresia, there will be indented mitochondria with irregular shape, reduction in cristae and changes in the matrix density. In the terminal stages of atresia the mitochondrial membrane will rupture leaving the empty remnants of the mitochondrial structure. In the last stages, the nuclear membrane will show large number of indentations or rupture and the largest part of

the cytoplasm will consist small to very large vacuoles. All these features were recorded in the present study.

The early involvement of mitochondria in the apoptotic process suggested the role of an oxygen radical induced damage. As reported by Alonso-Pozos *et al*. (2003) there will be changes in the mitochondrial membrane potential and formation of permeability transition pores during atresia. Mitochondria are the sites of the oxygen free radical production and it is the first organelle to show degeneration. Through the permeability transition pores, the oxygen radical come out and induce cellular changes as reported by Harman (1972), Kitagawa *et al*. (1993) and Keefe *et al*. (1995).

Vacuolation of the cytoplasm observed in the present study is in agreement with the findings of Devine *et al*. (2000) and Silva *et al*. (2001). Devine *et al*. (2000) observed secondary lysosomes containing cell debris as the predominant feature observed in atretic follicles. As reported by Sugimoto *et al*. (1998) the cell debris may be composed of degraded apoptotic bodies. These apoptotic bodies will be phagocytosed by the neighbouring granulosa cells true to the observations of the present study. During phagocytosis lysosomes of the granulosa cells fuses with the apoptotic bodies to form secondary lysosomes (Peluso *et al*., 1980 and Devine *et al*., 2000) in rats. Blebbing of the cytoplasm and alterations in the shape of the granulosa cells observed in the present study gets support from Peluso *et al*. (1980). According to Kerr *et al*. (1972) during apoptosis plasma membrane will introflex forming deep incisions called blebbing leading to very irregular appearance. Apoptotic bodies observed in the present study confirm with the findings of Depol *et al*. (1997).and Sugimoto *et al*. (1998). The apoptotic bodies are membrane enclosed particles containing extracellular materials (Depol *et al*. 1997).

Initially, inspite of the intraflexing and blebbing of the plasma membrane, the cellular permeability remains unaltered at the beginning and the organelles maintain their morpho-functional integrity. Later the cells will bleb and bud into spheroidal subunits surrounded by membranes. They contain portions of the cytoplasm and nucleus. They are called apoptotic bodies. Later they were phagocytosed by the neighbouring granulosa cells leading to the formation of multiple small vacuoles as reported by VanWezel *et al*. (1999) in bovines.

The loopiness and undulation of the basal lamina is observed in atretic buffalo ovarian follicles. Similar observations were found in the present study (Irving-Rodgers *et al*., 2001). In addition, a large number of highly condensed electron dense 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 absence of macrophages in the present study was in confirmity with the findings of Inoue *et al*. (2000) and Nourani *et al*. (2005). Phagocytosis of the apoptotic bodies and cell debris by macrophages were detected only during later stages of atresia (Sugimoto *et al*., 1998).

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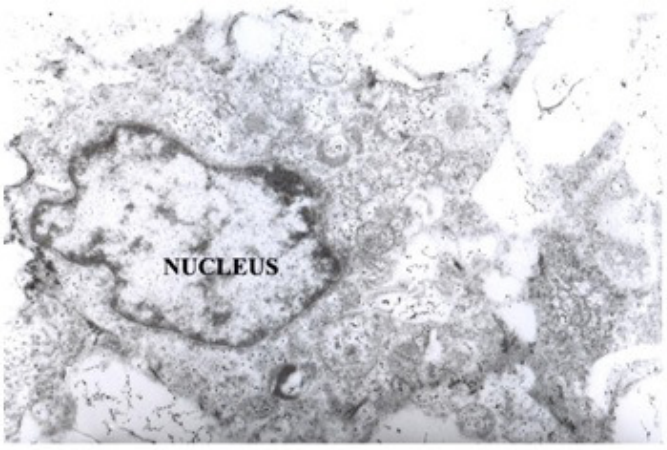

FIGURE 1.

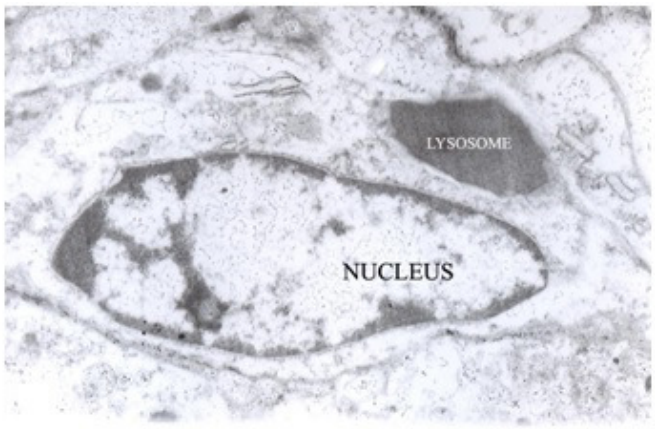

FIGURE 2

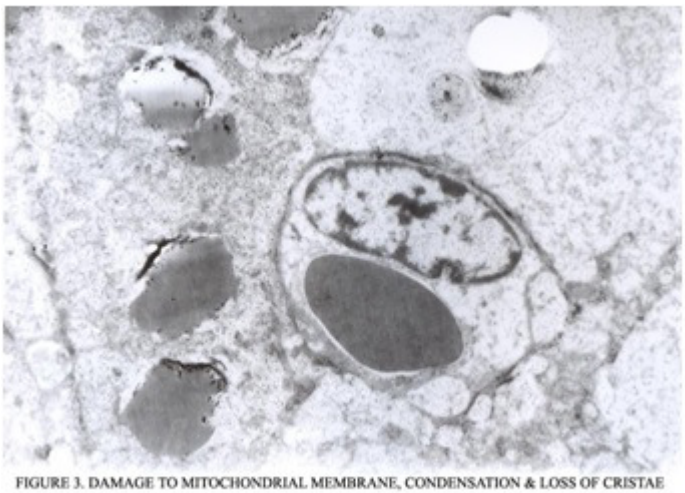

FIGURE 3. DAMAGE TO MITOCHONDRIAL MEMBRANE, CONDENSATION & LOSS OF CRISTAE

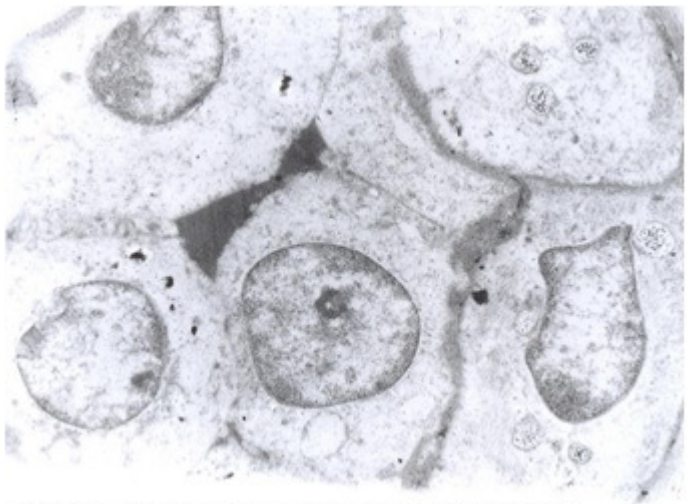

FIGURE 4. SHOWING ALTERATIONS IN SHAPE OF GRANULOSA CELLS

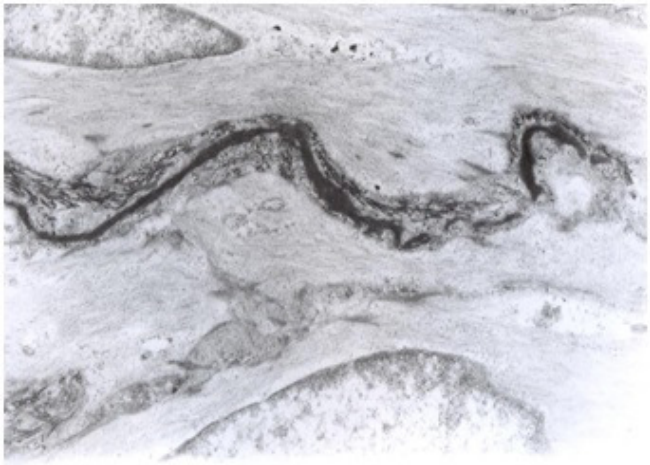

FIGURE 5. RIBBON LIKE APPEARANCE OF ELECTRON DENSE STRUCTURES

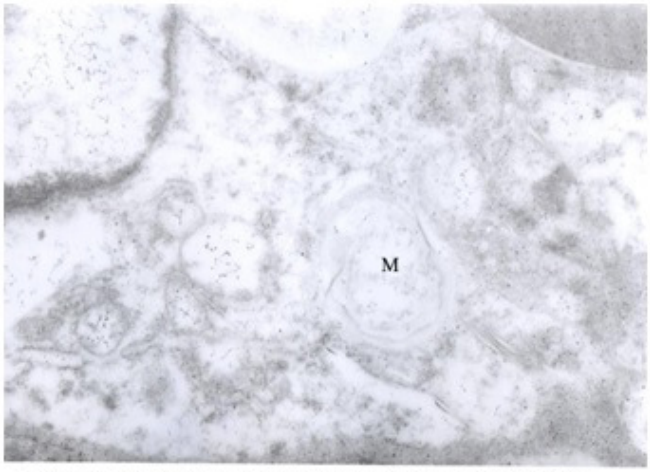

FIGURE 6. LOOPINESS OF BASAL GRANULOSA CELL LAYER AND MITOCHONDRIA (M)