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Apoptosis and Ovarian Follicular Atresia in Mammals

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1. Introduction

The reproductive performance of any mammalian species can be enhanced by enriching nutrition, regulating environmental factors like photoperiod, temperature, humidity etc., through selective breeding, endocrine manipulations and better management practices (Sharma, 2000; Maillet *et al.*, 2002; Bussiere *et al.*, 2002; Iwata *et al.*, 2004, 2005; Rudolf, 2007; Bhardwaj and Sharma, 2011). The endocrine regulation despite being the most complicated is very effective in improving reproductive output of the species. In females, follicular growth and estrous cyclicity are intricately linked (Craig *et al.*, 2007; Sharma and Batra, 2008). Of the thousands of oocytes and primordial follicles present in neonatal ovary only a small fraction i.e. approximately 0.001% are ovulated throughout the active reproductive life span of mammals (Tabarowski *et al.*, 2005; Sharma and Bhardwaj, 2009). Follicular atresia is a wide spread phenomenon that limits the number of ovulations and thus restricting the full reproductive potential of a species. It results in extensive loss of germ cells during development, prenatal, neonatal, prepubertal, pubertal, estrous cycle, pregnancy, lactation and post reproductive life of mammals (Guraya, 1997, 1998; Sharma, 2000; Manabe *et al.*, 2003, 2004; Bhardwaj and Sharma, 2011). Follicular atresia is a natural fertility regulatory mechanism that can best be exploited for increasing or decreasing the fertility of the species by decreasing or increasing the frequency of atresia. It is, therefore a lot of research papers have been published on morphology, histochemistry, biochemistry and endocrinology of follicular atresia (Williams and Smith, 1993; Guraya, 1998, 1999; Monniaux, 2002; Sharma, 2003; Sharma and Batra, 2008; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma, 2011). However, the mechanism of atresia still needs to be further explored and analysed for its effective implementation in fertility regulation programme. The molecular mechanism of atresia can be best explained on the basis of apoptosis (Palumbo and Yeh, 1994; Sharma, 2000; Yu *et al.*, 2004; Sharma and Bhardwaj, 2009). Apoptosis, a type of physiological cell death is the antithesis of mitosis involved in the regulation of tissue homeostasis (Collins and Lopez, 1993; Schwartzman and Cidlowski, 1993). It affects the single scattered cells in the midest of living tissues without eliciting an inflammatory response and is influenced by growth factors and hormones. Many studies on the morphological changes that occur in granulosa cells and theca-interstitial cells of follicles progressing through atresia have documented that apoptosis is, in all likelihood, the primary mechanism by which cell loss is mediated during follicle degeneration (Tsafriri and Braw, 1984; Hirshfield, 1991; Tilly, 1996). The earliest descriptions of apoptosis, the physiological cell death, in the ovary was made in

1885 on the morphological analysis of granulosa cells during the degeneration of antral follicles in the rabbit ovary (Flemming, 1885). A process referred to as 'Chromatolysis' was proposed as a mechanism by which granulosa cell loss was mediated, and in retrospect these observations closely matched all of the morphological criteria now known to be the hallmarks of apoptosis (Kerr *et al.*, 1972, 1994). In the ovary, the mechanisms underlying decisions of life and death involve cross dialogue between pro-apoptotic and pro-survival molecules (Hussein, 2005). Apoptosis operates in ovarian follicles throughout fetal and adult life. During fetal life, apoptosis is restricted to the oocytes, whereas in adult life, this phenomenon is frequently observed in granulosa cells of secondary and antral follicles (Hussein, 2005).

Apoptosis, a genetically regulated cell suicide is an energy requiring process observed commonly during early embryonic development. Apoptosis permits the safe disposal of cells at the point in time when they have fulfilled their biological functions (Hussein, 2005). The ovary represents the paradigm of effective apoptosis during active reproductive period of the animal. Apoptosis is central to many aspects of the ovary and executed by several molecular pathways. Bcl-2 (B-cell lymphoma 2) family, TNF(Tumor necrosis factor), caspases and Transforming growth factor-β TGF-β proteins constitute well established mechanisms of apoptosis (Hussein, 2005). Morphologically, apoptosis is characterized by cell shrinkage, membrane blebbing and cytoplasmic fragmentation in oocytes (Wu *et al.*, 2000; Chaube *et al.*, 2005), granulosa cells (Sharma and Sawhney, 1999; Sharma, 2003; Sharma and Bhardwaj, 2007, 2009) and corpus luteum (Sharma and Batra, 2005). A unique biochemical event in apoptosis is the activation of a Ca^{+2}/Mg^{+2} dependent endogenous endonuclease. This enzyme cleaves genomic DNA at internucleosomal regions, resulting in chopping of DNA in to fragments of 180-200 base pairs (bp). These DNA fragments can be visualized as a distinct ladder pattern by agarose gel electrophoresis. The presence of this DNA pattern in cells is considered a hallmark of apoptosis (Schwartzman and Cidlowski, 1993; Hsueh *et al.*, 1994; Yang and Rajamahendran, 2000). In addition to the detection of oligonucleosomes in isolated DNA, the occurrence of apoptosis is also inferred from the characteristic morphological appearance of degenerating cells, together with the detection of fragmented DNA in single cells *in situ* through the use of the 3' end-labeling technique (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling [TUNEL] (Gavrieli *et al.*, 1992; Palumbo and Yeh, 1994; Sharma and Bhardwaj, 2009). The early development of mammalian ovarian follicles is poorly understood. Although follicle stimulating harmone FSH and luteinizing harmone LH are essential for follicular development from the secondary stage onwards, but the onset of growth of primordial follicles is independent of gonadotrophin hormones (Tilly, 1996). Factors initiating the transition of quiescent primordial follicles to the pool of growing follicles are still unknown. Studies on animals have indicated that several locally produced growth factors are involved in the multifactorial regulation of the growth of primary and secondary follicles (Durlinger, 2000). According to recent findings, the loss of follicles by atresia would be counteracted by the formation of new primordial follicles from germ line stem cells (Johnson *et al.*, 2004), a condition still controversial and is yet to be confirmed (Bristol Gould *et al.*, 2006; Liu *et al.*, 2007). Throughout active reproductive life, follicles leave the resting repository pool to enter the growing pool on a regular basis and pass through subsequent developmental stages under the influence of stage specific subset of intra-ovarian regulators and endocrine factors like growth factors, cytokines and gonadal steroids (Gilchrist *et al.*, 2004, Pangas, 2007). At

different developmental stages, follicles behave differently in response to factors promoting follicular cell proliferation, growth, differentiation and apoptosis and very few reach upto ovulation (Tilly *et al.*, 1991; Jiang *et al.*, 2003; Craig *et al.*, 2007).

A large number of follicles undergo atresia in the late pre-antral to early antral stages. During this hormone dependent growth phase, a selection operates whether to allow the growth of the follicles upto the pre-ovulatory stage or not (Durlinger *et al.*, 2000). Gonadotropins are survival factors that prevent atresia (Uilenbroek *et al.*, 1980; Chun *et al.*, 1994). In addition, the growth factors like epidermal growth factor, transforming growth factor-α, basic fibroblast growth factor (Tilly *et al.*, 1992), insulin like growth factor (Chun *et al.*, 1994); and cytokine interleukin (IL)-1β (Chun *et al.*, 1995), also check onset of atresia. The activity of catalase in follicular fluid and granulosa cells exhibited a declining trend from healthy to atretic follicles. Catalase is an ubiquitous enzyme found in all known organisms. It catalyzes the breakdown of hydrogen peroxide to water and molecular oxygen (Aebi, 1984 and Vohra, 2002). The activity of enzyme superoxide dismutase shows a declining trend from healthy to slightly atretic to atretic follicles both in the follicular fluid and granulosa cells. The superoxide dismutase (SOD) family of metalloenzymes are antioxidants that protect cells from the deleterious effects of the oxygen free radical superoxide (Fridovich, 1975, 1986). SOD catalyzes the conversion of superoxide anion in the oxygen and hydrogen peroxide. Hydrogen peroxide is further converted to oxygen and water by catalase and peroxidases. Trace elements have also traditionally been known to play an important role in cellular homeostasis which entails the tight regulation of cell death. Although zinc known as microelement prevents or suppresses apoptosis, several published reports have clearly demonstrated that zinc may actively induce cell injury and cell death (both apoptosis and necrosis) in malignant as well as normal cells (Iitaka *et al.*, 2001; Bae *et al.*, 2006; Wiseman *et al.*, 2006; Rudolf, 2007; Bhardwaj and Sharma, 2011). Zinc has been shown to induce apoptosis as well as necrosis by altering calcium homeostasis via the generation of oxidative stress, with subsequent activation of mitogen activated protein kinases (MAPKs), via p53 – dependent and p53 independent signaling or through its interaction with the cytoskeleton (Feng *et al.*, 2002; Chen *et al.*, 2003). The role of reactive oxygen species (ROS) and anti-oxidants in relation to female reproductive function has, in contrast, received relatively little attention, although there are evidence of both physiological and pathological effects (Guerin *et al.*, 2001; Chaudhary *et al.*, 2004).. Yang *et al.*, (1998) have found high levels of hydrogen peroxide in fragmented embryos and unfertilized oocytes, whilst Paszkowski and Clarke (1996) have reported increased antioxidant consumption (suggesting increased ROS activity) during incubation of poor quality embryos. Recent studies indicate that apoptosis of granulosa cells shows a close relationship between estradiol and progesterone titre in the follicular fluid (Yu *et al.*, 2004; Sharma and Batra, 2008; Sharma *et al.*, 2008). The level of insulin like growth factor-1 IGF-1 but not insulin like growth factor-II IGF-II is the crucial factor in deciding whether a follicle will mature or undergo atresia (Yu *et al.*, 2004). The role of trace elements as pro-apoptotic or anti-apoptotic factors is not well known till date. The cascade of morphological and biochemical alterations need to be studied to rescue the germ cells or somatic cell losses.

A large population of ovarian follicles in mammals is lost through a wide spread phenomenon of follicular atresia that limits the number of ovulations thus restricting the full reproductive potential of a species (Sharma, 2000; Yu *et al.*, 2004; Tabarowski *et al.*, 2005;

Slomczynska *et al.*, 2006; Sharma and Bhardwaj, 2009). Follicle atresia is a wide spread degenerative phenomenon by which follicles lose their structural integrity and oocytes are lost from the ovaries other than the process of ovulation (Guraya, 1997; Sharma, 2000). Follicular atresia decreases the number of ovulations and the follicle wall components transmutate into a steroidogenically functional interstitial gland tissue (Sharma and Guraya, 1992; Manabe *et al.*, 2003) that helps in the endocrine regulation of ovarian physiology. Atresia affects the follicles at all stages of development but extensive loss of germ cells occurs during early development, prenatal, neonatal, prepubertal, pubertal, estrous cycle, pregnancy, lactation and post reproductive life of mammals (Guraya, 1998; Sharma, 2000; Tabarowski *et al.*, 2005). During early development primordial follicle population is the most affected whereas in the active reproductive phase the frequency of atresia is maximum in antral follicles (Danell, 1987; Guraya *et al.*, 1994, Guraya, 2000; Sharma, 2000) The modulation of frequency of atresia can regulate the fertility of the animal (Sharma, 2000). Follicle atresia is characterized by the appearance of pyknotic granulosa cells in intact membrana granulosa; free floating large sized granulosa cells with pyknotic nuclei; hyalinization of granulosa cells; chromolysis of the granulosa cell nuclei and their enucleation; loosening of the intercellular matrix; delamination of intercellular matrix; colloidal, opaque and cloudy follicular fluid; detachment of cumulus-oophorous complex from mural granulosa cells; appearance of RBCs in the follicle; invasion of connective tissue fibres within the follicle and huge accumulation of follicular fluid resulting in the cyst formation (Sharma, 2000; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma, 2011).

2. Apoptosis in normal cycling mature ovary

In prepubertal and mature mammalian ovaries the preantral atretic follicles, the atretic oocyte is characterized by shrinked ooplasmic contents surrounded by a peripheral zone of lipoidal components which is strongly sudanophilic. This zone in a few atretic oocytes is so dense that it occuludens the central mass. (Sharma *et al.*, 1992; Guraya *et al.*, 1994). Initially, pyknosis in granulosa cells was restricted only to specific area which consequently prolonged and large zone of atretic pyknotic granulosa cells was formed, which results in loosening of membrana granulosa (Sharma *et al.*, 1992). These pyknotic granulosa cells give positive test for lipids and 3-β-HSDH, indicating their active role in steroidogenesis (Guraya *et al.*, 1994). Pyknosis of granulosa cells as the first sign of atresia is evocative of the general plan and path of atretogenic changes in mammals (Guraya *et al.*, 1987; Tilly *et al.*, 1996; Burke *et al.*, 2005; Tatone *et al.*, 2008; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma, 2011). The various nuclear and ooplasmic contents are drastically affected in atretic preantral follicles. The organelles showing abnormal morphology and distribution increase in number and proceed towards disintegration and then lead to an accumulation of lipids in the oocytes and degenerating granulosa cells because of liberation of cytosolic membrane bound lipids (Byskov, 1978). In the atretic preantral follicles the granulosa cells develop histochemical characteristics of steroidogenic cells as evidenced by weak 3-β-HSDH activity (Sharma, 2000). In antral follicles, apoptosis is characterized by the presence of pyknotic nucleus in the membrana granulosa, appearance of spaces and release of fragments of pyknotic nuclei in the peripheral zone of antrum in type 1b (Sharma *et al.*, 1992; Guraya *et al.*, 1994; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma,2011). The granulosa cell glyco-conjugates are changed during follicular atresia in the pig and rat ovaries as shown with lectins (Sharma and Guraya 1992; Kimura *et al.*, 1999). The levels of fibronectin, laminin,

type IV collagen, proteoglycans, insulin like growth factor II/mannose 6 phosphate receptors, and matrix metaloproteinases 2 and -9 increased whereas 450 aromatase and connexin 43 decreased within the wall of granulosa cells during follicular atresia in sheep (Huet *et al.*, 1997, 1998, Sharma and Guraya 1998a, b). Guraya *et al.*, (1994) have demonstrated that the degenerative signs also appear in cumulus cells of antral atretic follicles, whereas atresia proceeds gradually, membrana granulosa thins off and theca hypertrophy enhances in antral atretic follicles (Sharma *et al.*, 1992; Guraya *et al.*, 1994). The appearance of pyknotic granulosa cells as the first sign of atresia in goat strongly advocate the concept that phenomenon of atresia in bovine species is similar (Zimmerman *et al.*, 1987; Wezel *et al.*, 1999; Hastie and Haresign, 2006). The chromophilic pyknotic granules observed in goat atretic follicles were similar to that of DNA - positive masses observed in sheep (Hay *et al.*, 1976, Hay and Cran 1978; Zhou and Zhang, 2005). However such granular material is negligible in cow (Guraya, 1997). The delamination of mural granulosa cells from the basal lamina leads to disruption causing a decline in oxygen carrying blood transudate that decreases the metabolic pace and results in onset of atresia (Guraya, 1985, 1998; Sharma and Guraya, 1998 b, c). However, Hay *et al.*, (1976) suggested that it is because of decline in estrogen synthesis and release. In advance stage of atresia, there is a tendency towards accumulation and storage of sudanophilic lipids in the atretic oocytes (Guraya, 1973 a). The mural layers acquire more lipids as compared to cumulus cells or peripheral granulosa cells. Similar trends were reported in hamster and rabbit (Guraya and Greenwald, 1964), thus indicating granulosa heterogeneity in morphology and physiology (Parshad and Guraya 1983; Sharma and Guraya, 1990). The NADH and NADPH-dependent tetrazolium reductase activity in goat further endorse this concept. The theca interna of atretic antral follicles undergo hypertrophy and develop lipid droplets largely comprised of phospholipids which are associated with steroidogenesis in mammals (Guraya, 1973 b, 1974 a, b, 1977, 1978 a,b; Nicosia, 1980). This hypertrophied theca interna in atretic follicles of goat finally constitute conspicuous masses of interstitial gland tissue which show strong 3-β-HSDH activity (Guraya, 1971, 1973; Guraya *et al.*, 1994; Sharma and Batra, 2008). These lipid droplets are largely phospholipids and triglycerides which change to cholesterol, its esters and phospholipids, and remain associated with theca type interstitial gland tissue. Various carbohydrates, proteins and enzyme histochemistry were used as an index to indicate whether atresia has initiated or not (Guraya, 1984, 1985; Sharma and Guraya, 1992, 1997; Burke *et al.*, 2005). The follicles undergoing atresia disintegrate oocytes and granulosa cells are reabsorbed while theca interna hypertrophy and acquire morphological and histochemical characteristics of a steroidogenic tissue (Sharma and Batra, 2008).

3. Structure and ultrastructure of the process

Intrafollicular paracrine steroid interactions are dependent on FSH and LH which regulate follicular development and oestrogen synthesis and release (Guraya, 1985, 1998; Ireland, 1987; Richards *et al.,* 1993; Gore-Langton and Armstrong, 1994; Hillier, 1994; Guthrie and Cooper, 1996; Maillet *et al.,* 2002; Burke *et al.,* 2005). Alterations in the production of steroid in the antral fluid are the first biochemical manifestations of atresia. (Guraya, 1985, 1998; Hillier, 1985; Greenwald and Terranova, 1988; Westhof *et al.,* 1991; Gore-Langton and Armstrong, 1994; Greenwald and Roy, 1994; Quirk *et al.,* 2006). Variations in normal and atretic follicular fluid concentration of androgens and that of progesterone are not significantly different (Hillier, 1985; Westhof *et al.,* 1991; Moor, 1977; Gore-Langton and

Armstrong, 1994; Slomczynska *et al.,* 2006). Hormonal profile of normal and atretic follicles depends largely on the stage of advancement of atresia. Normal1y it is oestrogen to progesterone ratio which determines whether a follicle will mature or undergo atresia (Moor *et al.,* 1978; Ireland and Roche, 1982, 1983 a, b; Guraya, 1997; Yu *et al.,* 2004; Sharma *et al.,* 2008). The estrogen level is highest in the preovulatory healthy follicles of pig, sheep, cow, and goat as compared to atretic ones (Eiler and Nalbandov, 1977; Moor *et al.,* 1978; Carlson *et al.,* 1981; Fortune *et al.,* 1988; Burke *et al.,* 2005; Sharma *et al.,* 2008). Proliferation, migration, differentiation and cellular death constitute the most important stages in the development and growth ovarian follicles and is actively involved in turnover of ovarian tissues. The cellular death basically involves two pathways: necrosis or apoptosis (Wyllie *et al.,* 1980; Kressel and Groscurth, 1994). Necrosis is induced as the result of injuries or environmental pathological influences and produces a series of cellular alterations that begin with the changes in cellular membrane permeability with consequent disruption of cytoplasmic structures and ensuing nuclear degeneration (Pol De *et al.,* 1997). On the other hand, apoptosis is the process of cellular self destruction which also involves active process of intracellular synthesis (Wyllie *et al.,* 1980; Cohen and Duke, 1984) and is controlled by cellular genes. In granulosa cell apoptosis, the primary events observed were the nuclear compaction, the chromatin collapses in to large irregular masses surrounded by nuclear envelope, and plasma membrane introflexes forming deep incisions that confer to the cell a very irregular appearance (Sharma and Bhardwaj 2009; Bhardwaj and Sharma, 2011). Despite this, the cellular organelles maintain their morpho-functional integrity. Subsequently the cell fragments into spheroidal subunits surrounded by membranes (apoptotic bodies) that contain portions of cytoplasm and nucleus that are finally phagocytosed by neighbouring cells or by macrophages. The cellular death in granulosa cells therefore is distinguishable on accounts of histological and ultrastructural morphology, and pattern of changes in cell organelles and the chromatin. In necrosis, chromatin was altered at the end in a disorderly fashion, whereas during apoptosis it was precociously excised in a regular sequence. The apoptotic changes observed in the degenerating granulosa cells from goat ovarian follicles revealed diminished size, withered surface morphology and pyknosis (Hay and Cran, 1978; Hirshfield, 1983; Guraya, 1985; Kaur and Guraya, 1987; Tilly, 1996; Manabe *et al.*, 2003, 2004; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma, 2011). The goat granulsoa cells resemble rat atretic granulosa cells in terms of nuclear condensation and cytoplasmic shrinkage as well as presence of apoptotic bodies (Hurwitz *et al.,* 1996). The undulations of nuclear membrane and pinching off of the apoptotic bodies strongly advocate the concept that the apoptosis is the basic mechanism involved in the phenomenon of atresia of granulosa cells in mammals. The vacuolization of condensed chromatin material within the nucleus was the specific positive indicator of apoptosis (Figs. 1, 2). Various studies, conducted during atresia of ovine and caprine follicles, cumulatively indicate that degenerative changes are restricted only to membrana granulosa layers that lie adjacent to the antral cavity during early phases of atresia (Sharma *et al.,* 1992). The detachment or delamination of mural granulosa cells from the basal lamina in the initial phases induce atresia due to disruption of oxygen and nutritional milieu carying blood transudate to the cells (Guraya, 1985, 1998; Sharma and Guraya, 1998 b, c; Sharma, 2000, 2003). Ultrastructurally, typical apoptotic granulosa cells from the antral follicle of goat show compaction and segregation of chromatin, condensation of the cytoplasm maintaining the integrity of organelles, and subsequent fragmentation in to membrane bounded apoptotic bodies (Figs.3, 4). Necrotic cells are characterized by irregular

clumping of chromatin, swelling and dissolution of organelles, rupture of the cell membrane, and finally disintegration of all cellular components. Ultrastructural changes in degenerating granulosa cells of goat ovaries strongly support the morphological hallmarks of apoptotic cells. First, the nucleus and cytoplasm became condensed, then the condensed cells were fragmented but retained the integrity of organelles, i.e. mitochondria, and nuclear and plasma membrane. Since cell debris in atretic follicles increased in number following the appearance of apoptotic bodies and had condensed nuclei similar to apoptotic bodies, the debris is possibly comprised of degraded apoptotic bodies. The investigations on fine morphology of granulosa cells *in vivo* showed similar characteristic features as observed after gonadotrophins and steroid treatment in rat *in vitro*, thus, confirming the role of hormones in structural modification of granulosa cells at different phases of the cycle (Balboni and Zecchi, 1981; Yu *et al.*, 2004; Sharma *et al.*, 2008; Sharma and Batra, 2008).
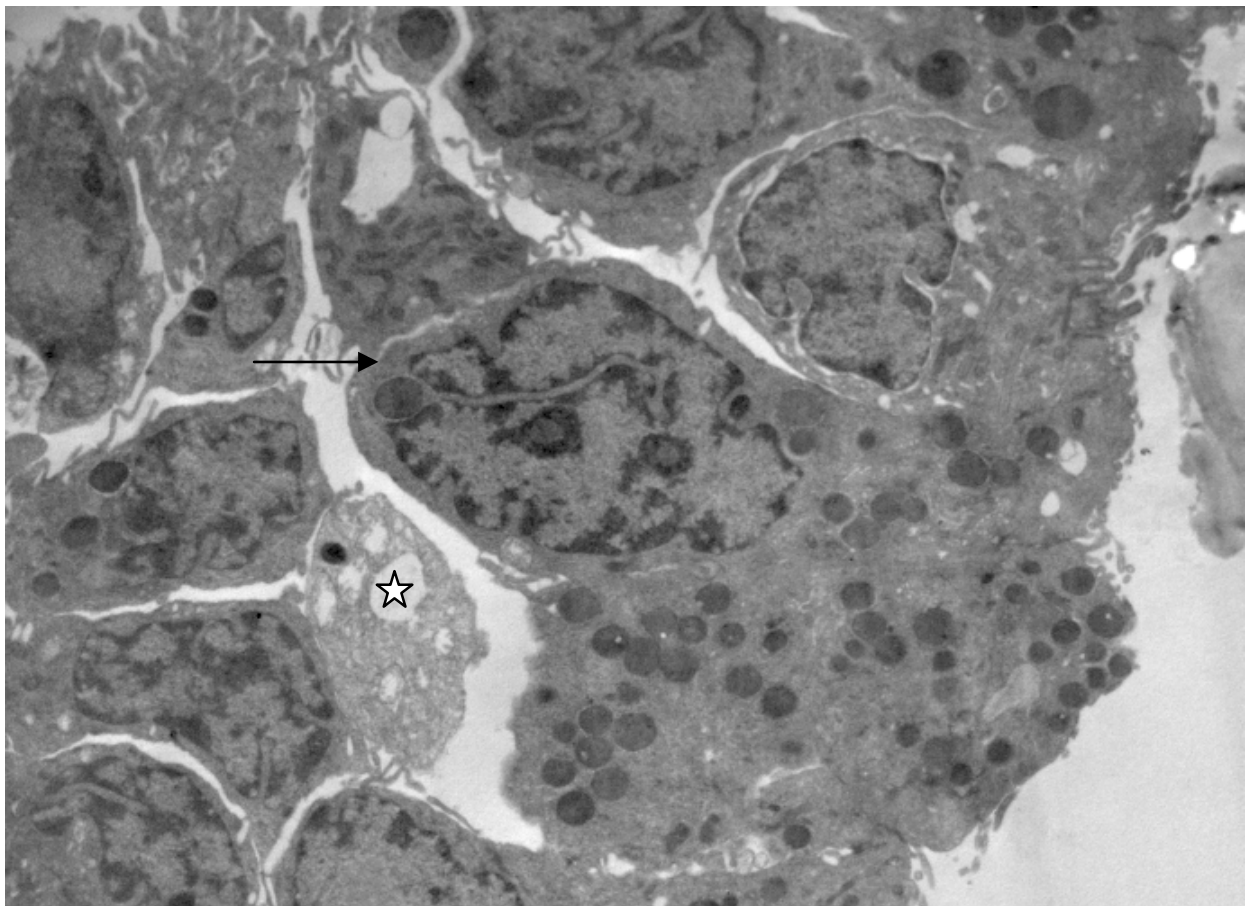

Fig. 1. Electron micrograph of apoptotic granulosa cells showing increased indentation (arrow) and vacuoles (star) of different shapes within condensed chromatin material.

Withdrawal of typical intercellular microvilli or interacting surfaces with the advancement of atresia, the appearance of lysosomal vacuoles and lipid droplets of varied dimensions observed during atresia in goat, further support the histological and histochemical characteristics of follicular atresia in mammals (Motta, 1972; Parshad and Guraya, 1983; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma,2011). The presence of lysosomal vacuoles in the degenerating granulosa cells of goat during advanced stage of atresia further support the findings of Sharma and Guraya (1997) who have reported that lysosomal

vacuoles appear in the cytoplasm which subsequently destabilize plasma membrane completely leading to the extrusion of interacellular contents making it more hyaline.
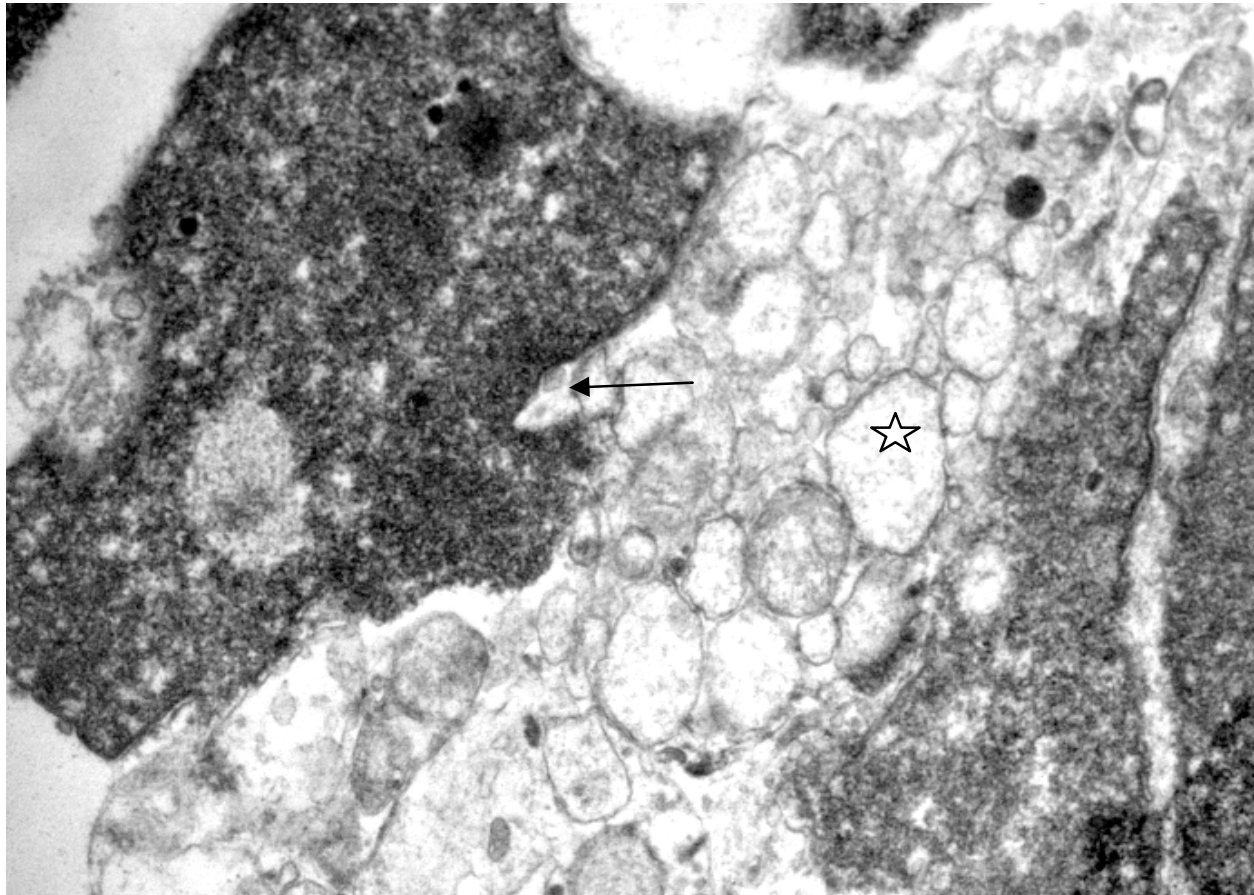

Fig. 2. Electron micrograph of apoptotic granulosa cells showing uneven wavy nuclear envelope (arrow) and increased vacuolization (star) of cytoplasm.

The presence of hyaline granulosa cells in the mural and antral layers of membrana granulosa and free floating in follicular fluid were observed in goat ovary (Sharma and Bhardwaj, 2007). The similar distribution of cells were also observed in primates and other mammals (Byskov, 1974; Balboni and Zecchi, 1981; Bill and Greenwald, 1981; Sharma and Guraya, 1992, 1997). The increased undulation and indentations of the nuclear membrane and pinching off of the nuclear fragments suggests that apoptosis is involved in initiation and execution of cell death during atresia in goat. The increase in the frequency and dimensions of nuclear pores as well as flattening of the nuclear membrane observed in goat follicles are similar to the earlier findings on ultrastructure of apoptotic granulosa cells in rat and cow (Coucouvanis *et al.*, 1993; Grotowski *et al.*, 1997; Isobe and Yoshimura, 2000; Yang and Rajamahendran, 2000; Inoue *et al.*, 2003), thereby suggesting a common plan of apoptosis in bovine species. The membrane bound pyknotic chromatin material carrying apoptotic vesicles were observed lying within the cytoplasm during the advanced stages of atresia which endorse the concept that apoptotic bodies are formed from condensed chromatin material packed in small vacuoles limited by the nuclear membrane. In a few cells, the presence of condensed cytoplasm in contrast to hyaline one was possibly due to the differential functional impairment of the cytoplasmic membrane. Sharma and Guraya

(1992) have reported changes in glycoconjugates and carbohydrates of atretic granulosa cells in rat and have postulated that changes in histochemical mapping of negatively charged moieties induces uncoupling of membrane interactions subsequently leading to impairment of membrane permeability characteristics that finally lead to atresia or cell death due to apoptosis. The alterations in acidic phospholipids phosphatidyl serine content that acts as apoptosis inducing agent (Krishnamurthy *et al.,* 2000), modulates the membrane chemistry leading to a change in its permeability to water molecules. The cell becomes larger and hyaline if the permeability is enhanced whereas the contents become pyknotic and electron dense if the permeability decreases. Recent studies have demonstrated that apoptosis involves cleaving of DNA in several animal species. Internucleosomal DNA fragmentation has been considered to be characteristic of apoptosis and is one of the earliest event (Schwartzman and Cidlowski, 1993; Okamura *et al.,* 2001; Hastie and Haresign, 2006).
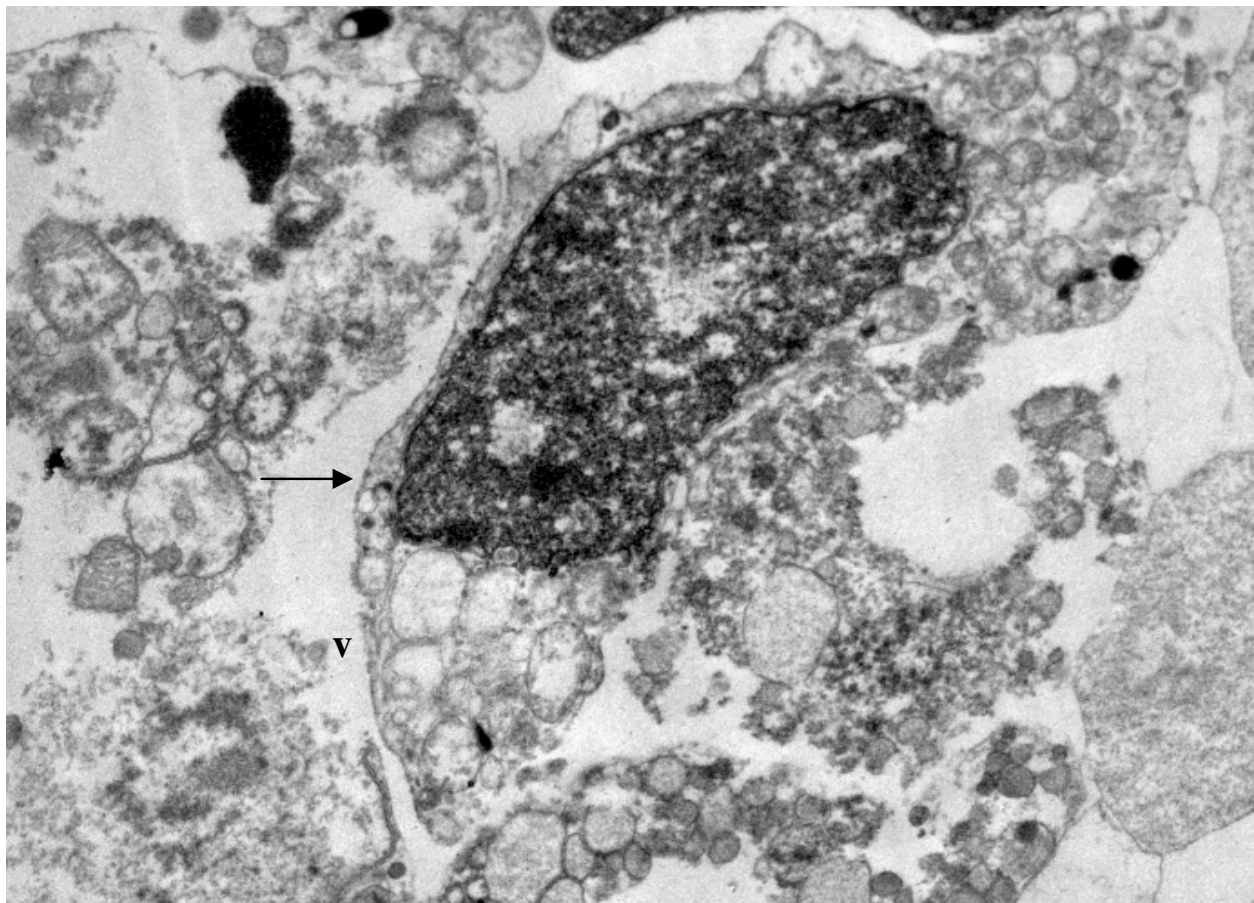

Fig. 3. Electron micrograph of apoptotic granulosa cells showing the pyknotic chromatin material adhering to the periphery of nuclear membrane (arrow) and vacuoles (v) of different shapes and sizes were observed within condensed chromatin material.

In addition to the detection of oligonucleosomes in extracted DNA, the occurrence of apoptosis may also be inferred from the characteristic morphological appearance of degenerating cells, together with the detection of fragment DNA in single cells *in situ* using TUNEL (Gavrieli *et al.,* 1992; Palumbo and Yeh, 1994; Bristol and Gould *et al.,* 2006; Sharma and Bhardwaj, 2009; Bhardwaj and Sharma,2011). Using *in situ* 3' end labeling (TUNEL), which can detect apoptosis precisely at the single cell level without disruption of the tissue

morphology (Gavrieli *et al.,* 1992; Palumbo and Yeh, 1994; Liu *et al.*, 2007; Sharma and Bhardwaj, 2009), the specific morphological features of granulosa cell death in follicular atresia (nuclear pyknosis, karyorrhexis, and formation of apoptotic bodies) can be related to the physiological process of apoptosis. The relationship is supported by a combination of biochemical, classic histological evidences, and *in situ* histochemical localization of DNA fragmentation. Different cellular details were observed in atretic and healthy follicles classified by morphological criteria, including cells with a single shrunken and dense nucleus (pyknotic appearance) and cells with marginated chromatin and/or nuclear fragmentation. According to Lussier *et al.,* (1987), non atretic follicles should have intact and normal granulosa layers with the mean pyknotic index per class varying from 0.13 percent to 0.67 percent. However, in another study in cows (Ireland and Roche,1983), pyknotic cells were observed in the granulosa cell layer in 30-60 percent of estrogen-active large follicles.
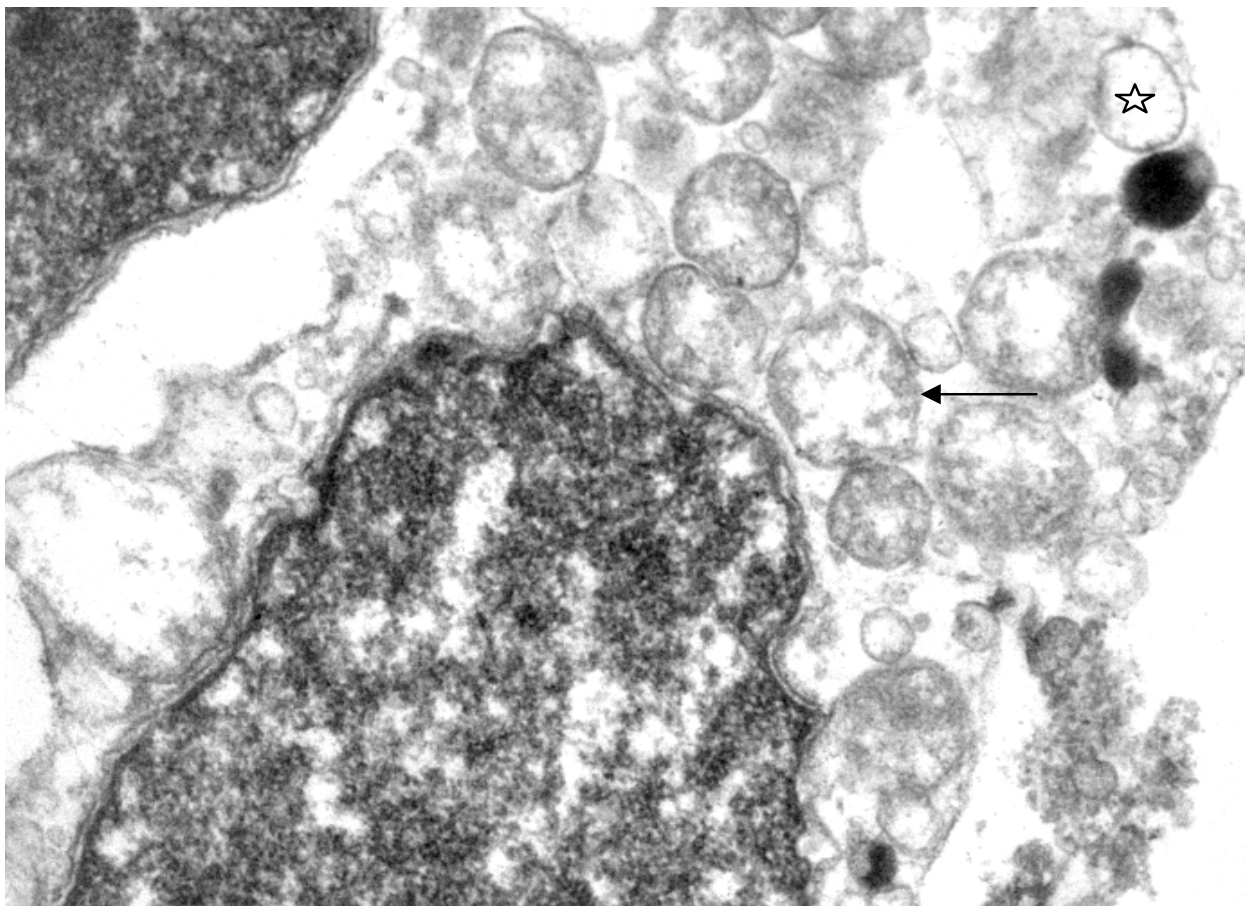

Fig. 4. Electron micrograph of granulosa cells revealing vacuolated cytoplasm (star) and mitochondria (arrow).

Thus, the mere presence of pyknotic cells in the granulosa cell layer does not imply that they are atretic. However, the morphological and biochemical results strongly indicate that apoptosis may occur to a certain level during normal follicle growth and development and that apoptotic death of granulosa cells may be detectable before other morphological and biochemical signs of degeneration in goats. Alkaline phosphatase activity in follicular fluid and granulosa cells exhibited a declining trend from healthy to slightly atretic and atretic follicles. The biochemical estimation of alkaline phosphatase endorse the earlier

histochemical mapping of alkaline phsophatase activity opining the possible role of alkaline phsophatase in active transport of nutrients and secretary material across the membrane (Verma and Guraya, 1968; Sangha and Guraya, 1988/89; Sharma, 2000). The association of alkaline phosphatase positive sites with theca interna indicates the involvement of this enzyme in steroid metabolism and transport (Britenecker *et al.,* 1978; Gilchrist *et al.,* 2004). The decline in levels of alkaline phosphatase in follicular fluid and granulosa cells of atretic follicles may be tangibly due to increased vascularity and changed morphology and biochemistry of granulosa cells for steroid hormone synthesis (Guiseppe, 1983; Gilchrist *et al.,* 2004; Pangas, 2007; Tatone *et al.,* 2008). Acid phosphatase activity in follicular fluid and granulosa cells exhibited an increasing trend from healthy to slightly atretic to atretic follicles. The increase in Golgi complex and lysosomes in atretic follicles/cells is possibly attributable to the rise in acid phosphatase activity. The ultrastructural modifications associated with the lysosomal accumulation during atresia which is further increased due to luteinization wherein chief protein synthesizing cells transmutate to steroidogenic cells may be attributable to the increase in acid phosphatase activity in the granulosa cells (Dorrington *et al.* 1975; Armstrong and Dorrington, 1976; Sangha and Guraya, 1988/89). The degenerative/transformative changes involved in reshaping of ovarian subcellular components that facilitate differentiation during follicular development while bringing about lysis and formation of apoptotic vesicles may be responsible for the rise in lysosomal activity (Sangha and Guraya, 1988/89; Sharma, 2000). The increase in acid phosphatase enzyme activity observed in the follicular fluid and granulosa cells of atretic follicles may also be related to some mechanism for the secretion of steroids (Sawyer *et al.,* 1979; Dimino and Elfont, 1980; Pangas, 2007). It has been reported that acid phosphatase activity is higher in active and regressing corpora lutea, provides a lurking possibility that during follicular atresia the rise in acid phosphatase activity may not be associated exclusively with regression but also with the formation of interstitial gland tissue. Catalase is generally associated with superoxide dismutase, constituting a reciprocally protective set, while catalase is inhibited by oxyradicals, and SOD is inhibited by H_2O_2 (Lapluye, 1990). If H_2O_2 produced by SOD, action on oxyradicals is not removed immediately it will react with super oxide radicals (Haber-Weiss reaction) giving rise to highly reactive hydroxyl radicals (Michiels *et al.,* 1994). However, with the increase in H_2O_2 concentration, the catalase contribution for its degradation concomitantly increased (Verkek and Jond Kind, 1992). Michiels *et al.,* (1991) reported a 30 percent increase in survival when catalase was injected in combination with SOD, whereas the survival was only 21 percent and 4 percent for SOD and catalase respectively, when independently injected in human fibroblasts. Singh and Pandey (1994) reported an increased catalase activity in the ovary of metaoestrous rats *Pari pasu* with a decline in H_2O_2 production in the mitochondria and microsomal fraction. In addition to its effects on oxygen free radical metabolism, SOD has been shown to influence cell functions by increasing the levels of the second messenger cGMP (Ignarro *et al.,* 1987; Schmidt *et al.,* 1993; Burke *et al.,* 2005). There are three known forms of SOD with specific subcellular and extracellular distributions (Ravindranath and Fridovich, 1975; Crouch *et al.,* 1984; Redmond *et al.,* 1984; Tibell *et al.,* 1987). The manganese-associated form of SOD is localized in mitochondria of cells, whereas the copper/zinc associated form is found in the cytoplasm. Furthermore, there is an extracellular form of SOD that is secreted from cells. All three forms of SOD are expressed in the ovary (Laloraya *et al.,* 1988; Shiotani *et al.,* 1991; Sato *et al.,* 1992; Hesla *et al.,* 1992, Tilly and Tilly, 1995) and the pattern of expression appears to

be related to gonadotropin induced follicular development and luteal steroidogenesis and regression (Laloraya *et al.*, 1988; Hesla *et al.*, 1992; Sato *et al.*, 1992; Tilly and Tilly, 1995; Pangas, 2007). Furthermore, SOD effectively blocks gonadotropin-induced ovulation (Miyazaki *et al.*, 1991). The activity of enzyme glutathione peroxidase in follicular fluid and granulosa cells shows a declining trend from healthy to slightly atretic to atretic follicles. It has been reported that glutathione peroxidase (GPx) catalyzes the breakdown of H_2O_2 with much more affinity than the catalase (Gul *et al.*, 2000). The major protection against both lipid peroxide and H_2O_2 is reported to be achieved by GPx (Halliwel and Gutteridge, 1985). Land and Verdetti (1989) reported decreased level of GPx with age in kidney and liver in rats, whereas Imre *et al.*, (1984) and Hazelton and Lang (1985) reported a decrease in GPx activity with age in liver and kidney and many other tissues of mice. It has been reported that GPx activity remained constant in the caudate putamen and the temporal cortex but decreased in the substantial nigra and the thalamus in rats (Benzi *et al.*, 1989).

4. Conclusion

Thus, the importance of understanding the mechanistic machinery of apoptosis is vital because programmed cell death is a component of both health and disease, being initiated by various physiologic and pathologic stimuli. Moreover, the widespread involvement of apoptosis in the pathophysiology of disease lends itself to therapeutic intervention at many different checkpoints. Therefore, understanding the mechanisms of apoptosis and other variants of programmed cell death, at the molecular level provides deeper insight into various disease processes and may thus influence therapeutic strategy.

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Zoology

Edited by Dr. María-Dolores García

The present book is not a classical manual on Zoology and the reader should not expect to find the usual treatment of animal groups. As a consequence, some people may feel disappointed when consulting the index, mainly if searching for something that is considered standard. But the reader, if interested in Zoology, should not be disappointed when trying to find novelties on different topics that will help to improve the knowledge on animals. This book is a compendium of contributions to some of the many different topics related to the knowledge of animals. Individual chapters represent recent contributions to Zoology illustrating the diversity of research conducted in this discipline and providing new data to be considered in future overall publications.

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