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Mechanisms of atresia in ovarian follicles

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Abstract

There are thousands to millions of follicles in the mammalian ovary, and the majority (99.9%) are eliminated by a process known as atresia. This phenomenon occurs in any stage of follicular development, through an apoptotic manner or the degenerative process of necrosis. Thus, a better understanding of the mechanisms involved in atresia is necessary to avoid the great follicular loss that occurs *in vivo* and to maximize female reproductive potential. The present review focuses on aspects related to follicular population and atresia, mechanisms of atresia (apoptosis or the degenerative process of necrosis), techniques used to analyze atresia in ovarian follicles, and the occurrence of the atretic process during different follicular stages.

Keywords: apoptosis, atresia, follicle, necrosis, ovary.

Introduction

The mammalian ovary represents a dynamic organ that provides an adequate environment for the production of several substances, such as hormones and growth factors, and releases viable female gametes (Johnson, 2003). There are thousands to millions of ovarian follicles that are the structural and functional units of the mammalian ovary, which support an appropriate environment for oocyte growth and maturation (Cortvrindt and Smitz, 2001). Despite the great number of follicles present in the ovary, most do not reach ovulation (about 99.9%), and instead die by atresia during growth and maturation (Markström et al., 2002).

Although atresia results in the loss of many ovarian follicles, this is a crucial event for the maintenance of mammalian ovarian homeostasis, which assures animal cyclicity (Amsterdam et al., 2003). In follicles, this process can occur through the apoptosis or the degenerative process of necrosis (for details see session 3). However, apoptosis is the most frequent form of physiologic cellular death (van Cruchten and van den Broeck, 2002) leading to significant follicular loss. Further knowledge of the intracellular mechanism, as well as the factors that regulate atresia, will contribute to a better comprehension of this process, which can facilitate the development of strategies to minimize the great follicular loss that occurs *in vivo*.

This review outlines the current understanding of these aspects related to follicular population and atresia, the mechanism of atresia (apoptosis or degenerative process of necrosis), techniques used to analyze atresia in ovarian follicles, and the occurrence of the atretic process during different stages of follicular development.

Follicular population and atresia

Folliculogenesis is an event that begins in prenatal life for most species and can be defined as the process of follicular assembly, growth, and maturation, beginning with the formation of the primordial follicle and finishing with the preovulatory follicle. The ovarian follicle is composed of an oocyte surrounded by somatic cells (granulosa and theca cells). According to the degree of development, follicles can be classified as preantral (primordial, intermediate, primary, and secondary) and antral follicles (tertiary and preovulatory), as shown in Fig. 1.

The female gametes are stocked in the ovary, especially as primordial follicles, which are composed of an immature oocyte surrounded by a single layer of flattened pre-granulosa cells. Primordial follicles remain relatively inactive in ovaries until recruitment into the growing follicle population (van den Hurk and Zhao, 2005), a process known as follicular activation (Nilsson and Skinner, 2004). The follicles enter into a pre-programmed course of development and maturation after activation, which is necessary for the success of ovulation and fertilization. Alternatively, the follicles can die by atresia.

Although there is a great follicular population present in the mammalian ovary (for example: 1,500 follicles in mouse - Shaw et al., 2000; 35,000 in goat - Lucci et al., 1999; 114,000 in domestic cat - Lima, 2006; 160,000 in sheep - Driancourt, 1991; 235,000 in cow - Betteridge et al., 1989; 2,000,000 in woman - Erickson, 1986), almost all follicles do not reach ovulation (99.9%), but rather die by a process called atresia. Even as a natural phenomenon, atresia significantly reduces the number of oocytes ovulated, decreasing the female reproductive potential. Thus, a better understanding of the mechanisms of atresia in ovarian follicles is necessary in order to minimize this great follicular loss.

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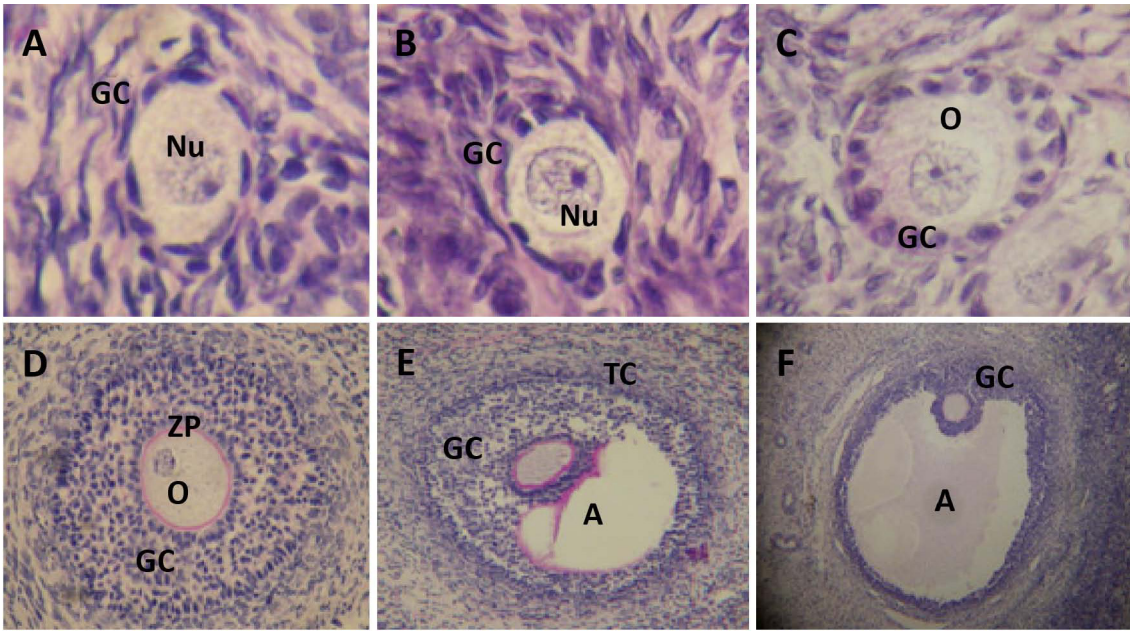

Figure 1. Histological sections containing ovarian follicles after staining with PAS-haematoxylin (400x). Preantral follicles: (A) primordial; (B) intermediate; (C) primary and (D) secondary. Antral follicles: (E) tertiary and (F) preovulatory. Nu: oocyte nucleus; O: oocyte; GC: granulosa cells; ZP: zona pelucida; A: antrum; TC: theca cells.

Mechanisms of atresia in ovarian follicles

Apoptosis

Apoptosis is a form of programmed cell death and has also been implicated in a spectrum of processes associated with normal functions of the ovary and follicular development, such as atresia and corpus luteum regression (Hussein, 2005). This process is observed in ovarian follicles throughout fetal and adult life. Apoptosis is a genetically determined and active event, i.e., dependent on the balance of pro-and anti-apoptotic genes and requires energy (Hussein, 2005). Apoptosis is mediated by active intrinsic mechanisms and extrinsic factors (Johstone et al., 2002), such as oxidative stress, irradiation, activation of gene promoters of apoptosis, damage to DNA, cytokines, viral coat proteins, or the withdrawal of cell growth factors (Johnson, 2003).

The initiation, execution, and regulation of apoptosis involve various biochemical factors, and the caspase family of enzymes plays a central role in the apoptosis-signaling network. Caspases are members of the highly conserved family of cysteine proteases with aspartate specificity. Caspases are expressed as pro-enzymes that undergo proteolytic processing to generate the activated form after apoptotic stimulus. There are 14 types of caspases identified as caspase-1 to caspase-14 (Tibbets et al., 2003). Some members of the family function specifically in cellular death by apoptosis and are subdivided into initiator (caspases-2, -8, -9, and -10) and executor or effector caspases (caspase-3, -6, and -7; Strasser et al., 2000). Expression of caspase-3 has been found in ovarian leukocytes and in follicular cells of atretic follicles (Berardinelli et al., 2004; Tsai et al., 2005). Initiator caspases are cleaved in response to apoptotic stimuli and activate the effector caspases (Green, 2003). During apoptosis, the effector caspases cleave numerous proteins located in the cell membrane, nucleus, and cytoplasm. The activation of caspase-activated DNase (CAD) to facilitate DNA degradation is one of the important functions mediated by caspases in the apoptotic process (Nagase et al., 2003).

There are two main apoptotic pathways: (1) the extrinsic or death receptor pathway and (2) the intrinsic or mitochondrial pathway. However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (Igney and Krammer, 2002). The initial apoptotic stimulus strongly influences the pathway that is activated. The extrinsic and intrinsic pathways converge on the same terminal, or execution pathway. This pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, cross-linking of proteins, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Elmore, 2007). An overview of the two cellular death signaling pathways is presented in the following sections and is schematically illustrated in Fig. 2.

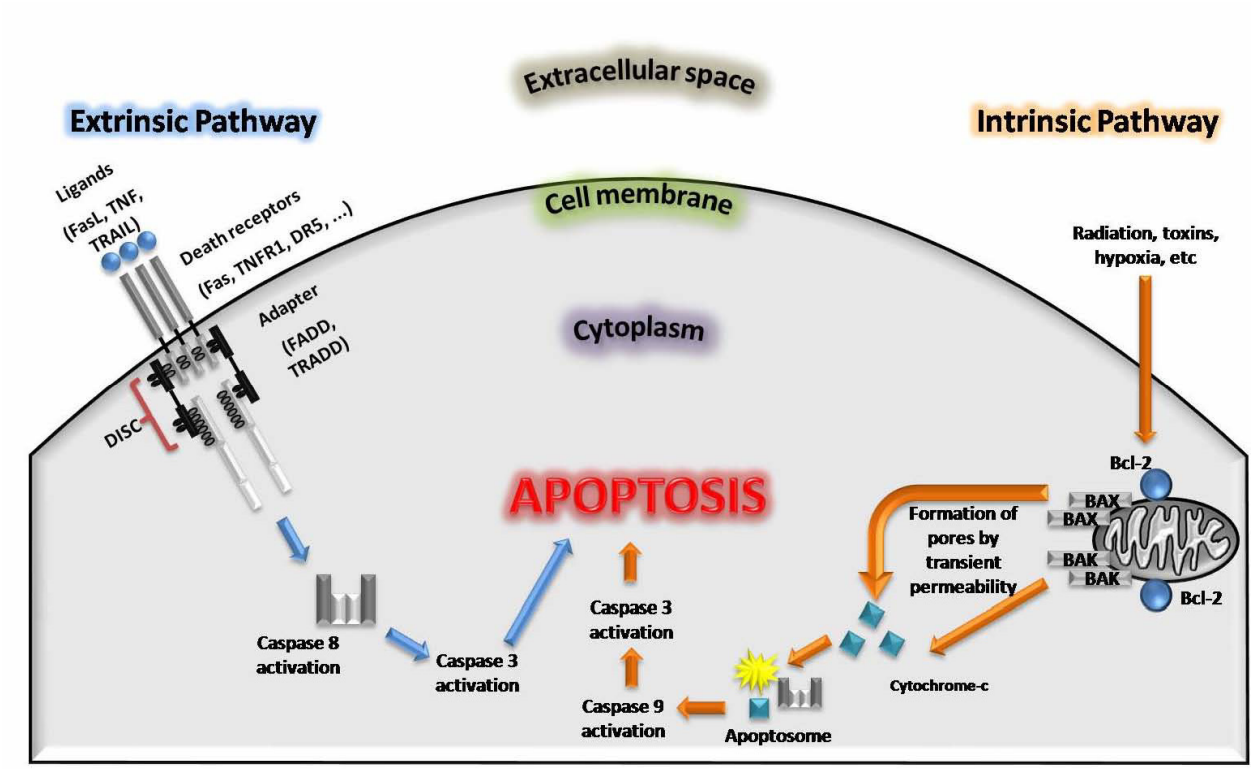

Figure 2. Two apoptotic pathways: membrane receptors (extrinsic) and mitochondrial (intrinsic). The extrinsic pathway can be induced by members of the TNF family of cytokine receptors, such as Fas, TNFR1 and DR5. These proteins recruit adapter proteins, including FADD, TRADD, which then binds pro-caspases. The intrinsic pathway can be induced by release of cytochrome-c from mitochondria, induced by various stimuli, including elevations in the levels of pore-forming pro-apoptotic Bcl-2 family proteins such as Bax. In the cytosol, formation of apoptosome occurs. Each pathway activates its own initiator caspase (8, 9, 10) which in turn will activate the executioner caspase 3. Adapted from: Elmore, 2007.

Extrinsic apoptotic pathway (membrane receptors)

Cells activate extrinsic apoptotic machinery in response to several conditions, such as signaling through apoptosis-related membrane receptors, which send apoptotic messages after binding to their respective cell death ligands. Caspase activation through cell death receptors is mediated by a subset of the tumoral necrosis factor (TNF) receptors superfamily, which includes TNF receptor type 1 (TNFR1), Fas/CD95 (a membrane-associated polypeptide), and the TNF-related apoptosis-inducing Ligand (TRAIL) receptors, DR-4 and DR-5 (Slot, 2004; Contassot et al., 2007). The expression of Fas and FasL, as well as their functions in the ovary have been shown by Fujino et al. (2008) and Manabe et al. (2008), suggesting that the Fas system is involved in apoptosis in this organ. Additionally, Porter et al. (2001) showed higher concentrations of Fas and FasL in bovine atretic follicular cells than in healthy follicles.

Subsequent apoptotic signaling is mediated by the cytoplasmic domain of the death receptor, which contains a region termed the death domain (DD). Adapter molecules, like Fas-associated protein with death domain (FADD) or TNF receptor-associated protein with death domain (TRADD), bind to the activated death receptor, forming the death-inducing signaling complex (DISC). When bound to the DISC, several pro-caspase-8 molecules are recruited, resulting in cross-activation by (auto) proteolysis. Caspase-8 is a key initiator caspase that activates the downstream caspase cascade, and initiates the apoptotic program in the death receptor pathway (Reed, 2000), following activation in the DISC (Slot, 2004). In some cell types, a high concentration of caspase-8 in the DISC can process downstream effect or caspase-3 directly. This latter caspase leads the signaling of apoptosis to the nucleus, where an endonuclease is released and degrades DNA at each 180-200 kilobase pair fragments (Hussein, 2005).

Intrinsic pathway of apoptosis (mitochondria)

Mitochondria are not only important for amplifying extrinsic apoptotic pathway, but also for transmittance of death signals caused by intrinsic stress stimuli and apoptosis developmental instructions (Joza et al., 2002). Mitochondrial integrity and the release of cytochrome-c into the cytosol are primarily under the control of the Bcl-2 family members. The Bcl-2 family of

proteins can be divided into anti-apoptotic members, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Boo, survivin, and pro-apoptotic members, such as Bax, Bak, Bok, and Diva. The Bcl-2 expression is found in the granulosa cells of both fetal and adult ovaries (Hussein, 2005; Hussein et al., 2006). Choi et al. (2004) showed a correlation between decreases in the level of Bcl-2 mRNA with the incidence of apoptosis in isolated granulosa cells which were cultured under different hormonal treatment conditions. They considered Bcl-2 to have a critical role in inhibiting the granulosa cell apoptosis pathway. Survivin is one member of the apoptosis inhibitor protein family that has been shown to bind and inhibit the cell death effectors, caspase-3 and -7 (Shin et al., 2001). In the ovary, survivin acts in granulosa cells as a bifunctional protein associated with the regulation of the cell cycle and inhibition of apoptosis (Johnson and Bridgham, 2002). Bax is a pro-apoptotic protein involved in granulosa cell apoptosis (Tilly et al., 1995) and is an important regulator of follicle growth, but is dispensable for follicle atresia in mice. In addition, a defect in folliculogenesis was shown following Bax deletion (Greenfeld et al., 2007).

The key functions related to the cytochrome-c release from the mitochondrial intermembrane compartment into the cytosol have not been elucidated (Suzuki et al., 2000). One hypothesis is that Bcl-2 family members may insert inside the outer mitochondrial membrane to form large pore channels that allow the passage of molecules (Reed and Kroemer, 2000). Cytosolic cytochrome-c triggers the formation of the mitochondrial apoptosome, which is indicative of apoptosis and consists of cytochrome-c, Apaf-1 (Apoptotic Protease Activating Factor), and caspase-9 (Joza et al., 2002). The caspase-9 serves as the apical caspase of the mitochondrial pathway (Reed, 2000). Cytochrome-c binds to the Apaf-1 adapter protein, which recruits pro-caspase-9. This pro-caspase is cleaved into the active form, caspase-9, which proteolytically activates caspase-3, resulting in cell death. The link between the death receptor-activated caspase-8 (extrinsic pathway) and mitochondrial cytochrome-c release (intrinsic pathway) is created by a pro-apoptotic member, termed Bid. This member is cleaved by caspase-8 and is transferred to the mitochondria, where Bid acts in concert with the other apoptotic members of Bcl-2 family (Bax and Bak) to induce the release of cytochrome-c (Slot, 2004).

Another protein which is expressed in the apoptotic granulosa cells of atretic follicles is 53 protein (p53; Tilly, 1996). This protein functions as a transcription factor in response to DNA damage, inducing either growth, arrest or apoptosis (Slot, 2004). The p53 is known to activate the transcription of genes as Bax, Apaf-1, Fas, as well as to repress transcription of Bcl-2 genes (Bourdon et al., 2003). However, independent of its transcription control of these genes, p53 has also been shown to engage the apoptotic program by directly activating Bax to permeabilize mitochondria (Bras et al., 2005).

Morphological alterations in apoptotic cells

Independent of the particular stimulus and pathway, the morphologic features that characterize apoptosis are pycnosis and karyorrhexis in the nucleus, as well as condensation, swelling, loss of cytoplasmic detail, and fragmentation in the cytoplasm (Zeiss, 2003). The condensed chromatin appears as crescents along the periphery of the nuclear membrane or as spherical bodies within the nucleus. The cytoplasmic condensation induces shrinking of the cell (Hussein, 2005). Subsequently, the nuclear and plasma membranes become convoluted, and small masses of condensed chromatin undergo fragmentation along with condensed cytoplasm to form "apoptotic bodies". The apoptotic bodies are bound to the plasma membrane, and often contain functional mitochondria and other organelles. The phosphatidyl serine residues that are normally localized to the inner membrane are relocated to the outside of the cell membrane prior to fragmentation. These residues of phosphatidyl serine on the apoptotic bodies serve as a signal to the neighboring healthy cells to perform phagocytosis and remove the cellular debris (Bhatia, 2004). There is essentially no inflammatory reaction associated with the process of apoptosis nor with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and (3) the engulfing cells do not produce anti-inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

Necrosis

Generally, necrosis is initiated by non-cellular mechanisms, such as ischemia, ATP depletion (Bhatia, 2004), and traumatic insults, which lead to irreversible cellular damage (McCully et al., 2004). In addition to passive mechanisms, studies have suggested that "active" mechanisms, such as Na^+ overloading, Ca^{2+} accumulation, and changes in mitochondria permeability, can also participate in the necrotic process (Barros et al., 2001b; Padanilam, 2003).

The biochemical pathway that leads to necrotic cell death is not well known. In ischemic or hypoxic injury, energy depletion occurs by defective ATP production associated with the rapid consumption of ATP by Na^+-K^+ pumps and through hydrolysis as well as ATP loss. The necrotic volume increase associated with necrotic cell death is initiated by an influx of Na^+ and release of ATP due to membrane leakage (Padanilam, 2003). The increased Na^+ levels in the cytosol activate the Na^+-K^+-ATPase pump, resulting in dissipation of ATP. In the early stages of the injury, a simultaneous

efflux of K^+ maintains ion homeostasis. Severe depletion of ATP leads to failure in the pump-leak balance mechanism, leading to an influx of Na^+ and water that results in swelling and collapse of cell.

In addition, the reactive oxygen species have been suggested to mediate necrotic volume increase, and Na^+ influx is initiated by the binding of free radicals to ion channels, including nonselective Ca^{2+} channels (Barros et al., 2001b). The increased levels of Na^+ activate the Na^+-K^+-ATPase pump and consume ATP, activating the nonselective Ca^{2+} channels that result in massive cytosolic Ca^{2+} accumulation. High levels of Ca^{2+} can participate in ATP depletion by activating the Ca^{2+}ATPase pump and mitochondrial depolarization. The increased levels of Ca^{2+} activate endonucleases to degrade DNA and cellular proteases to degrade several structural and signalizing proteins (Wang, 2000). Figure 3 illustrates the biochemical events occurring in passive necrosis.

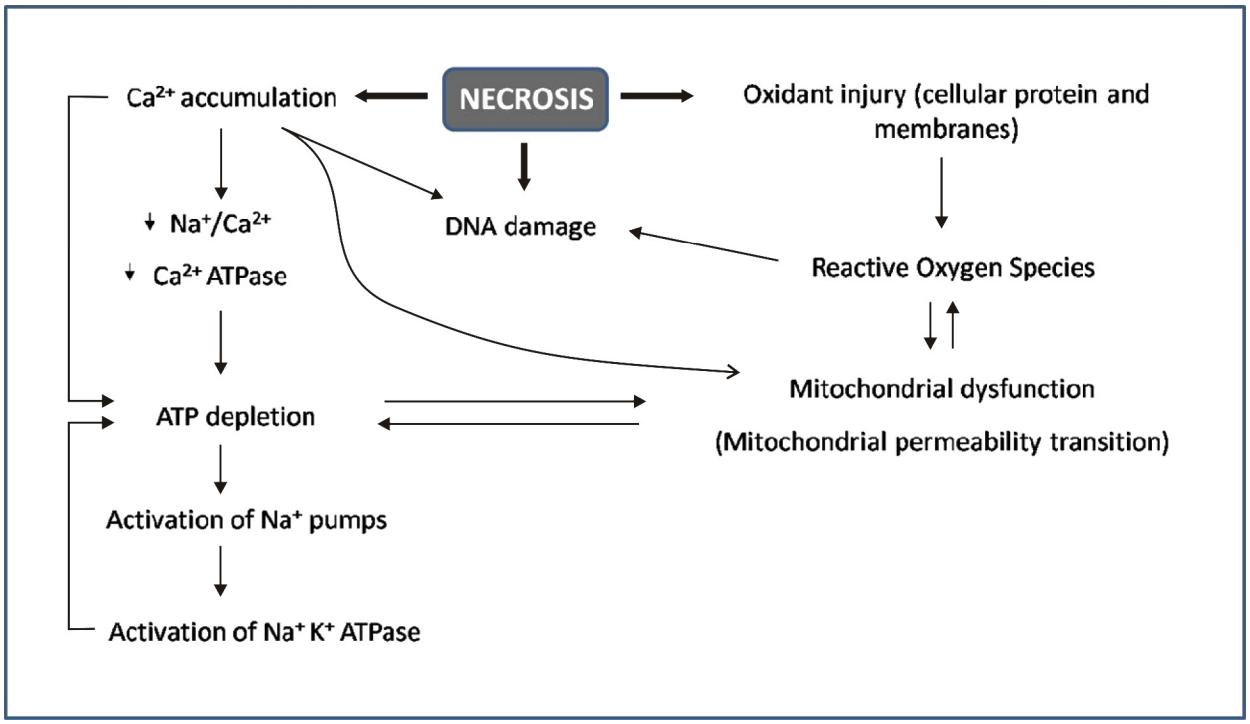

Figure 3. Cell death by necrosis caused by a sequence of bioquimical events. Adapted from: Bhatia, 2004.

Morphological alterations in necrotic cells

The morphology of a necrotic cell is very distinct from that of a cell undergoing classic apoptosis, with ultrastructural changes occurring in both the cytoplasm and the nucleus. The main features are chromatin flocculation, swelling and degeneration of the entire cytoplasm and the mitochondrial matrix, blebbing of the plasma membrane, and eventual shedding of the cytoplasmic content into the extracellular space (Scaffidi et al., 2002). In contrast to apoptosis, the chromatin is not packed into discrete membrane-bound particles, but may form unevenly textured and irregularly shaped clumps. During necrosis, mitochondria undergo inner membrane swelling and disintegration (Barros et al., 2001a). Polyribosomes are dissociated and dispersed throughout the cytoplasm, imparting a dense and granular appearance of the cytoplasmic matrix. Moreover, dilation and fragmentation of the cisterns of the rough endoplasmic reticulum and Golgi apparatus are frequently observed (Berridge et al., 2000). It is also important to note that cytomorphological changes like follicular atresia caused by either apoptosis or necrosis karyolysis, pyknosis and karyorrhexis can occur in necrosis, which is not exclusive to apoptosis (Cotran et al., 1999).

Although the mechanisms and morphologies of apoptosis and necrosis differ, there is overlap between these two processes. Evidence indicates that necrosis and apoptosis represent morphologic expressions of a shared biochemical network described as the "apoptosis-necrosis continuum" (Zeiss, 2003). For example, two factors that will convert an ongoing apoptotic process into a necrotic process include a decrease in the availability of caspases and intracellular ATP (Denecker et al., 2001). Whether a cell dies by necrosis or apoptosis depends in part on the nature of the cell death signal, the tissue type, the developmental stage of the tissue and the physiologic milieu (Zeiss, 2003). Table 1 illustrates the differences between the processes of apoptosis and necrosis.

Techniques used for analysis of follicular atresia

Different techniques have been used to detect after cryopreservation and/or *in vitro* culture of ovarian

follicles during the different stages of development. However, each technique has advantages and disadvantages which may make it acceptable to use for one application but inappropriate for another (Otsuki et al., 2003).

For apoptosis detection, different techniques may be utilized, such as: 1) morphological analysis (laser confocal microscopy and Transmission Electronic Microscopy – TEM; Staldemann and Lassman, 2000); 2) evaluation of DNA fragmentation (enzyme-linked imumunosorbent assay - ELISA and terminal deoxynucleotidil transferase-mediated deoxyuridine triphosphate biotin nick end-labeling – TUNEL; Linde et al., 2000); 3) analysis of DNA content (flow cytometry); 4) evaluation of the translocation of phosphatidilserin residues located in the inner mitochondrial membrane (Wiegele et al., 1998); 5) analysis of gene expression and caspases involved with apoptosis (RT-PCR, northern and western blot, and immunohistochemistry; Kiechle and Zhang, 2002). With respect to techniques for cytomorphological alterations, the TEM is considered the gold standard to confirm apoptosis, which better defines the subcellular changes. However, the main disadvantages of TEM are that it is costly, time consuming, and the ability to only assay a small region at a time. The TUNEL has been used as a principal method to identify and quantify the apoptotic cells in atretic follicles during luteal regression (Zhang et al., 2008) because it is very sensitive and fast (it takes about 3 hours). The disadvantages are cost and the unknown parameter of how many DNA strand breaks are necessary for detection by this method. This method is also subject to false positives from necrotic cells and cells in the process of DNA repair and gene transcription (Elmore, 2007). For membrane alterations detection, the externalization of phosphatidyl serine residues on the outer plasma membrane of apoptotic cells allows detection via Annexin V in tissues, embryos or cultured cells (Bossy-Wetzel and Green, 2000), which can be visualized with fluorescent microscopy. The advantages are sensitivity (can detect a single apoptotic cell) and the ability to confirm the activity of initiator caspases. The disadvantage is that the membranes of necrotic cells are labeled as well (Elmore, 2007). Another method for detecting apoptosis is through the detection of some factors such as the caspase activation (Gurtu et al., 1997). The major disadvantage is that the integrity of the sample is destroyed thereby eliminating the possibility of localizing the apoptotic event within the tissue or determining the type of cell that is undergoing apoptosis. Another disadvantage is that caspase activation does not necessarily indicate that apoptosis will occur (Elmore, 2007). Among these techniques, the most used for apoptosis detection is TUNEL (evaluation of nuclear alterations, such as DNA fragmentation) and immunohistochemistry.

For evaluation of cell death by necrosis, several techniques are used, such as classical histology (staining with hematoxilin-eosin or Periodic Schiff Acid-hematoxilin), laser confocal microscopy, TEM (Martinez-Madrid et al., 2007), that allow for visualization of the presence of vacuoles, especially with TEM, which can also analyze organelle damage and the integrity of basal and nuclear membranes. Some vital fluorescent staining can also be used, like propidium iodide and Lucifer Yellow, which penetrate damaged membranes of necrotic follicles (Thomas et al., 2001; Choi et al., 2007).

Atresia during the different stages of follicular development

Ovarian follicles of all growth stages undergo atresia due to either apoptosis or necrosis (Chen et al., 2005; Valdez et al., 2005). According to the follicular stage, there is different susceptibility of follicular compartments to atresia. In preantral follicles, the atresia is most commonly observed in the oocyte. However, during the advanced stages of development (e.g., in antral follicles), atresia occurs both in the oocyte and the granulosa cells (Silva et al., 2002).

The balance of several substances may influence the decision of cell death during the different follicular stages, such as endocrine factors (Follicle Stimulating Hormone - FSH and Luteinizing Hormone - LH) and paracrine factors (Kit Ligand - KL, Insulin-Like Growth Factor-1 - IGF-1, Epidermal Growth Factor - EGF, Fibroblast Growth Factor-2 - FGF-2, Vascular Endothelial Growth Factor - VEGF, and activin). Additionally, there are atretogenic factors, which include TNF-α, androgens, IL-6, and free radicals (Markström et al., 2002). Furthermore, the importance of several pro- and anti-apoptotic genes, such as p53, Bcl-2, Bax, Fas, FasL and survivin, and their roles in follicular atresia have been shown in some reports (Fujino et al., 2008; Pru et al., 2009). Thus, we will focus on investigations in the following sections, which report the process of atresia after *in vitro* culture or cryopreservation of ovarian follicles from different stages of development.

Atresia in preantral follicles

Several human studies have demonstrated that apoptosis occurs in ovaries even before birth, and has been identified in 13 to 32 week old fetuses during pregnancy (Markström et al., 2002; Albamonte et al., 2008). The vast majority of oogonia and oocytes are lost during embryonic, neonatal and adult life through apoptosis (Kim and Tilly, 2004) and are not destined to produce mature oocytes for fertilization. Arguments that this is a selection mechanism designed to remove abnormal oocytes from the follicle pool are cogent but observations of single cell atresia in oocyte nests during primordial follicle formation (Pepling and Spradling, 2001) indicates that other factors such as somatic cell support may regulate this process.

Studies performed with animal ovarian models have suggested that the viability of primordial

(quiescent) and primary (initial growth) follicles is determined by survival factors derived from the oocyte. During these stages of development, cell death results specially from an insufficient availability of growth factors, such as KL, EGF, IGF-1, leukemia inhibitor factor (LIF), or growth and differentiation factor-9 (GDF-9). In primordial follicles, oocyte apoptosis is probably responsible for further follicular degeneration. This oocyte apoptosis was demonstrated by Reynaud and Driancourt (2000) in rodents, which described the importance of KL and the interaction of KL with other factors, such as EGF and FGF-2 in preventing apoptosis. Another study demonstrated that the interaction between KL and the KL receptor, c-kit, is important to prevent follicular degeneration and the rescue of the follicles, thus avoiding oocyte death (Driancourt et al., 2000). *In vitro* studies have demonstrated that KL inhibits apoptosis in oocytes of mouse primordial follicles by increasing the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-cL, and reducing the expression of the pro-apoptotic factor Bax (Jin et al., 2005). Other studies have demonstrated that the oocytes had no visible signs of degeneration after addition of 50 or 100 ng/ml of KL even after 20 days of culture (Klinger and De Felici, 2002).

The EGF is also known as an *in vivo* and *in vitro* survival factor (Markström et al., 2002). Some authors showed that low concentrations of EGF promote an inhibition of granulosa cells apoptosis or the reduction of follicular atresia levels in swine, bovine and caprine (Gutierrez et al., 2000; Mao et al., 2004, Zhou and Zhang, 2005). Furthermore, EGF was tested at different concentrations (1 or 10 ng/ml) for *in vitro* culture of caprine preantral follicles, leading to increased follicular survival after 7 days of culture which was demonstrated by the maintenance of follicular ultrastructure (Celestino et al., 2009). With respect to IGF-1, Mao et al. (2004) observed that the percentage of apoptotic granulosa cells in swine preantral follicles treated with 10 and 100 ng/ml of IGF-1 was lower than those treated with 0 and 1 ng/ml of IGF-1. Moreover, addition of EGF, IGF-1, or EGF + IGF-1 inhibited apoptosis in granulosa cells and stimulated proliferation of these cells to antrum formation after 8 days of culture. Furthermore, FGF-2 can also influence follicular atresia through the inhibition of apoptosis after culture of rat preantral follicles (McGee et al., 1999). Another factor derived from the oocyte that is important for small follicle survival is GDF-9. In mice, lack of this factor prevents the development of the follicles from the primary to early secondary stages, causing follicular atresia (Dong et al., 1996). In an *in vitro* study which tested different concentrations of GDF-9 in the culture of caprine preantral follicles, the concentration of 200 ng/ml maintained follicular survival after 7 days, without signs of atresia after analyzing ultrastructural integrity by TEM (Martins et al., 2008).

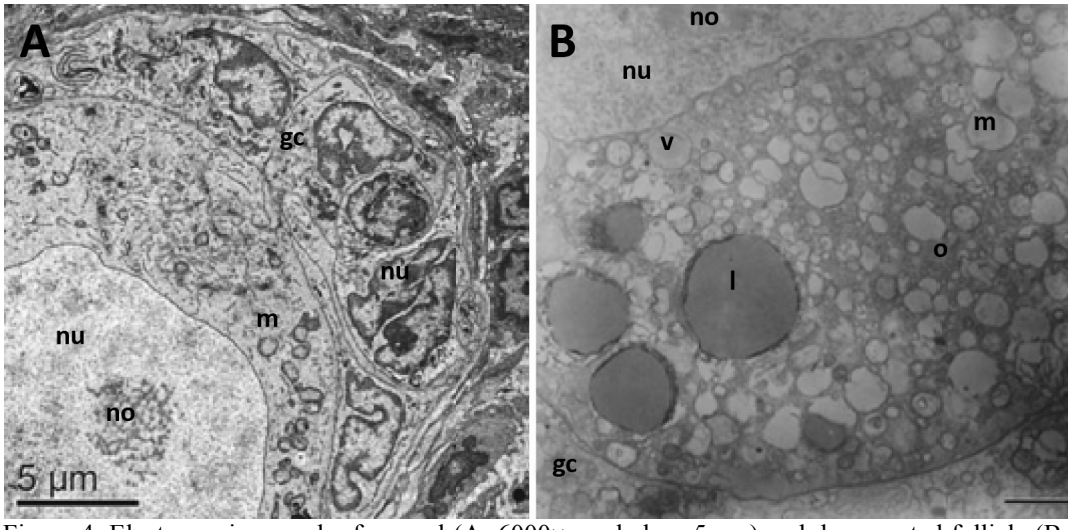

Figure 4. Electron micrograph of normal (A; 6000×; scale bar: 5 µm) and degenerated follicle (B; 7000×; scale bar: 2µm) after culture of caprine ovarian tissue in medium containing FSH + FGF-2 and control medium (Minimal Essential Medium), respectively. In Figure 4A, note the homogeneous cytoplasm with numerous rounded mitochondria and the basement membrane integrity. In Figure 4B, note the extreme vacuolization and the great holes present in the cytoplasm, indicative of degeneration. gc, granulosa cell; l, lipid droplet; m, mitochondria; no, nucleolus; nu, nucleus; o, oocyte; v, vesicles. (Reproduced with permission from Matos et al., 2007a).

Matos et al. (2007a, b) verified that ultrastructural integrity of caprine preantral follicles was

maintained after using FSH or the association between FSH and FGF-2 in the culture medium (Fig. 4A). Nevertheless, follicles cultured only in control medium (without addition of any hormone or growth factor) exhibited a high rate of degeneration by necrosis, which was characterized by a large vacuolization in the cytoplasm (Fig. 4B). However, another study showed that in the early stage of development, FSH and its mediator cAMP did not have any effect on apoptosis of the rat isolated preantral follicles cultured *in vitro* (McGee et al., 1997). In the same way, the Anti-Müllerian Hormone (AMH) stimulated follicular growth but did not inhibit apoptosis (McGee et al., 2001). Nevertheless, Visser et al. (2007) recently demonstrated that AMH had an important role in follicular growth and death, serving as a survival factor for small follicles.

The susceptibility to atresia, besides the relation to the stage of follicular development, depends on the conditions of *in vitro* culture, thus varying the pathway of cellular death, i.e., apoptosis or necrosis. After using the TUNEL technique to detect apoptosis in preantral follicles cultured *in vitro*, Silva et al. (2006) demonstrated that addition of 100 ng/mL of activin to the medium significantly reduced the number of atretic follicles enclosed in ovarian tissue, but not for isolated follicles. Among the atretic follicles cultured in the ovarian cortex, less than 30% exhibited DNA fragmentation (Fig. 5), while this phenomenon occurred especially in granulosa cells of isolated preantral follicles. The authors suggested that this incidence could occur most likely due to a reduced access to oxygen and nutrient in follicles cultured in ovarian cortex, thus favoring the occurrence of necrosis as a pathway of degeneration. Contrarily, isolated follicles which have better access to nutrients and oxygen died via an apoptotic pathway.

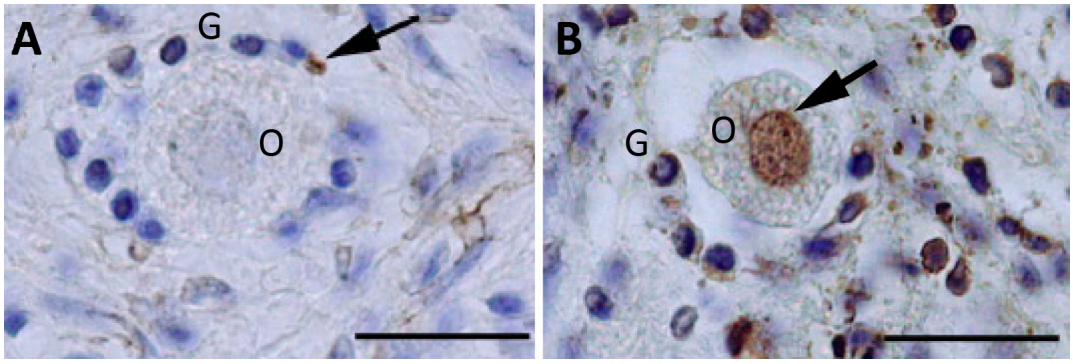

Figure 5. DNA fragmentation detected using TUNEL technique in caprine ovarian follicles cultured *in vitro* for 5 days in the presence of activin. Arrows represent DNA fragmentation in granulosa cell (A) and oocyte (B). O, oocyte, G, granulosa cells. Bars: 25 µm. (Reproduced with permission from Silva et al., 2006).

In addition to *in vitro* culture, cryopreservation studies have also been performed with ovarian follicles to observe the percentage of atresia and the most predominant pathway of cell death. Martinez-Madrid et al. (2007) showed that after cryopreservation of human ovaries with their vascular pedicle, no primordial or primary follicle was found to be positive for TUNEL or active caspase-3. Nevertheless, TEM revealed that some follicles exhibited morphological alterations that were suggestive of necrosis, such as oocyte nuclear membrane rupture and mitochondrial swelling. In another study, a high apoptosis rate was observed after cryopreservation of mouse ovaries followed by graft, with loss of approximately half of the number of primordial follicles present in the graft tissue (Liu et al., 2002). Apoptosis also may be involved in follicular damage during freezing and thawing of the ovary (Rimon et al., 2005). In addition, Tirelli et al. (2005) observed a significant increase in apoptosis of sow granulosa cells that were frozen slowly. They suggested the increase in apoptosis was probably caused by physical alterations due to low cultured, isolated follicles. In a more recent study in temperature, high salt concentration and impairment of antioxidant metabolism. Similar results were reported by Rimon et al. (2005), who demonstrated a higher incidence of apoptosis in frozen–thawed human ovarian tissue compared with fresh ovarian tissue. In contrast, other investigations and previous report have shown that the incidence of apoptosis in cryopreserved tissue was not significantly different from fresh controls (Hussein et al., 2006; Mazoochi et al., 2008).

Choi et al. (2007) demonstrated a significantly higher proportion of apoptosis and necrosis in cryopreserved ovaries than in fresh ones after 1 and 5 days of culture, respectively. Most likely, the absence of apoptosis after 5 days of culture was due to the phagocytosis of apoptotic cells by healthy neighboring cells. Nevertheless, the cryopreservation procedure may lead to a death process by cooling through degeneration (necrosis), and not by an apoptotic pathway with caspase activation. Haidari et al. (2008) showed no significant differences between the survival rates or the ultrastructural changes of vitrified and non-vitrified, which apoptosis was assessed in the preantral follicles of

vitrified mice and then cultured for 10 days, no signs of apoptosis were found by morphological and ultrastructural studies. However, real-time RT-PCR demonstrated that the vitrification affected the expression of some genes related to apoptosis (Mazoochi et al., 2009).

Atresia in antral follicles

In antral follicles, atresia in granulosa cells close to the antrum is an important sign of cell death (Amsterdam et al., 2003). In addition, the follicular selection depends largely on granulosa cell apoptosis (Rung et al., 2006). It has been established that granulosa cell death during follicular atresia and luteolysis results from apoptosis (Chen et al., 2005).

Among the factors locally produced that are important to the survival of rat, early antral follicles include IGF-1, EGF, FGF-2, VEGF, activin, and cytokine IL-1β. However, some locally produced factors are the most potent survival factors in the latest stages of follicular development. For instance, the IL-1β is more efficient in preventing apoptosis in preovulatory follicles than in early antral stages. In addition, early antral follicles express FSH receptors and are dependent on this stimulus for survival. Due to a lack of FSH support, several follicles never progress beyond this stage of development (Hirshfield, 1991). On the contrary, the stimulation of LH receptors has a limited effect on the survival of rat follicles in this stage (Chun et al., 1996). Studies with rats, sheep, cows (Palumbo and Yeh, 1994; Yang and Rajamahendran, 2000) and goats (Yu et al., 2003) demonstrated that FSH alone or synergistically with other factors, such as IGF-1 and VEGF, reduced the rates of apoptotis in granulosa cells cultured *in vitro* (Kosaka et al., 2007). In rats, both gonadotropins FSH and LH inhibited the apoptosis level in isolated preovulatory follicles, as this apoptosis suppression is mediated by endogenous IGF-1 (Chun et al., 1994). Moreover, GH inhibited apoptosis in preovulatory follicles through the stimulation of endogenous IGF-1 production (Eisenhauer et al., 1995).

In addition to growth factors and hormones, the involvement of determinant genes in follicular atresia has been reported, especially in those follicles subordinate in a specific follicular wave. For example, death of subordinate and nonovulatory dominant follicles is mediated via proapoptotic pathways and granulosa cell survival is mediated in part by protein kinases: A (PKA), B (PKB) and C (PKC), and by another group of serine/threonine kinases: mitogen-activated protein kinases (MAPKs; Johnson, 2003; Ryan et al., 2007). A study performed by Forde et al. (2008) verified important roles of some genes in antral follicle survival, such as EphA4, CCND2, and GADD45. EphA4 is a receptor tyrosine kinase and a class A type receptor. It is a member of the ephrin family and binds both class A and complexes (COCs) with signs of early atresia are more class B ephrins (Frisen et al., 1999). EphA4 has a role to play in cell-cell adhesion via the cadherin family of molecules (Cheng et al., 2002). Another study demonstrated that enhanced expression of the proliferative gene CCND2 and the anti-apoptotic gene GADD45B in granulosa cells may support further growth of the dominant follicle (Mihm et al., 2008). Similarly, the gene product GADD45 may act as a potential survival factor in the growing dominant follicle, as it is involved in DNA damage repair and control of genomic stability, and has also anti-apoptotic properties (Sheikh et al., 2000; De Smaele et al., 2001).

Gonadotropins and growth factors mediate their biological effects through binding to cell surface receptors, which results in enzymatic phosphorylation cascades (signal transduction pathways) that transmit signals from outside the cell to the nucleus. One major survival pathway involves the activation of Akt (protein kinase B), a serine/threonine kinase which is a common mediator of cell survival and proliferation. Activation of the Akt pathway causes general inhibition of pro-apoptotic factors, such as the forkhead transcription factors, Bad and caspase 9, all of which are known to mediate apoptosis (Datta et al., 1999; Cardone, 2000). One of the most characterized pathways of the MAPK group is the extracellular-regulated kinase (Erk), which also regulates cell proliferation, differentiation, and survival, depending on the cellular context (i.e., the type and duration of stimulus, cell type, and any additional signaling pathways: Zhang and Liu, 2002). Ryan et al. (2007) showed that higher levels of the genes Akt and Erk may confer a developmental advantage on the future dominant follicles by promoting survival at the time when circulating FSH concentrations decline and by regulating key processes, such as follicle growth and estradiol production. Furthermore, both Akt and Erk pathways have been implicated in promoting granulosa cell proliferation and survival.

The incidence of apoptosis in cumulus cells may be a good indicator of oocyte developmental competence (Corn et al., 2005; Yuan et al., 2005) due to the bidirectional communication established between cumulus cells and oocytes through gap junctions (De Loos et al., 1991). It was shown in different cell lines as well as various organs that gap junctions play a negative role in apoptosis (Lin et al., 1998; Krutovskikh et al., 2002). Specifically, the connexin 43 (Cx43) was shown to inversely correlate with apoptosis, acting in fact as a survival factor (Lin et al., 2003). The phenomenon of reduced expression of Cx43 in apoptotic follicles was also observed in the porcine, bovine and avian granulosa cells (Johnson et al., 1999; Krysko et al., 2004; Chang et al., 2005). Cumulus cells play an important role in regulating the maturation of the nucleus and cytoplasm in the oocyte (Tanghe et al., 2002) and in protecting oocytes against oxidative stress-induced apoptosis (Tatemoto et al., 2000). Some authors consider that cumulus oocyte developmentally competent (Bilodeau-Goeseels and

Panich, 2002) because of the similarity between structural changes during oocyte degeneration and those occurring in the oocyte of the dominant follicle prior to the LH surge (Hyttel et al., 1997). Others authors, however, have reported that COCs with no signs of atresia yield higher blastocyst rates (Corn et al., 2005; Yuan et al., 2005). In prepubertal goats, apoptosis in cumulus cells was negatively related to the morphology of COCs (Anguita et al., 2009).

Final considerations

This review evidenced the complexity of the mechanisms regulating atresia in ovarian follicles during different stages of follicular development. Clearly, regulation of follicular atresia involves some mechanisms of extra and intra-ovarian control, which are dependent on each phase of follicular development. Elucidation of these mechanisms can contribute to a better comprehension of the processes involved in the ovarian folliculogenesis and, consequently, provide large number of viable and mature oocytes, which can be destined for different *in vitro* techniques of reproduction. In addition to these applications in reproduction, a better knowledge regarding the mechanism of cell death can contribute to therapies for neoplasic and degenerative diseases.

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