Ciência Animal, v.31, n.3, p.112-128, 2021.

QUALITATIVE AND QUANTITATIVE ASPECTS OF ATRESIA DURING MAMMALIAN FOLLICULOGENESIS

(Aspectos qualitativos e quantitativos da atresia durante a foliculogênese em mamíferos)

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RESUMO

Durante a foliculogênese ovariana, cerca de 99,9% dos folículos morrem pelo processo de atresia folicular. O processo de atresia pode ocorrer pelas vias degenerativa ou apoptótica. Este processo compromete todos os estágios de desenvolvimento folicular, sendo os folículos antrais os mais afetados. Por outro lado, os folículos pré-antrais são mais resistentes, em função de sua menor taxa metabólica, bem como do número reduzido de células e camadas de células somáticas, células da granulosa e/ou da teca. Embora folículos pré-antrais sejam menos afetados pelo processo de atresia, quando este evento ocorre, pode-se classificá-lo de duas formas, degeneração do tipo I e degeneração do tipo II. Na degeneração do tipo I, o oócito é o compartimento mais comprometido, apresentando núcleo picnótico, embora suas células da granulosa apresentem-se bem organizadas e sem picnose nuclear. Já na degeneração do tipo II, os folículos apresentam oócito retraído e células da granulosa edemaciadas, desorganizadas e sem aderência à membrana basal e ao oócito. É importante destacar que a degeneração do tipo I é mais comum em folículos primordiais e primários, enquanto folículos secundários apresentam mais degeneração do tipo II, ou seja, a medida que os folículos evoluem, a degeneração do tipo II é mais frequente. Considerando todos os aspectos aqui relacionados, essa revisão de literatura abordará aspectos relacionados aos processos de foliculogênese e atresia folicular, bem como as substâncias que induzem a atresia durante a foliculogenese *in vitro* e *in vivo* e, ainda, os métodos e parâmetros para análise da atresia em folículos ovarianos. Isto se deve a necessidade de desenvolvimento de protocolos mais eficientes de recuperação dos folículos ovarianos, prevenindo essa grande perda folicular e otimizando as possibilidades de utilização desses materiais biológicos no futuro.

Palavras-chave: Folículos ovarianos, fatores de crescimento, métodos de análises, vacúolos.

ABSTRACT

During ovarian foliculogenesis, about 99.9% of follicles die by the follicle atresia process. The atresia process can occur via degeneration or apoptosis. This process compromises all the follicle development stages, with antral follicles being those most affected. On the other hand, the preantral follicles are more residents, since their slow metabolic rate, as well as the reduced number and layers of somatic cells, granulosa and/or thecal cells. Although preantral follicles are less affected by the atresia process, when the event occurs, we can classify it in two ways, type I or type II degenerated follicles. In the type I degeneration the oocyte is the most compromised compartment, showing the picnotic nuclei, although its granulosa cells presented well organized and no picnosis. Already in the type II degeneration, the follicles presented shrunken in the oocyte, and swollen, disorganization and no adhered granulosa cells from the basal membrane and oocyte. It is important to highlight that type I degeneration is the most common in primordial and primary follicles, while in secondary follicles present more type II degeneration, which means that the as follicle evolve, type II degeneration is more frequent. Considering all the aspects related here, this literature review will address aspects related to the folliculogenesis and the process of follicle atresia, as well as substances that induce atresia during *in vitro* and *in vivo* folliculogenesis and methods and parameters for analyzing atresia in ovarian follicles. This is due to the need to develop more efficient ovarian follicle recovery protocols, which can prevent this great follicular loss and optimizing the possibilities of use of these biological materials in the future.

Key words: ovarian follicles, growth factors, methods of analysis, vacuoles

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INTRODUCTION

In the mammalian ovary there are hundreds of thousands of follicles at birth. However, despite all of this oocyte capital, many do not reach ovulation (about 99.9%), and instead die by atresia during growth and maturation (WILLIAMS and ERICKSON, 2012). Atresia can occur in both pre and postnatal life and at all stages of follicular development and includes apoptosis of granulosa cells (TINGEN *et al.,* 2009). In ovarian follicles, this process can occur through apoptosis or the degenerative process of necrosis.

Apoptosis is an active cellular event that is dependent on transcription and protein synthesis (SVANBERG and BILLIG, 1999), and is characterized by numerous membrane-bound apoptotic bodies with condensed cytoplasm (with or without nuclear fragments). The histological features of apoptosis are: (i) condensation of the nuclear chromatin into a sharply circumscribed mass; (ii) swirling of nuclear and cellular outlines; (iii) fragmentation of the nucleus and cell, and production of membrane-bound apoptotic bodies; and (iv) phagocytosis of apoptotic bodies by macrophages (INOUE *et al.,* 2011).

Generally, necrosis is initiated by non-cellular mechanisms, such as ischemia, adenosine triphosphate (ATP) depletion (BHATIA, 2004), and traumatic insults, which lead to irreversible cellular damage (YEUNG *et al.,* 2017). In this case, 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 (HOU *et al.,* 2016).

Upon the consideration of the importance of cellular changes related to death that involve ovarian cells, this literature review will address aspects related to the folliculogenesis and the process of follicle atresia, as well as substances that induce atresia during *in vitro* and *in vivo* folliculogenesis and methods and parameters for analyzing atresia in ovarian follicles.

DEVELOPMENT

Folliculogenesis and the process of follicle death

Folliculogenesis is an event that begins in prenatal life for most species and can be defined as the process of follicle assembly, growth and maturation, beginning with the formation of the primordial follicle and ending with the preovulatory follicle (van den HURK and ZHAO, 2005; GOUGEON, 2010). This event results from a complex balance between proliferation, differentiation and cell death, of both the somatic (granulosa and/or theca cells) and germ (oocyte) cell follicle compartments. Follicle is considered the morphological and functional unit of the mammalian ovary, providing an ideal environment for growth and maturation of the oocyte (WANG *et al.,* 2017), besides producing some substances essential to its maintenance and development (HERNANDEZ-MEDRANO *et al.,* 2012).

During folliculogenesis, changes in follicular morphology are observed and comprise oocyte growth, differentiation and proliferation of granulosa cells, as well as the appearance of theca cells (BRISTOL-GOULD and WOODRUFF, 2006). Considering the morphological changes that occur during folliculogenesis, follicles can be divided into two major phases of development: 1) preantral phase (Fig. 1A), which is subdivided into activation of primordial follicles and growth of the primary and secondary follicles; and 2) antral phase (Fig. 1B),

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divided into initial and terminal growth of tertiary follicles and formation of the *De Graaf* or preovulatory follicle (ARAÚJO *et al.,* 2014). It is noteworthy that preantral follicles represent about 90% of the ovarian follicular population (AERT and BOLS, 2010) and that 95% are primordial follicles (McGEE and HSUEH, 2010), which constitute a reserve *pool* of gametes throughout the reproductive lifespan of females (QU *et al.,* 2000). Primordial follicles leave the quiescence *pool* and begin to grow; nevertheless, only a small number of these follicles, about 0.01%, ever ovulate.

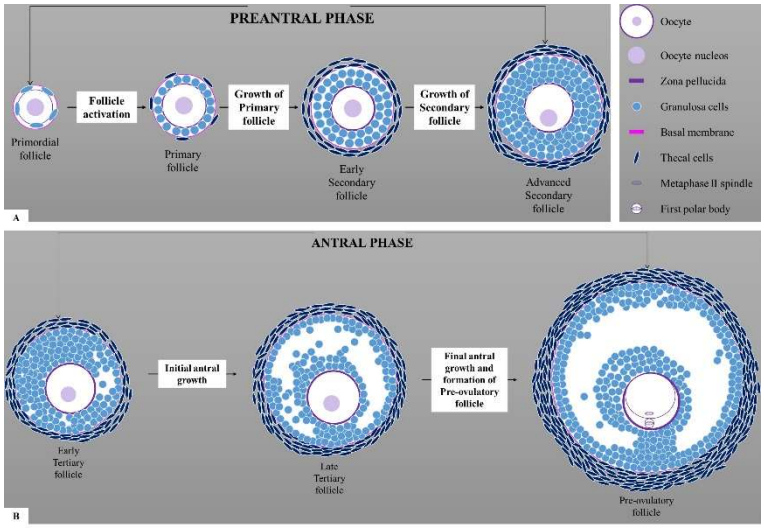

Figure 01: Schematic sequence of complete follicular development. Preantral phase (A) and Antral phase (B). **Obs.:** Adapted from Araújo *et al*. (2014).

The follicular development through the stages of folliculogenesis is characterized by high rates of proliferation and atresia, which depend on a balance between stimulatory and inhibitory substances. However, the decrease in the number of follicles at each stage of development indicates that the control of follicle survival or atresia during folliculogenesis is not the same at each stage (MARKSTRÖM *et al.,* 2002).

Atresia is a natural process characterized by reabsorption of one or more ovarian follicles before a state of maturity has been reached. In follicles, this process can occur through the apoptotic way or the degenerative process of necrosis. However, apoptosis is the most frequent form of physiologic cellular death (ELMORE, 2007), leading to significant follicle loss. Tab. 01 shows the differences between apoptosis and necrosis. 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 (McGEE and HSUEH, 2010). 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*.

The early stages of follicular atresia are characterized by pyknotic granulosa cells showing chromatin condensation and margination with sharply circumscribed, uniformly dense crescents, as well as cytoplasmic condensation beyond the presence of cell debris in the follicular antrum. Moreover, in this process numerous membrane-bound apoptotic bodies with condensed cytoplasm (with or without nuclear fragments) are also observed (ELMORE, 2007). In the later stages, the basal lamina disintegrates, the number of granulosa cells is reduced, and

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the follicle collapses. At this stage, apoptotic granulosa cells are believed to be removed from the atretic follicle by viable ovarian cells and macrophages (CELESTINO *et al.,* 2009). It is unclear which ovarian cell types are involved in removal of the apoptotic granulosa cells.

Table 01: Differences between apoptosis and necrosis.

APOPTOSIS **Programmed cell death** **(physiologic or pathologic)**	**NECROSIS** **Accidental cell death** **(pathologic)**
Single cells or small clusters of cells	Extensive tissue damage resulting in an intense inflammatory response
Cell shrinkage due to condensation of cytoplasm and convolution	Karyolysis (the complete dissolution of the chromatin matter of a dying cell due to the activity of DNase)
Pyknosis (the irreversible condensation of chromatin in the nucleus of a cell)	Pyknosis (the irreversible condensation of chromatin in the nucleus of a cell)
Karyorrhexis (fragmentation of the nucleus)	Karyorrhexis (fragmentation of the nucleus)
Formation of numerous membrane-bound vesicles (apoptotic bodies) with condensed cytoplasm (with or without nuclear fragments)	Swelling of organelles and of the cells, resulting in cell lysis due to loss of membrane integrity
DNA fragmentation	Swelling and degeneration of the entire cytoplasm and the mitochondrial matrix
Intact cell membrane	Eventual shedding of the cytoplasmic content into the extracellular space
No inflammation	Ischemia
Phagocytosis of apoptotic bodies by macrophages	ATP depletion
Requires ATP	Traumatic insults, which lead to irreversible cellular damage
	Chromatin flocculation
	Blebbing of the plasma membrane
	Random digestion of DNA

DNA = Desoxi nucleotide acid.

Features and sensitivity levels of ovarian follicles

Follicular atresia occurs in both pre and postnatal life and at all stages of follicular development. The susceptibility to atresia, either by apoptosis or necrosis degenerative process depends on the stage of follicular development, being predominant in the antral phase. According to the follicle stage, there is different susceptibility among follicle compartments to atresia. In preantral follicles, atresia is more commonly observed in the oocyte. Nevertheless, over follicle development, the more advanced stage, atresia occurs both in the oocyte and granulosa cells, being granulosa cells first to become atretic.

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Preantral follicles

Primordial and primary follicles show both type I and type II degeneration. Type I degeneration is characterized by a presence of an oocyte with a pyknotic nucleus and well-organized granulosa cells without pyknotic nuclei. While on type II degeneration, the oocyte and granulosa cells are equally affected, and retraction of the oocyte and swollen granulosa cells detached from basement membrane can be observed (SILVA *et al.,* 2002). In primordial and primary follicles there is a higher incidence of type I degeneration, which could be a consequence of improper growth activation (MHAWI *et al.,* 1991), since after the oocyte activation, there is organelle multiplication and an increase on the uptake of nutrient (SILVA *et al.,* 2002). It can also be provoked by reduced oxygen and nutrient diffusion for preantral follicles within ovarian cortex (SILVA *et al*., 2006). Moreover, degeneration of type II is more common in the secondary follicles.

The higher sensitivity of secondary follicles to degeneration may be due to the fact that these follicles are at a stage of growth, showing higher morphological evidence of biosynthetic activity and nutrient uptake. In addition, it is important to note that not necessarily pyknotic bodies in the granulosa cells or rupture of the basement membrane in degenerated follicles are observed (SILVA *et al.,* 2002). Other studies also reported that primordial and tertiary follicles at initial and advanced stages of atresia may contain an intact basement membrane (SILVA *et al.,* 2002), including after *in vitro* culture (ARAÚJO *et al*., 2010).

Antral follicles

The first sign of degeneration in tertiary follicles is observed with the increase of the number of dead granulosa cells (HIRSHFIELD, 1986; 1988), which are characterized by pyknotic nuclei and the presence of cell debris in the follicular antrum (SVENSSON *et al.,* 1999). In contrast to preantral follicles, the tertiary follicles show only degeneration of granulosa cells and can contain an intact basement membrane (SILVA *et al.,* 2002). In the adult human (GOUGEON, 1986) and in pregnant and non-pregnant cattle ovary (De los REYES *et al*., 2006), the degree of atresia has been estimated to be highest in antral follicles >5 and 6mm in diameter, respectively. It could confirm that pregnancy is associated to increase proportion of follicle atresia.

Tilly and Hughes teams suggested that granulosa cell apoptosis, at least in part, are involved the induction of follicular atresia. They found granulosa cells with DNA fragmentation entering into apoptosis in atretic follicles (HUGHES *et al.,* 1991; TILLY *et al.,* 1991). Since then, numerous researchers have attempted to confirm the primary trigger of apoptotic stimuli and the intracellular signal transduction pathway engaged in granulosa cell apoptosis during follicular atresia. To date, many apoptotic factors involved in follicular atresia, including cell death ligands and receptors, pro- and anti-apoptotic factors, growth factors and cytokines have been identified.

In the porcine ovary, apoptosis is induced in granulosa cells located in the inner surface of granulosa layer, but not in cumulus cells, oocytes, inner or extra theca layers in the early stages of atresia (MANABE *et al.,* 2000; NAKAYAMA *et al.,* 2000). At the more advanced stages, most granulosa cells undergo apoptosis and the granulosa layer was irregular and disorganized, the basement membrane showed a total destruction, and apoptosis is started in

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theca layers and cumulus cells, showing lymphocyte infiltration (De los REYES *et al*., 2006). Thus, follicle apoptosis of large antral follicles is induced by the neighboring granulosa cells.

Some early and advanced atretic follicles exhibited heterogeneous staining for P450 aromatase but this staining completely disappeared with ongoing atresia (MLODAWSKA and SLOMCZYNSKA, 2010). In swine, apoptosis in individual atretic follicles was correlated with substantial decreases in follicular fluid estrogen levels (TILLY *et al.,* 1992), which has previously been attributed to decreased aromatase activity (MAXON *et al.,* 1989). Moreover, follicular atresia has been associated with low amounts of P450 aromatase mRNA in bovine early and advanced atretic follicles (BAO *et al.,* 1997) and with loss of mRNA for P450 aromatase in caprine granulosa cells (YUAN *et al.,* 2008).

Factors directly affecting follicle atresia

Several factors, which influences the physiological state of the ovarian donor may affect follicle atresia, both *in vivo* and *in vitro* (Fig. 02). Because of that, it much be consideration to development of a culture system that might be able to maintain follicular growth and avoid follicles loss (ARAÚJO *et al*., 2014, 2015).

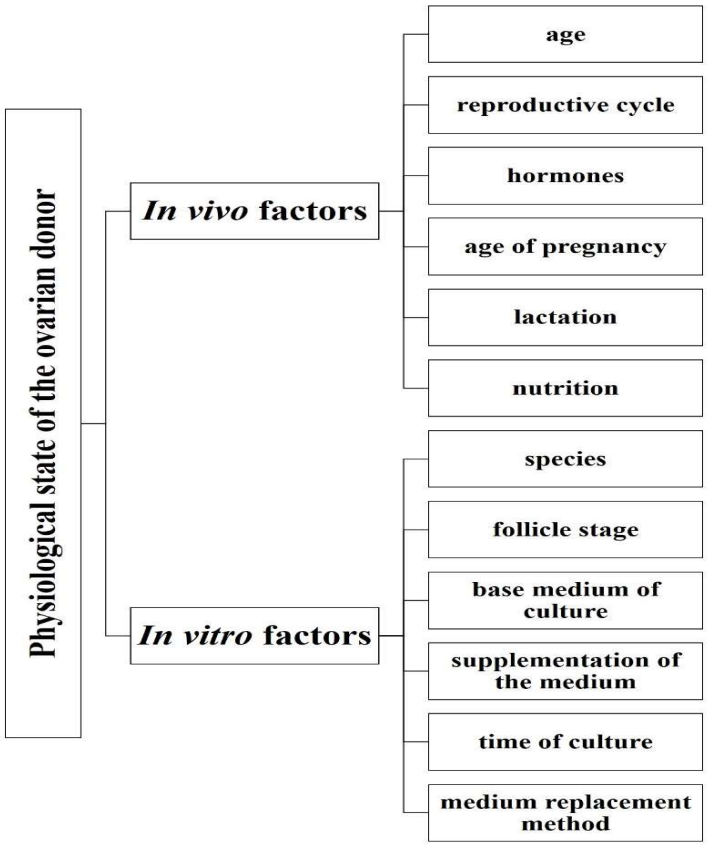

Figure 02: *In vivo* and *in vitro* factors that influences the physiological state of the ovarian donor and may affect mammalian follicle atresia.

Obs.: Source: Own author.

In vivo, studies have showed that the size of antral follicle, as well as the age of pregnancy influence in the follicular atresia rate. In rats, for example, Tabarowski *et al.* (2005) demonstrated that from day 5 of pregnancy, the follicles sporadically exhibited apoptotic bodies observed at the periphery of the antral cavity and/or in the region linking cumulus oophorus

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complex (COC) with the follicular wall. In addition, expansion of the COC or resumed meiosis were not observed in those follicles. In cattle, the largest follicles might be atretic during pregnancy (De los Reyes *et al*., 2006). Concerning the ovarian *in vitro* development, it may be affected if follicles are collected from postpartum cows, for example. After *in vitro* culture, viability of preantral follicles from postpartum cattle have decreased (FIGUEIREDO *et al.,* 1994). Therefore, *in vitro* follicle development and atresia can either induced or avoided depending on several aspects.

Substances that induce atresia during *in vitro* and *in vivo* folliculogenesis

The susceptibility to atresia, besides the stage of follicle development, depends on the conditions of *in vitro* culture, which thus determines the pathway of cellular death, i.e. apoptosis or necrosis. In addition, problems related to prolonged culture are chromosomal instability and shortening of the telomere, which may lead to a loss in the function of genes that are important for development (CUNHA *et al.,* 2014).

Some studies assessed the influence of the addition of solutions containing coconut water to the *in vitro* culture medium for caprine primordial follicles (MARTINS *et al.,* 2005). It was observed that base medium (Minimum Essential Medium; MEM) without or with low proportions (5 or 10%) of coconut water enabled high follicle survival and activation rates. Nevertheless, an increase of follicle degeneration was found when pure coconut water was employed as culture medium. On the other hand, beneficial effects of the addition of insulin-transferrin-selenium, pyruvate, glutamine, hypoxanthine and bovine serum albumin to the culture medium were observed, which reduced follicle degeneration (SILVA *et al.,* 2005).

Studies investigated the importance of antioxidants (alpha-tocopherol and ternatin; LIMA-VERDE *et al.,* 2009) and protein compounds (fetal or estrus female serum; BRUNO *et al.,* 2008). It was observed that these substances had no effect on follicle activation and growth, as well as they did not provide maintenance of ultrastructural integrity of caprine preantral follicles. Other studies showed that, instead preventing atresia, 3-indol-acetic acid (IAA – MATOS *et al.,* 2006), an auxin present in coconut water, and some intraovarian factors such as the bone morphogenetic protein-6 (BMP-6; ARAÚJO *et al.,* 2010) induced atresia in goat primordial follicles after *in vitro* culture of ovarian cortical tissues for seven days. Although IAA and BMP-6 promoted proliferation of granulosa cells, which is critical for primordial follicle activation and growth, these compounds caused several abnormalities in the follicles, such as large number of vacuoles, reduction and/or absence of organelles in the ooplasm, as well as irregular or fragmented nuclear and cytoplasmic membranes, and no oocyte and granulosa cells contact (MATOS *et al.,* 2006; ARAÚJO *et al.,* 2010).

In antral follicles, even though vascular endothelial growth factor (VEGF) has been showed as a factor that promotes in vitro maturation, both from preantral (ARAÚJO *et al.,* 2011) and antral follicles (ARAÚJO *et al.,* 2017), the injection of its antagonist (VEGF-A Trap) can impair ovulation and the subsequent development and functional capacity of the corpus luteum (HAZZARD *et al.,* 2002). This antagonist caused a decrease of granulosa and thecal cell proliferation (ABRAMOVICH *et al.,* 2010), and a consequently increase in the number of apoptotic granulosa cells (ABRAMOVICH *et al.,* 2006). In addition, it was observed an increase in the spontaneous DNA fragmentation with an exhibition of multiples internucleosomal fragments of 180-bp (ABRAMOVICH *et al.,* 2006), and an increase of the

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levels of the protein p17, an active fragment of caspase 3, suggesting that VEGF is involved in preventing apoptosis (ABRAMOVICH *et al.,* 2010). Similarly, the TNF-α induced apoptosis in antral follicles (KAIPIA *et al.,* 1996) and in granulosa cells (PRANGE-KIEL *et al.,* 2001) of murine and swine, respectively. Ovarian follicles cultured in these conditions are currently being used to investigate the pathways that control apoptosis and follicular atresia (TILLY *et al.,* 1991; 1992; PARBORELL *et al.,* 2002), since that factor are potential causative of follicular atresia.

METHODS AND PARAMETERS FOR ANALYZING ATRESIA IN OVARIAN FOLLICLES

In vitro and *in vivo* culture

In vitro studies have shown that growing follicles are more sensitive to atresia than primordial follicles. In addition, as previously mentioned, with the increase of follicle diameter, secondary follicles become more sensitive to atresia. The technique of *in vitro* culture is used for the evaluation of follicular quality after cryopreservation, since the damage caused by follicular cryopreservation is not always observed immediately after heating. For this reason, several hours of *in vitro* culture are needed before an analysis of follicular viability. The restoration of cellular metabolism, which can be detected via the enzymatic activity, may report on the normal functioning of the cell. In addition, *in vitro* short-term (24h) culture allows a better analysis of follicular quality after cryopreservation (RODRIGUES *et al.,* 2006, SANTOS *et al.,* 2007) and apoptotic DNA cascade of follicle cells (ABRAMOVICH *et al.,* 2010). It is also possible to evaluate the ability to complete follicular development after cryopreservation by *in vivo* culture, i.e., by ovarian transplantation, since it allows the resumption of metabolic activity (SANTOS *et al.,* 2007).

Techniques for the evaluation of follicular quality

Different techniques have been used to detect follicle atresia caused by either apoptosis or necrosis after *in vitro* culture of ovarian follicles during the different stages of development.

For apoptosis detection, different techniques may be utilized, such as: 1) morphological analysis (laser confocal microscopy and transmission electron microscopy; TEM); 2) evaluation of DNA fragmentation (enzyme-linked imumunosorbent assay - ELISA and terminal deoxynucleotidil tranferase-mediated deoxyuridine triphosphate biotin nick end-labeling - TUNEL); 3) analysis of DNA content (flow cytometry); 4) evaluation of the translocation of phosphatidilserin residues located in the inner mitochondrial membrane; 5) analysis of gene expression and caspases involved in apoptosis (RT-PCR, northern and western blot, and immunohistochemistry). For evaluation of cell death by necrosis, it can be used classical histology (staining with hematoxilin-eosin or periodic acid schiff-hematoxilin), laser confocal microscopy, and TEM. Regardless of the type of atresia, the characteristic changes of cell death can be assessed by the techniques briefly described below:

Morphological analysis by Classical histology

Under the light microscope, the most common signs of atresia are: shrinkage of the cytoplasm of the oocyte; detachment of granulosa and/or theca cells; pycnotic bodies in the

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nuclei of oocytes; cytoplasmic vacuolization, which depends on the sectioning thickness used (Fig. 03). This technique is limited since it does not allow assessing the integrity of cytoplasmic organelles, being need other more accurate techniques such as transmission electron microscopy (ARAÚJO *et al*., 2010) and laser confocal microscopy, both described below.

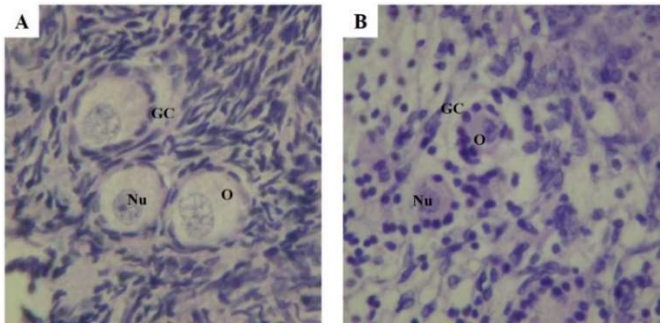

Figure 03: Histological section of (A) normal follicles from uncultured tissue and, (B) atretic follicles after culture in the presence of 50 ng/ml of bone morphogenetic protein-6 (BMP-6).

Obs.: O: Oocyte; NU: Oocyte nucleus; GC: Granulosa cells. Staining with periodic acid Schiff-hematoxylin, 400x. Source: Own author.

Morphological analysis by TEM

Morphological analysis by TEM allows for a marked improvement in the evaluation of ovarian tissue integrity. The main features observed during the identification of atretic ovarian follicles are an increased number of vacuoles in oocytes and granulosa cells, the absence of organelles, as well as irregular or fragmented nuclear and cytoplasmic membranes. In addition, fragmented granulosa cells with no oocyte contact are also observed (MATOS *et al.,* 2006; ARAÚJO *et al.,* 2010, Fig. 04).

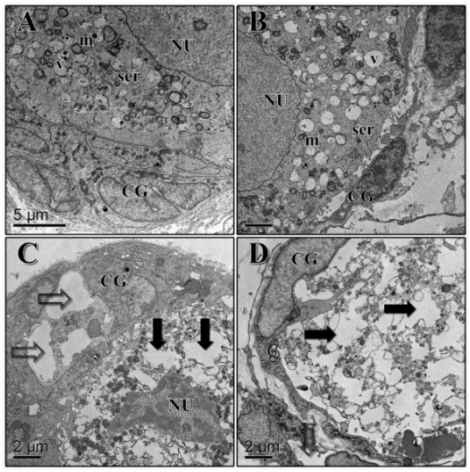

Figure 04: Electron micrograph of caprine preantral follicle from (A) an uncultured control (5800x), (B) MEM^{+} culture, (C) 1ng/mL of BMP-6 culture, and (D) 50ng/mL of bone morphogenetic protein-6 (BMP-6) culture (8000x) for 7 days.

Obs.: Homogeneous cytoplasm with numerous rounded mitochondria is characteristic of non-cultured follicles and cultures with only MEM (3A and 3B, respectively). Extreme vacuolization and great holes are present in the cytoplasm, indicative of degeneration (3C and 3D; solid arrow). Note the empty space in degenerated granulosa cells after *in vitro* culture with BMP-6 (3C and 3D; open arrow). NU: Oocyte nucleus; GC: Granulosa cells; m: Mitochondria; ser: Smooth endoplasmic reticulum; v: Vesicle. Reproduced with permission from Araújo *et al.* (2010).

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Morphological analysis by Fluorescence or Laser confocal microscopy

In the confocal microscope, using fluorescent probes, membrane alterations can be visualized, since the externalization of phosphatidylserine 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). The integrity of the plasma membrane and metabolism can also be evaluated using the fluorescent markers ethidium homodimer-1 or calcein-AM, respectively. Whilst the later probe detects intracellular esterase activity of viable cells, the first labels nucleic acids of non-viable cells with plasma membrane disruption (LOPES *et al.,* 2009). The advantages are sensitivity (can detect a single apoptotic cell), the ability to confirm the activity of initiator caspases, and to use only a common fluorescence microscope, as Eclipse 80i (Fig. 05). The disadvantage is that the membranes of necrotic cells are labeled as well and caspase activation does not necessarily indicate that apoptosis will occur (ELMORE, 2007).

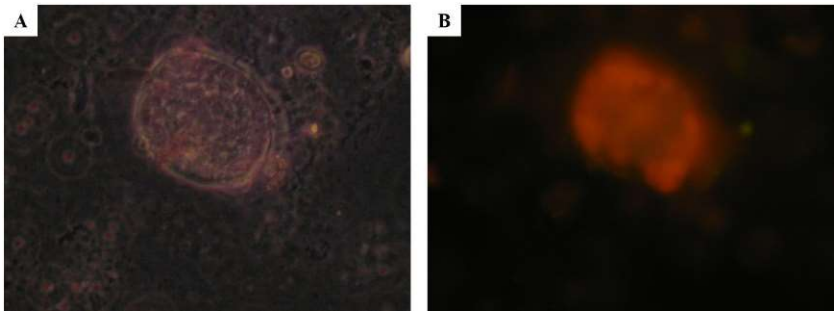

Figure 05: Fluorescence micrograph of caprine preantral follicle cultured from 50 ng/ml of bone morphogenetic protein-6 (BMP-6) for 7 days. (A) bright field and (B) dark field marked in red by ethidium homodimer-1.

Obs.: 400x. Source: Own author.

Evaluation of DNA fragmentation by ELISA

Nuclear DNA fragmentation by apoptosis or necrosis can be quantified by ELISA from the release of BrdU into the cytoplasm of apoptotic cells. The principle of the assay is that cells are incubated with non-radioactive thymidine analogue BrdU, which is incorporated into the genomic DNA. Thus, BrdU-labeled DNA fragments are released from the cells into the cell cytoplasm during apoptosis. These DNA fragments are detected immunologically by the ELISA technique using an anti-DNA-antibody bound to capture the DNA fragments (HONG *et al*., 1991).

Evaluation of DNA fragmentation by TUNEL

In order to identify cells undergoing apoptosis via detection of terminal fragments of DNA (portion 3'-OH), typically related to the fragmentation of nuclear DNA, the TUNEL allows the morphological identification of apoptotic cells. In this context, the TUNEL has been used as a principal method to identify and quantify the apoptotic cells in atretic follicles, since it is very sensitive and fast, it takes about 3 hours (ZHANG *et al.,* 2008). 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). Even considering these

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issues, among these techniques, the TUNEL and immunohistochemistry are still the most used for apoptosis detection (SILVA *et al.,* 2006).

Analysis of DNA content (flow cytometry)

Recently, the flow cytometry has established analysis of the apoptotic markers bound to cell compartments in different tissues. Basically, a flow cytometer consists of fluidics, optics and electronics, as it measures cells in suspension that flow in single-file through an illuminated volume where they scatter light and emit a fluorescence that is collected, filtered and converted to digital values for storage on a computer (OCHATT, 2006).

Evaluation of the translocation of phosphatidylserine residues

The residues of phosphatidylserine located in the inner mitochondrial membrane 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: (i) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (ii) they are quickly phagocytized by surrounding cells thus likely preventing secondary necrosis; and, (iii) the engulfing cells do not produce anti-inflammatory cytokines (SAVILL and FADOK, 2000; KUROSAKA *et al.,* 2003). Combined to that, ischemia impairs cellular energetic metabolism which decreases the activity of the Na^+/K^+-ATPase pump with a gain in sodium (BONZ *et al.,* 1998). During mild ischemia, mitochondrial matrix swells moderately due to the uptake of sodium (GARLID, 1996).

Analysis of gene expression and caspases involved in apoptosis

Caspases are family of highly conserved cysteine proteases that mediate the course of apoptotic cell suicide. Caspases are the main effector molecules in ovarian apoptosis. They are activated in two ways in the granulosa cells: (i) cell surface receptors; and (ii) members of the Bcl-2 family of proteins. In the ovary, caspase-3 is expressed in luteal and thecal cells of healthy corpus luteum as well as in the granulosa cells of atretic follicles. It is absent in granulosa cells of healthy follicles. This expression is regulated by gonadotrophin and may be altered as part of the apoptotic process in the granulosa cells (BOONE and TSANG, 1998).

FINAL CONSIDERATIONS

This work demonstrated the complex mechanism that regulates atresia, either by apoptosis or necrosis, in different stages of follicular development, and the techniques used to evaluate these parameters. Elucidation of these mechanisms can contribute to a better comprehension of ovarian folliculogenesis and may help us to control follicular development. As a result, this may allow for a greater number of viable oocytes that will be destined for different *in vitro* techniques of assisted reproduction. In addition to these applications in reproduction, a better knowledge regarding the mechanism of cell death can contribute to the design of new therapeutic modalities. These treatments may include ovarian disorders

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characterized by excessive cell degeneration and infertility, such as premature ovarian failure and polycystic ovary syndrome.

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