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Acquisition of gonadotropin dependence by early antral follicles and the challenges to promote their growth *in vitro*

Aquisição da dependência de gonadotrofinas pelos folículos antrais iniciais e os desafios para promover o seu crescimento *in vitro*

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Abstract: This review aims to discuss the main factors involved in the development of early antral follicles until gonadotropin dependence. This follicular phase is characterized by intense proliferation of granulosa cells, formation of a fluid-filled cavity, morphological differentiation of cumulus cells, mural granulosa cells and recruitment of theca cells. The interaction between oocyte, granulosa and theca cells is crucial for follicular growth and hormone production. Growth factors produced by the oocyte, such as growth and differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15), regulate granulosa cell proliferation and differentiation and antral cavity development, as well as stimulate the production of follicle-stimulating hormone (FSH) receptors in granulosa cells. In response to FSH, granulosa cells secrete C-type natriuretic peptide (CNP), which acts through its receptor to increase cyclic guanosine monophosphate (cGMP) production and consequently follicular development. Granulosa cells also produce insulin-like growth factor-1 (IGF-1) and increase aromatase enzyme activity, which results in greater sensitivity to gonadotropins and follicular steroidogenesis. The absence of IGF-1 signaling causes cessation of follicular growth at the early antral stage. Many other local factors are involved in the regulation of follicular development. Therefore, this review brings relevant data for a better understanding of the mechanisms involved in the control of early antral follicle growth, emphasizing the role of endocrine and paracrine factors, the oocyte-granulosa cell interaction and the processes of follicular atresia. The challenges for the establishment of efficient culture systems for *in vitro* growth of early antral follicles are also discussed.

Keywords: Oocyte; granulosa cells; theca cells; gonadotropins; cumulus cells; folliculogenesis

Resumo: Esta revisão tem como objetivo discutir os principais fatores envolvidos no desenvolvimento de folículos antrais iniciais até a dependência de gonadotrofinas. Essa fase

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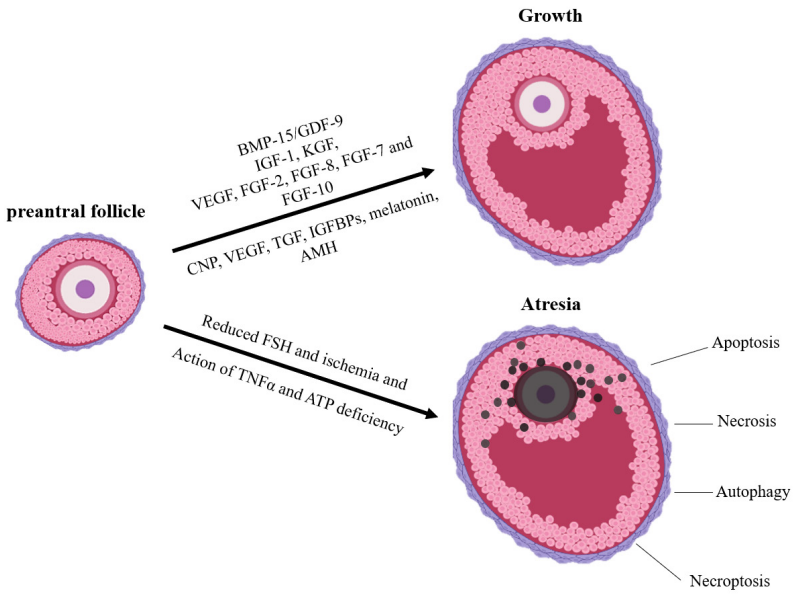
folicular é caracterizada por intensa proliferação de células da granulosa, formação de uma cavidade preenchida por líquido, diferenciação morfológica das células do *cumulus*, células da granulosa murais e recrutamento de células da teca. A interação entre oócito, células da granulosa e da teca é determinante para o crescimento folicular e produção hormonal. Fatores de crescimento produzidos pelo oócito, fator de crescimento e diferenciação-9 (GDF-9) e proteína morfogenética óssea-15 (BMP-15), regulam a proliferação e diferenciação de células da granulosa, e o desenvolvimento da cavidade antral, bem como estimulam a produção de receptores do hormônio folículo estimulante (FSH) nas células da granulosa. Em resposta ao FSH, as células da granulosa secretam o peptídeo natriurético tipo C (CNP), que atua através de seu receptor para aumentar a produção de monofosfato de guanosina cíclico (GMPc) e consequentemente o desenvolvimento folicular. As células da granulosa também produzem o fator de crescimento semelhante à insulina 1 (IGF-1) e aumentam a atividade da enzima aromatase, o que resulta em maior sensibilidade às gonadotrofinas e esteroidogênese folicular. A ausência de sinalização do IGF-1 causa cessação do crescimento folicular no início do estágio antral. Muitos outros fatores locais estão envolvidos na regulação do desenvolvimento folicular. Por tanto essa revisão traz dados relevantes para uma melhor compreensão dos mecanismos envolvidos no controle do crescimento de folículos antrais iniciais, enfatizando o papel dos fatores endócrinos e parácrinos, a interação oócito-células da granulosa e os processos de atresia folicular. Os desafios para o estabelecimento de sistemas de cultivo eficientes para o crescimento *in vitro* de folículos antrais iniciais também são discutidos.

Palavras-chave: Oócito; células da granulosa; células da teca; gonadotrofinas; células do *cumulus*; foliculogênese



1. Introduction

Oocyte developmental competence refers to the ability of a female gamete to mature, to be fertilized, and to support embryonic development until the blastocyst stage[1]. According to Dode et al.[2], this competence is acquired gradually during preantral and early antral follicular growth. To reinforce this information, oocytes from 3.0 mm antral follicles are able to complete nuclear maturation *in vitro*, while those from smaller follicles (1.0 and 2.0 mm) have reduced competence[3-4]. This non-competence of oocytes from small antral follicles is due to the reduced expression of genes that activate signaling pathways to increase the oocyte's ability to respond to the increase in gonadotropins[5]. Responsiveness to gonadotropins enables the follicles to grow until selection and dominance[6-7].

In the course of follicular development, proliferation and morphological differentiation of granulosa cells are of great importance to prepare the follicle to respond to gonadotropins and to create a favorable environment for oocyte development[8]. Granulosa cells produce several autocrine and paracrine factors that may be involved in oocyte growth and antrum formation[9]. Additionally, oocyte-derived factors stimulate the expression of follicle-stimulating hormone receptors (FSHR) in granulosa cells, enabling them to become responsive to gonadotropins[10]. The follicle-stimulating hormone (FSH) induces proliferation and viability of the oocyte-cumulus-granulosa complex and may also induce granulosa cell differentiation[11]. Furthermore, oocyte-derived factors also stimulate antral cavity formation by increasing the expression proteoglycans, as a result of interaction with FSH[9]. Thus, understanding the endocrine, paracrine and autocrine mechanisms that control the interaction between follicular cells and the oocyte during early antral follicles favors the development of strategies to promote their development *in vitro*[12].

The present review provides an overview of the main factors that control the development of early antral follicles up to gonadotropin dependence, i.e., regulation of granulosa cell proliferation, steroidogenesis, atresia, interaction between oocyte and granulosa cells, as well as strategies to promote the development of early antral follicles *in vitro.*

2. Endocrine control of early antral follicle development

Follicle development from the preantral to the early antral stage is mainly controlled by intraovarian regulators, but it can be stimulated by FSH. The specific receptors for FSH are expressed in granulosa cells of secondary and early antral follicles[13]. When secondary follicles are formed, granulosa cells express FSHR, and theca cells express the luteinizing hormone receptor (LHR)[14]. In domestic and human species, antrum formation is observed when the follicles have around 0.2 mm[15] and become dependent on gonadotropin when they reach 3.0 mm in cow[16], 4.0 mm in sheep[17], 3.0 mm in goat[18] and 5.0 mm in human[19]. Follicular growth and maturation beyond this stage, which includes follicle recruitment, selection, dominance, and ovulation, is gonadotropin-dependent[20-21]. Acquisition of FSH dependence during this interval of growth is crucial to determining follicular fate, *i.e.*, growth or atresia. The C-type natriuretic peptide (CNP) is secreted by granulosa cells of secondary and antral follicles in response to FSH stimulation. CNP acts through its receptor (NPRB) expressed in

granulosa cells of secondary follicles and increases Guanosine 3',5'-cyclic monophosphate (cGMP) production to stimulate follicle development[33]. Gene expression analyses indicated increases in transcripts for CNP receptors (NPP and NPRB) during early folliculogenesis in mice, in association with increases in ovarian CNP peptides[33] (Figure 1).

Growth and differentiation factor-9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), both secreted by the oocyte, promote proliferation of granulosa cells and the recruitment of theca cells, events that are required for the transition of follicles from the primary to the secondary stage[34]. Factors produced by secondary follicles, including vascular endothelial growth factor (VEGF), transforming growth factor (TGF), insulin-like growth factor (IGF), fibroblast growth factor-2 and -7 (FGF-2 and FGF-7), BMPs and activin, are necessary for survival and further development. At the antral stage, locally synthesized peptides play a key role in the regulation of follicular development through endocrine and paracrine mechanisms[17, 35]. Among these peptides, those of the IGF system, including IGF-1, IGF-2 and the IGF binding proteins (IGFBPs) and some members of the FGF family, such as FGF-2, FGF-7 (or KGF), FGF-8 and FGF-10[35-36], appear to be critical for late-stage follicle development (Figure 1).

Fushii et al.[22] recently showed that cumulus-oocyte complexes (COCs) cultured with FSH have the antral cavity formed one day earlier than those that do not receive this hormone, showing the importance of FSH in follicular development. Intraovarian regulators, IGF, activin, oocyte-derived factors, and gap junction membrane channel protein play a central role in the acquisition of FSH dependence at the early antral stage of follicle development[13]. Theca-derived androgens bind to androgen receptors (ARs) in granulosa cells[23], thereby inducing FSHR expression and follicle growth during the preantral-to-antral transition[14, 24, 25]. The AR deficiency in the mice ovary induces granulosa cell apoptosis, arrests antral follicle growth and results in premature ovarian failure[26-28]. Thus, androgens play an important role in the growth, survival and acquisition of FSH dependence in early antral follicles[13] (Figure 1).

Anti-Müllerian hormone (AMH) is a product of granulosa cells from small antral follicles onwards that has an inhibitory or retarding role in the development of antral follicles. The AMH reduces follicle sensitivity to FSH, decreasing the expression of FSHR. It inhibits cyclic FSH-dependent recruitment and appears to play a role in all gonadotropin-independent follicular growth. Despite having a regulatory relationship between androgens and AMH, it is not possible to guarantee that their effects are mediated by estradiol, via testosterone aromatization[29] (Figure 1).

Melatonin is found in the follicular fluid of human antral follicles and has important roles in the control of follicle development[30]. Its receptors have previously been detected in granulosa cells of preantral and antral follicles[31]. Regarding the effects of melatonin, Barros et al.[32] demonstrated that this hormone is associated with meiotic competence of oocytes from early antral follicles. Melatonin maintains follicular survival, stimulates antral cavity formation and subsequent follicular and oocyte growth, as well as increases glutathione and metabolically active mitochondria levels after *in vitro* culture of sheep secondary follicles[32].

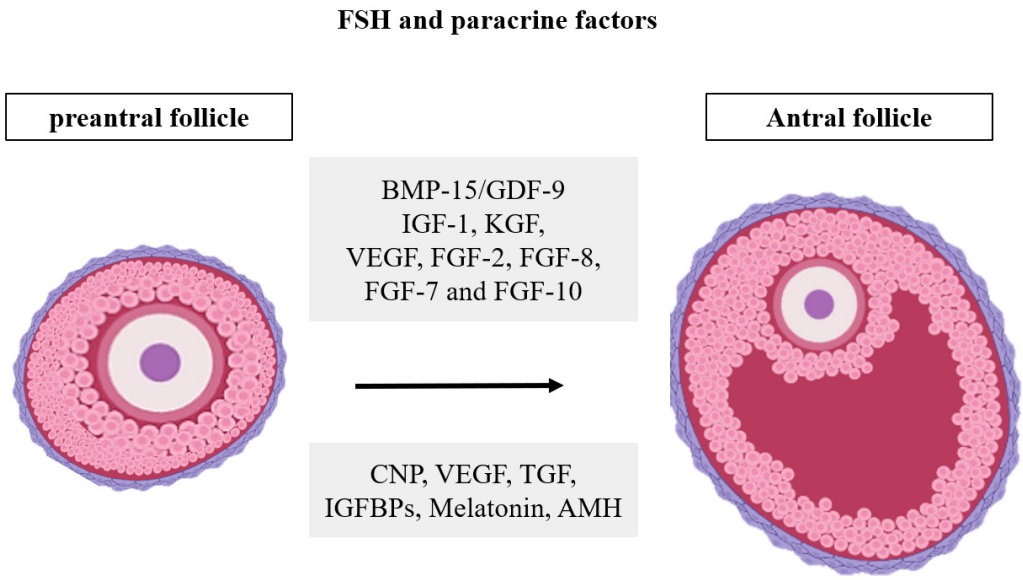


Figure 1. Factors that regulate the development of preantral follicles up to gonadotropin-dependent stages.

3. Oocyte-granulosa cell interaction during early antral follicle development

It is well known that during follicular development; members of the transforming growth factor beta (TGFβ) family and their receptors are involved in the control of oocyte growth and granulosa cell proliferation. Oocyte-derived TGFβ family members, such as GDF-9 and BMP-15, regulate granulosa cell proliferation and differentiation, as well as the development of the antral cavity[33-34]. Additionally, recent studies indicate that these factors regulate the expression of mRNA for LHR in cumulus cells[35]. Oocyte-derived GDF-9 promotes the growth of cumulus-oocyte (COCs), while BMP-15 induces the expression of choriogonadotropin receptor mRNA (LHCGR) in cumulus cells, and FSH receptor expression in follicles. Such factors contribute to follicular development and oocyte maturation[35-36]. GDF-9 and BMP-15 bind to type II BMP receptor[37] and recruit type I activin-like kinase (ALK)5[38] and ALK6[39] to regulate downstream SMAD proteins in granulosa cells. Studies indicate that GDF-9 enhances growth and differentiation of preantral follicles in culture [40] and promotes theca cell androgen biosynthesis and proliferation[41]. In addition to these factors, R-spondin2 protein is also an important paracrine factor that can promote granulosa cell proliferation[42]. The FGF-2 and their respective receptors are also involved in early antral follicle development since[36, 43-44] FGF-2 alone or in association with VEGF-A influence steroidogenesis and proliferation of buffalo granulosa cells, by regulating mRNA expression of cytochrome P450 19A1 (CYP19A1), proliferating cell nuclear antigen (PCNA), and Bcl-2 Associated X-protein (BAX)[44-45] (Figure 2). Furthermore, Mattar et al.[46] reported that VEGF-A and FGF-2 promote the formation of endothelial cell networks during *in vitro* culture of theca cells. These structures support successive follicular development up to the preovulatory stage.

Granulosa cells play a role in the development of antral follicles by promoting the development of the oocyte-granulosa cell complex and providing adenosine triphosphate

(ATP) to the oocytes[48]. Moreover, Yang et al.[48] demonstrated the influence of BPM-4, derived from theca cells, on steroidogenesis in early antral follicles. The CNP is also a stimulating factor for early antral follicles[30] (Figure 2). In association with the granulosa cells, the theca cells contribute to the synthesis of inhibin α; which is a hormone that inhibits the production of FSH[49]. Yang et al.[48] demonstrated the influence of BPM-4, which comes from the theca cells, on steroidogenesis and initial antral follicles.

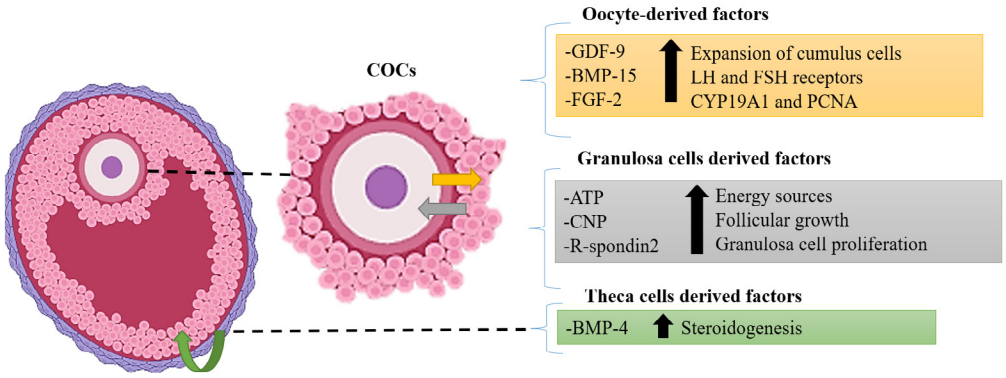


Figure 2. Oocyte-granulosa cell interactions during early antral follicle development.

4. Control of granulosa cell proliferation and estradiol production during early antral follicle development

In early antral follicles, granulosa cells are highly proliferative but susceptible to apoptosis. The factors secreted by the oocyte, like GDF-9 and BMP-15, regulate granulosa cell proliferation and survival[50-51]. Furthermore, granulosa cell proliferation is dependent on cyclin D2 to activate cyclin-dependent kinase (CDK) family members CDK2, CDK4 and CDK6[52]. In developing follicles, FSH stimulates granulosa cell proliferation and aromatization of androgens into estrogens. Estrogens also stimulate granulosa cell proliferation[53]. An increase in estradiol is associated with an increase in the expression of genes for aromatase, 3β-HSD and receptors for FSH and LH in granulosa cells (Figure 2)[54].

Neuronal neuropeptide Y (NPY) is strongly present in granulosa cells, and the abundance of mRNA for NPY is higher in early antral follicles than in late antral follicles. In addition, NPY increases the proliferation of granulosa cells via NPY receptor Y5 (NPY5R) and mitogen-activated protein kinase (MEK)[55]. Baddela et al. [56] reported that, in granulosa cells, hypoxia-inducible factor 1 (HIF1) transcriptionally regulates genes associated with steroidogenesis, such as steroidogenesis acute regulatory protein (StAR), 3B-hydroxysteroid dehydrogenase (HSD3B) and CYP19A1, and proliferation (CCND2 and PCNA). The onset of StAR mRNA expression occurs in early antral follicles of 1.0 mm in diameter[57]. Furthermore, FSH and LH, together with intraovarian cytokines, induce the expression of steroidogenic enzymes in granulosa cells, including StAR, CYP11a1, 3βHSD and CYP19a1, as shown in Figure 3[58]. The expression of mRNA for LHR is found in granulosa cells from follicles smaller than 5 mm[59].

Follicular steroidogenic potential involves an extensive and highly coordinated series of developmental stages. During this process, after intense granulosa and theca cell

proliferation (up to 100-fold), they differentiate into specialized endocrine cells. Ovarian steroids are synthesized by the cooperation of these cells. Theca cells synthesize androgens through the enzymatic activity of cytochrome P450 17A1 (CYP17A1)[53]. Follicles over 2 mm in diameter strongly expressed LH-R and CYP17A1 mRNAs in most thecal cells[55]. Androgens are then converted to estrogens by aromatase (CYP19) produced by granulosa cells. Moreover, progesterone is produced by granulosa cells and used by theca cells to synthesize androgens[60]. StAR, CYP11a1 and CYP19a1 are the key enzymes in the hormone synthesis process[61] (Figure 3).

Granulosa cells express estradiol receptors, contributing to follicular development[62]. Autocrine and paracrine activities of estradiol in granulosa cells stimulate aromatase enzyme activity, increasing gonadotropin sensitivity and expression of IGF-1[59]. In the ovary, IGF-I stimulates follicular steroidogenesis and increases estradiol production. The absence of IGF-I results in interruption of follicular growth in the preantral/early antral stage since these follicles do not respond to gonadotropin[60-61]. In granulosa cells, the stimulatory effect of FSH on CYP19 and protein kinase B (AKT) depends on IGF-I and on the expression and activation of IGF-IR[61]. Furthermore, FSH induces estradiol production via FSHR-cAMP-dependent signaling to induce transcription of the CYP19A1 gene[77]. After follicle recruitment, gonadotropins gradually reduce granulosa cell proliferation and induce their differentiation to produce estradiol[68] (Figure 3).

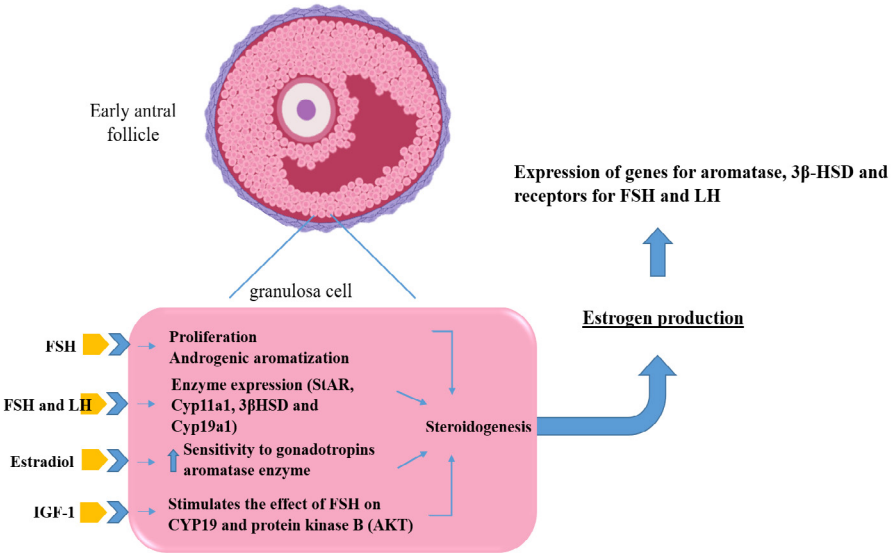


Figure 3. Influence of FSH, LH estradiol and IGF-I on granulosa cells to induce proliferation and production of enzymes involved in steroidogenesis.

5. Follicle atresia during development from early antral follicles

At birth, the ovaries contain thousands of follicles, but only a small portion develops up to ovulation, while the great majority (~99.9) are lost by atresia. Follicular atresia does not occur equally during follicular development, differing between preantral follicles and antral follicles[68]. Spanel-Borowski et al.[69] reported two types of atretic patterns in ovarian follicles, namely type A, in which the oocyte degenerates while granulosa cells remain intact, and

type B, in which the granulosa cells show signs of extensive degeneration while the oocyte remains initially unaffected. Type A is the predominant form of atresia in preantral follicles[70], while in late antral follicles only type B is observed, with apoptosis of granulosa cells in the presence of a more or less intact oocyte being characteristic of atresia in large antral follicles[69]. In early antral follicles, the first changes that indicate atresia occur in the oocyte, such as nuclear chromatin retraction and oocyte fragmentation, while changes are rarely found in the granulosa cells present in these follicles[70].

When the paracrine or endocrine environment is not suitable to support oocyte growth and/or proliferation and differentiation of follicular cells, atresia can occur through necrosis, necroptosis, autophagy and apoptosis pathways[71] (Figure 4). The necrosis and necroptosis pathways have similar morphological features and are characterized by an increase in cell volume, permeabilization and rupture of the plasma membrane, which lead to cell death[71]. Generally, necrosis is initiated by non-cellular mechanisms such as ischemia, deficiency in ATP levels and trauma, leading to irreversible cell damage[71]. Necroptosis is initiated by tumor necrosis factor-α (TNFα) and operated through protein kinase-1 and 3, which interact with its receptors-interacting serine/threonine-protein kinase 1 and kinase 3, respectively, as well as by the domain-like protein of mixed lineage kinase (MLKL)[71]. Zhou et al.[72] showed that the process of autophagy is involved with atresia in secondary and early antral follicles. Autophagy is an evolutionarily conserved form of intracellular process that involves damaged proteins and organelles for degradation and recycling (Figure 4).

It is believed that granulosa cell apoptosis in late antral follicles is triggered by insufficient FSH levels or reduced numbers of FSH receptors[73]. The absence of LH and the decline of FSH circulation cause a decrease in the growth of subordinate follicles, ultimately resulting in atresia[74]. FSH protects granulosa cells from oxidative damage and rescues granulosa cells from apoptosis. FSH is thought to rescue granulosa cells of antral follicles from apoptosis via activation of the phosphatidylinositol 3 kinase (PI3K)–AKT signal transduction pathway. The activation of the phosphoinositide 3-kinase (PI3K)/Akt, via binding of FSH to its receptor, leads to phosphorylation of the box O subfamily of forkhead transcription factors (FOXO), which influences, among other processes, the survival of granulosa cells[75].

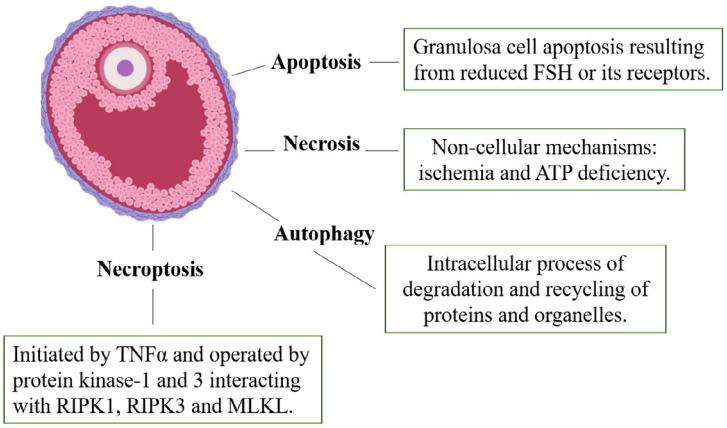


Figure 4. Mechanisms involved in early antral follicle atresia.

6. Strategies for in vitro development of early antral follicles

Several studies have investigated the relationship between follicular and oocyte size with the acquisition of oocyte developmental competence *in vitro*, and many studies have focused on the development of culture protocols that can support the development of oocytes from early antral follicles (Figure 5). Harada et al.[76] demonstrated, for the first time, that 90.0 to 99.0 µm oocytes from early bovine antral follicles (0.5 to 0.7 mm) can grow and acquire developmental competence *in vitro* in the presence of hypoxanthine and FSH. Likewise, Yamamoto et al.[77] demonstrated that, in addition to being able to grow and acquire developmental competence *in vitro*, oocytes (90.0 to 99.0 µm) from small bovine follicles were capable of producing offspring after undergoing maturation, fertilization and subsequent *in vitro* cultivation.

When culturing isolated antral follicles with a diameter between 0.2 and 0.5 mm, it was observed that, like the COCs (0.4 and 0.7), the follicles can also grow in *in vitro* culture, and that the oocytes can achieve meiotic competence[78]. In goat species, in addition to obtaining improved *in vitro* oocyte maturation, embryo production from oocytes from small antral follicles cultured *in vitro* was reported[79]. Cadenas et al.[80] showed that early antral follicles from goats cultured in a medium containing insulin (10 ng/mL) associated with growth hormone (50 ng/mL) are capable of maintaining the growth and maturation of oocytes *in vitro* at levels similar to in life. Similarly, when observing the effect of stimulation of recombinant human FSH (hrFSH) on early goat antral follicles, hrFSH improved antral follicle development in a concentration-dependent manner[81]. Lopes et al.[82] also demonstrated that early antral follicles isolated from goat ovarian stroma are able to grow and survive *in vitro* for a short period of time, after going through a vitrification process. Cordeiro et al.[83] have recently reported that the presence of N-acetyl-cysteine (NAC) in the medium culture of early antral follicles reduces the levels of reactive oxygen species (ROS) and maintains the integrity of oocytes during culture in cattle.

In vitro culture of COC and isolated follicles ensures bidirectional communication between oocytes and granulosa cells through transzonal projections (TZPs), which is crucial for the occurrence of molecular events necessary for follicle and oocyte development until ovulation. These events involve, in addition to chromosomal separation, characterizing nuclear maturation. Moreover, they involve the distribution of cytoplasmic organelles, the stock of mRNA, proteins and other factors that are essential for the oocyte to be able to resume meiosis and support fertilization and embryonic development[84].

Some studies have already reported the birth of live calves from oocytes obtained from early antral follicles, but the viability and developmental competence of these oocytes *in vitro* can be improved[77, 85-86]. Therefore, our research group has focused on the development of culture protocols that favor the acquisition of oocyte competence *in vitro*. Bezerra et al.[87] showed that follicular hemisections combined with cilostamide have a synergistic effect on the maintenance of oocytes in the germinal vesicle during *in vitro* culture. Barrozo et al.[88] showed that the presence of this NAC in the culture medium increased the percentage of meiotic resumption and the distribution of TZPs, as well as reduced ROS levels, indicating that

the inclusion of antioxidants is important to optimize the IVM systems. He et al.[89] suggested that the two-dimensional (2D) culture system is more suitable for the culture of oocytes from early antral follicles lasting up to four days, while in culture periods longer than four days, the three-dimensional (3D) system is more adequate.

Culture of isolated early antral follicles may also be a promising alternative for providing competent oocytes for use in *in vitro* maturation protocols[90], as this communication between oocytes and granulosa cells is maintained. However, the choice of follicle size is fundamental to the acquisition of competence in oocyte development. It has already been shown that oocytes from small antral follicles (1 and 2 mm) have significantly reduced competence compared to oocytes from larger antral follicles (>3 mm)[3-4]. Bezerra et al.[90] showed that the levels of mRNAs for transcripts involved in the oocyte development process, such as histone with oocyte-specific ligand (H1FOO), GDF-9 and poly (a) specific ribonuclease (PARN), increase in oocytes when follicles grow from secondary to small, medium and large antral follicles.

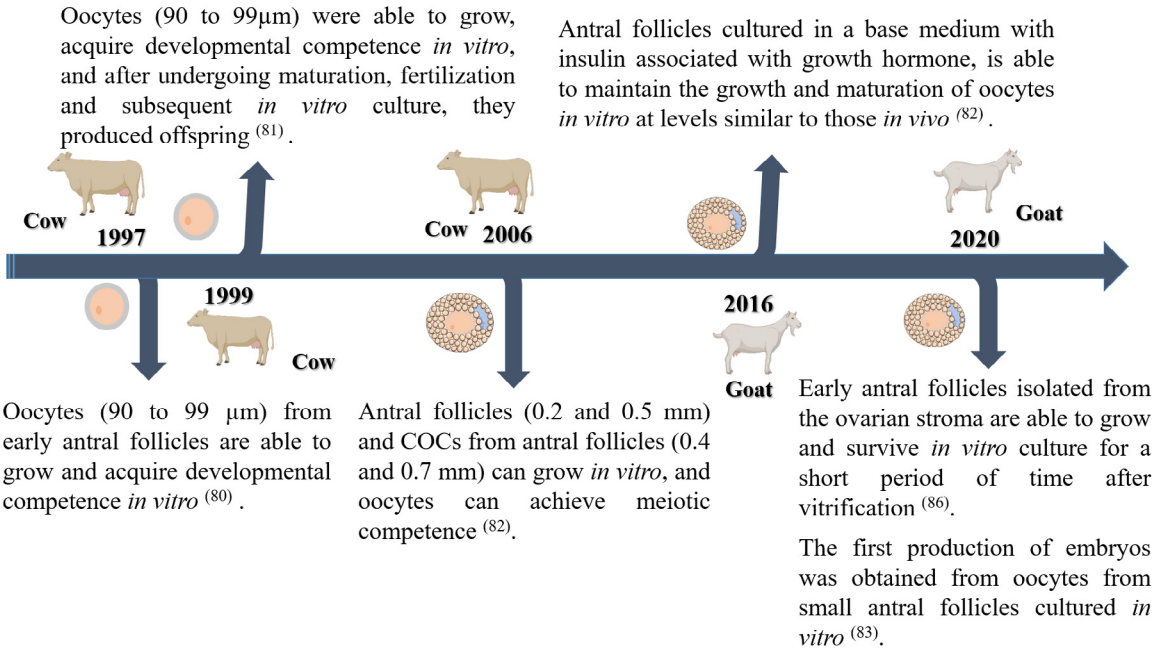


Figure 5. Schematic representation of the major advances with *in vitro* culture of early antral follicles.

7. Conclusions

The development of early antral follicles up to gonadotropin dependence involves a wide range of processes, which can be decisive for follicular growth, steroidogenesis and acquisition of oocyte competence. The interplay between oocyte and follicle cells directly influences follicle and oocyte fate. In addition, the *in vitro* culture of early antral follicles opens new prospects for the use of their oocytes for *in vitro* fertilization and for a better understanding of the mechanisms involved in the control of early antral follicles.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Conceptualization: J.R.V. Silva. *Funding acquisition*: J.R.V. Silva. *Project administration*: J J.R.V. Silva. *Supervision*: J.R.V. Silva. *Writing (original draft)*: E. C. Barbalho, D. R. Nascimento, L.G. Barrozo, L.R.F.M. Paulino and E. I. T. de Assis. *Writing (proofreading & editing)*: J.R.V. Silva.

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