

Morphological study of apoptosis in granulosa cells and ovulation in a model of atresia in rat preovulatory follicles

Short Communication

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Cite this article: da Silva Bitecourt F *et al.* (2018) Morphological study of apoptosis in granulosa cells and ovulation in a model of atresia in rat preovulatory follicles. *Zygote* 26: 336–341. doi: 10.1017/S0967199418000291

Received: 18 March 2018

Revised: 23 June 2018

Accepted: 11 July 2018

First published online: 2 October 2018

Keywords:

Atresia; Gonadotropin; Granulosa cell apoptosis; Ovulation; Preovulatory follicle

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Summary

Previous studies have established a model of atresia in preovulatory follicles after stimulation of immature rats with equine chorionic gonadotropin (eCG). This gonadotropin recruits a follicular pool and the deprivation of preovulatory luteinizing hormone (LH) surge induces the atresia in preovulatory follicles. The present study investigated the occurrence of ovulation and provided some morphological features of granulosa cell (GC) apoptosis of atretic follicles at 0, 48, 72 and 120 h after eCG stimulation. Histological sections of ovaries from untreated animals (0 h) showed primordial, primary, secondary and early antral follicles. After 48 h ovaries showed large antral follicles. Preovulatory follicles were observed at 72 h, and two out of five rats displayed cumulus–oocyte complexes (COCs) in the oviducts. All animals exhibited corpora lutea after 120 h. We observed increased estradiol (E₂) levels 48 h after eCG treatment that might trigger an endogenous preovulatory gonadotropin surge. Higher progesterone (P₄) level, which is the hallmark of a functional corpus luteum, was observed at 120 h. Atresia in secondary and antral follicles was observed by pyknotic granulosa cell nuclei in histology and positive immunolabelling for cleaved caspase 3. We also observed macrophages in secondary and antral follicles in atresia. Transmission electron microscopy revealed GCs with compacted chromatin against the nuclear envelope, nuclear fragmentation, cell shrinkage and fragmentation. No preovulatory follicles showed apoptosis of GCs. In conclusion, our results suggested the occurrence of an endogenous gonadotropin surge, promoting ovulation and preventing atresia of preovulatory follicles.

Introduction

Mammalian ovaries consist of follicles with oocyte surrounded by granulosa cells (GCs) and thecal cells (TCs). These follicles develop through primordial, primary, and secondary stages before acquiring an antral cavity (antral follicle) (Pangas *et al.*, 2015). Most follicles are lost by atresia and only a few follicles reach the preovulatory stage (Graafian follicle) and ovulate (Krysko *et al.*, 2008). Ovarian follicles undergo atresia mainly through GC death by apoptosis (Matsuda-Minehata *et al.*, 2006). Pituitary gonadotropins (follicle-stimulating hormone – FSH, and luteinizing hormone – LH) promote follicular development and prevention of atresia. FSH regulates gene expression and post-translational modification of proteins that inhibit apoptosis, stimulate proliferation, trigger steroidogenesis and promote cell differentiation, allowing GCs to respond appropriately to the preovulatory gonadotropin surge. Next, LH prevents atresia of preovulatory follicles and promotes ovulation (Erickson and Danforth, 1995).

In addition to the gonadotropins, many growth factors and intraovarian regulators such as Bcl-2 (Wang *et al.*, 2012), insulin-like growth factor (Hu *et al.*, 2004) and members of the TGF- β superfamily (inhibins and activins) (Hillier, 2009) also act on follicular development and the survival of ovarian follicles. Apoptosis of GCs occurs through extrinsic and intrinsic signalling pathways (Hussein, 2005; Matsuda *et al.*, 2012). Ligand binding to cell-surface receptors mediates the extrinsic pathway, which activates caspase-8, while the intrinsic pathway is triggered by the mitochondrial release of cytochrome *c* initiated by caspase-9. These initiator caspases activate executioner caspases, such as caspase 3, that degrade cellular components producing morphological changes observed in apoptosis (Johnson and Bridgham, 2002).

Previous studies have established a model to investigate the mechanisms of atresia in preovulatory follicles after stimulation of immature rats with equine chorionic gonadotropin (eCG) (Braw and Tsafiriri, 1980; Choi *et al.*, 2010; Dhanasekaran and Moudgal, 1989; Hughes and Gorospe, 1991). This model is obtained through recruitment of a follicular pool by a single injection of eCG, which displays FSH-like effects. After 48 h, the ovaries present many preovulatory follicles. Considering LH inhibits apoptosis in preovulatory follicles and promotes ovulation in cycling rats, the absence of a preovulatory LH surge in immature rats will induce atresia of preovulatory follicles. Therefore, the aim of the present study was to investigate the occurrence of ovulation and provide some morphological features of GC apoptosis of atretic follicles from this model of atresia in preovulatory follicles.

Materials and methods

Immature female Wistar rats (25 days old, five animals per group) were obtained from CEBIO/Federal University of Minas Gerais (Belo Horizonte, Brazil). The animals were maintained in a room with controlled temperature ($22 \pm 2^\circ\text{C}$) and a 12 h light: 12 h dark cycle, with free access to tap water and standard rat chow (Nuvilab, Brazil). The animals received a single subcutaneous injection of 20 IU eCG (NHPP, Torrance, CA, USA) to induce follicular development and atresia (Braw and Tsafiriri, 1980; Choi *et al.*, 2010; Dhanasekaran and Moudgal, 1989; Hughes and Gorospe, 1991). Animals were anaesthetised with ketamine–xylazine and euthanized at 0, 48, 72 or 120 h after gonadotropin stimulation. The ovaries were immediately removed and weighed. The right ovaries were frozen in liquid nitrogen for the dosage of ovarian steroids. Fragments of the left ovaries were fixed in phosphate-buffered formaldehyde for histology/immunohistochemistry. Next, they were serially dehydrated in graded ethanol baths, embedded in paraffin (Histotec Pastilles—Merck, Darmstadt, Germany), sectioned at $5\ \mu\text{m}$ and stained with haematoxylin and eosin (H&E). Images were acquired using an Olympus B53 microscope.

Immunodetection of cleaved caspase-3, an executioner caspase that promotes morphological changes observed in apoptosis, was performed by the avidin–biotin–peroxidase method using the Vectastain Universal Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA), as described previously (Gonzaga *et al.*, 2017), using anti-cleaved caspase-3 antibody (Cell Signaling Technology–Massachusetts) diluted 1:1000 dilution as primary antibody. Ovarian sections with $5\text{-}\mu\text{m}$ thick were dewaxed in xylene, hydrated through a series of descending concentrations of alcohol to water and endogenous peroxidase activity was blocked in 0.6% hydrogen peroxide in methanol for 30 min. After microwaving for antigen retrieval in 0.1 M citrate buffer pH 6.0, non-specific antibody binding was blocked with normal goat serum (10%). Sections with primary antibodies were incubated overnight at 4°C . Sections without primary antibodies were used as negative controls. Next, sections were incubated with biotinylated goat anti-rabbit secondary antibody (Dako–Carpinteria), diluted 1:100 for 30 min and with the avidin–biotin complex (1:100 dilution) for 30 min. The immunoreaction was visualized using 0.05% (p/v) diaminobenzidine containing 0.01% (v/v) hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.6. Immunostaining was performed in duplicate to confirm the results.

For transmission electron microscopy (TEM), ovarian fragments were fixed in Karnovsky's solution (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 M cacodylate buffer pH 7.4 overnight at 4°C . Fragments were postfixed in 2% (w/v) osmium tetroxide and 1.5% (w/v) potassium ferrocyanide for 2 h at room temperature. They were washed in distilled water and kept in 2% uranyl acetate (en bloc staining) overnight. Then, they were dehydrated and embedded in Epon 812 (EMS, Hatfield, PA, USA). Specimens were sectioned in 50 nm ultrathin sections, stained with Reynolds lead citrate and analyzed in a Tecnai G2-12 Spirit electron microscope (FEI Company, Hillsboro, Oregon, USA) at 80 kV.

Ovarian steroids extraction were performed as previously described (Pereira *et al.*, 2014), and testosterone (T), 17β -estradiol (E_2) and progesterone (P_4) levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercial kits (DRG Diagnostics, Marburg, Germany). The assays were performed according to the manufacturers' instructions and all samples were measured in duplicate.

Data were expressed as the mean \pm standard error of the mean (SEM), and differences were considered significant if *P*-values were < 0.05 . Data exhibited normal distribution by Kolmogorov–Smirnov test and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.

Results and Discussion

In the present study, we observed ovulation after the induction of follicular development in an well established model of atresia in immature rats treated with eCG (Braw and Tsafiriri, 1980; Choi *et al.*, 2010; Dhanasekaran and Moudgal, 1989; Hughes and Gorospe, 1991). Untreated animals (0 h) showed primordial follicles (Pr) surrounded by a single layer of flat follicular cells, primary follicles with a monolayer of cubic follicular cells and secondary follicles, which possessed more than one layer of follicular cells without visible follicle fluid (antrum) (Fig. 1A, B). Ovaries of untreated animals also exhibited antral follicles with oocytes surrounded by differentiated GCs and TCs, and an accumulation of liquid forming the antral cavity (Fig. 1C). After 48 h of stimulation with eCG, we observed large antral follicles (Fig. 1D). Preovulatory follicles were observed 72 h after eCG stimulation (Fig. 1E), and two out of five rats displayed cumulus–oocyte complexes (COCs) in the oviducts (Fig. 1F). All animals exhibited corpora lutea after 120 h (Fig. 1G). According to the literature, this treatment induces atresia in preovulatory follicles due to gonadotropin surge deprivation (Choi *et al.*, 2010; Hughes and Gorospe, 1991). So, the presence of corpora lutea (on days 4 and 5) after eCG treatment was rare (5%) in experimental animals (Kim *et al.*, 1999). In the present study, all animals had been ovulated 120 h after eCG stimulation, suggesting an endogenous gonadotropin surge had happened. The induction of atresia after eCG injection resembles the widely used protocols of ovulation based on exogenous gonadotropins stimulation (Luo and Zhu, 2000). When prepubertal rats are injected with eCG, a follicular pool recruitment will take place. After 48–50 h, if the rats are injected with human chorionic gonadotropin (hCG), which simulates endogenous LH surge, the animals will ovulate (Luo and Zhu, 2000).

We also measured T, E_2 , and P_4 from ovaries to evaluate the ovarian steroidogenic profile. T and E_2 reached to higher levels

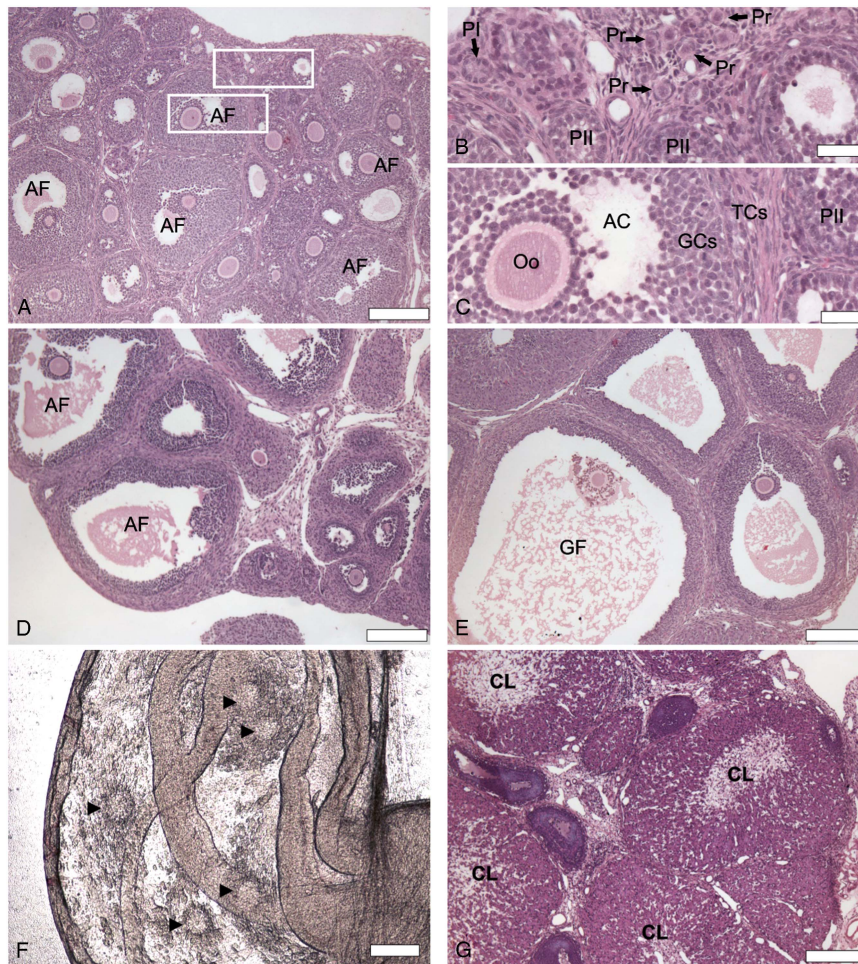


Figure 1. Histological sections (H&E) of rat ovaries showing follicular development 0 h (A–C), 48 h (D), 72 h (E) and 120 h (G) after stimulation with equine chorionic gonadotropin (eCG). (B) High magnification of selected area from (A) showing primordial follicles (Pr) surrounded by a single layer of flat follicular cells, primary follicle (PI) with a single layer of cubic follicular cells and secondary follicles (PII) with a multiple layers of follicular cells. (C) High magnification of a second selected area from (A) showing antral follicle (AF) with differentiated granulosa cells (GCs) and theca cells (TCs) surrounding oocyte (Oo) and beginning of antral cavity (AC) formation. (D) Antral follicle (AF). (E) GF: Graafian/preovulatory follicle (GF). (F) Cumulus–oocyte complex (arrowhead) in the oviducts 72 h after eCG stimulation, indicating that ovulation happened. (G) Corpus luteum (CL). Scale bars represent 200 μm (A, B, E–G) and 40 μm (C, D).

after 48 h of eCG treatment (Fig. 2A, B) and P_4 levels increased 7.8-fold at 120 h compared with 0 h (Fig. 2C). The increased E_2 levels 48 h after eCG treatment might contribute to promoting a positive feedback in the hypothalamus–pituitary axis, culminating in endogenous gonadotropin-releasing hormone (GnRH) and LH surges that triggered ovulation (Levine, 2015). The high level of E_2 exhibits a preovulatory positive feedback in the hypothalamus, as well documented in the literature (Richards *et al.*, 2015). This positive effect of E_2 on LH surge was first reported in 1971 with adult ovariectomized rats treated with exogenous E_2 (Caligaris *et al.*, 1971).

We also observed higher P_4 levels 120 h after eCG stimulation, indicating the presence of functional corpora lutea. If ovulation occurs, the remaining GCs and TCs will become a corpus luteum that is associated with cellular hypertrophy and increased P_4 synthesis and release. LH stimulates expression of P-450 cholesterol side chain cleavage (SCC), which converts cholesterol to pregnenolone, and 3β -hydroxysteroid dehydrogenase (3β -HSD), that converts pregnenolone to P_4 (Hunzicker-Dunn and Mayo, 2015).

In spite of the ovulation confirmed by COCs in the oviducts, hormonal dosage and corpus luteum in ovarian sections, we

observed atresia in early follicles. We considered atresia in any follicle showing pyknotic granulosa cell nuclei (Braw and Tsafri, 1980). Pyknotic bodies were randomly distributed throughout atretic follicles in the granulosa layer of follicles (Fig. 3A). We observed atretic follicles with fragmented oocytes showing loss of contacts between the oocytes and GCs (Fig. 3B). Macrophages were commonly found in atretic follicles. They were recognized by acidophilic cytoplasm and vesicular nucleus in the antral cavity or in the granulosa layer (Fig. 3A, B). Previous studies have shown the presence of macrophages in any phase of follicular development (Katabuchi *et al.*, 1996; Wu *et al.*, 2004). In atretic follicles, GCs in apoptosis might stimulate a recruitment of macrophages as reported in atretic follicles induced by nanosilver particles (Mirzaei *et al.*, 2017).

To confirm apoptosis in GCs, we used immunohistochemistry for cleaved caspase 3 and TEM, which is a standard method of detecting apoptosis of the cell. The GCs from atretic follicles showed positive staining for cleaved caspase-3, an effective marker of apoptosis (Fig. 3C, D), which is activated in both apoptotic extrinsic and intrinsic pathways (Johnson and Bridgham, 2002; Hussein, 2005), corroborating the hypothesis that apoptosis of GCs plays a pivotal role in atresia.

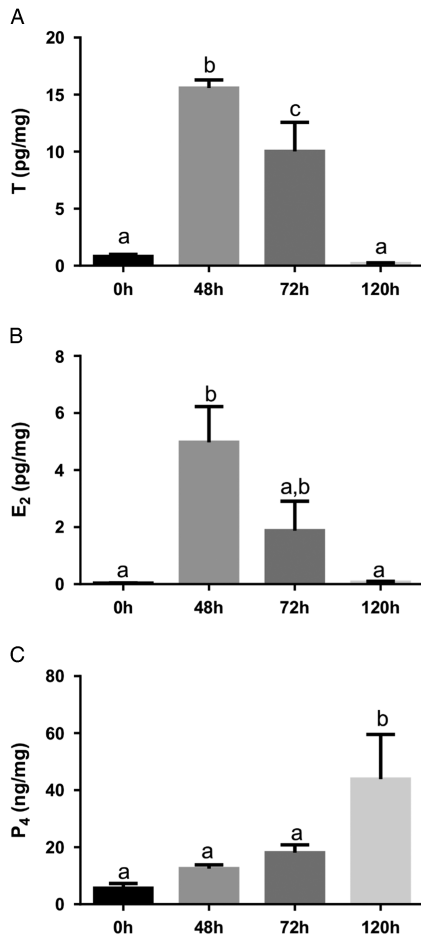


Figure 2. Ovarian testosterone (T; A), 17 β -estradiol (E₂; B) and progesterone (P₄; C) 0 h, 48 h, 72 h and 120 h after eCG stimulation. Values represent means \pm standard error of the mean (SEM) ($n=5$ /group). Common lowercase letters indicate that means do not differ significantly ($P < 0.05$).

Many studies used terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) to investigate the follicular atresia in immature rats treated with eCG (Asselin *et al.*, 2001; Boone *et al.*, 1997; Kim *et al.*, 1999; Zwain and Amato, 2000). This assay is widely used to identify and quantify apoptotic cells through detection of DNA fragmentation (Gavrieli *et al.*, 1992). However, TUNEL labels any cell with DNA cleavage, such as in autolysis and necrosis (Grasl-Kraupp *et al.*, 1995). Therefore, identification of apoptosis stills requires the demonstration of morphological and ultrastructural features. Ultrastructure of follicles revealed GCs with developed rough endoplasmic reticulum, Golgi apparatus, mitochondria and contacts such as tight junctions coupled adjacent cells (Fig. 4A, B). Atretic follicles exhibited GCs with a compacted chromatin aggregated against the nuclear envelope, nuclear fragmentation, cell shrinkage and fragmentation similar to apoptotic bodies (Fig. 4C, D). These cells with apoptotic characteristics were observed in secondary and antral stages any time after eCG treatment. No preovulatory follicle showed signs of chorionic gonadotropin (CG) apoptosis. Apoptotic GCs in some small antral follicles and no apoptotic cells in preovulatory follicles 48 h and 72 h after eCG stimulation were also reported previously, suggesting GCs from small follicles are liable to apoptosis and sensitive to atretogenic factors (Luo and Zhu, 2000). This gonadotropin inhibits the expression of target genes that induce apoptosis (Wang *et al.*, 2012).

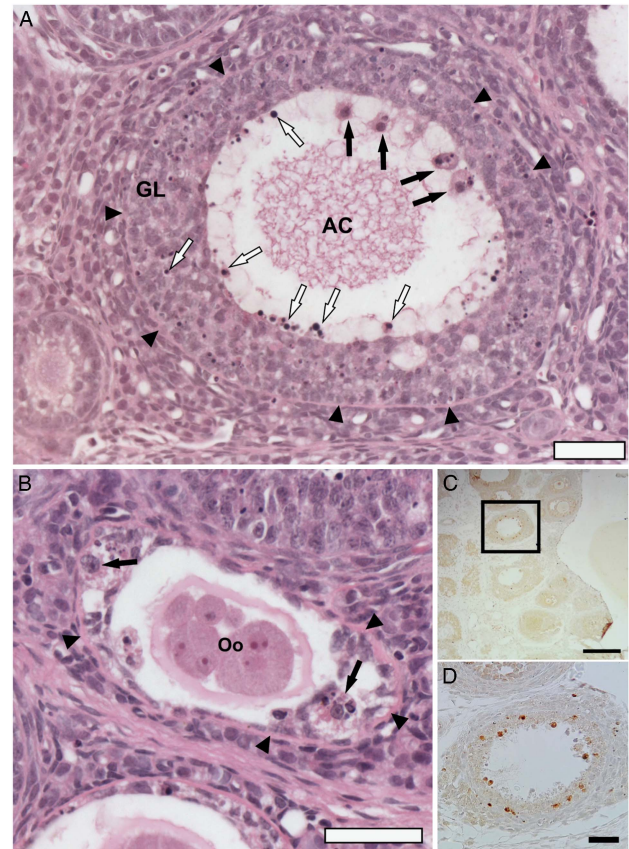


Figure 3. Apoptosis of granulosa cells (GCs) of atretic follicles from rat ovaries after eCG stimulation observed in ovarian sections stained by H&E (A, B) and immune labelling for cleaved caspase-3 by avidin-biotin-peroxidase method (C, D). (A) Antral follicle with pyknotic nuclei of GCs (white arrows) in antral cavity (AC) and in granulosa layer (GL), and the presence of macrophages (black arrows). (B) Secondary follicle showing fragmentation of the oocyte (Oo) and macrophages (black arrows). (C, D) Immunolocalization of cleaved caspase-3 in granulosa cells (brown staining). Arrowheads: basement membrane between theca and granulosa cells. Scale bars represent 200 μ m (C) and 40 μ m (A, B, D).

In the present study, the animals were immature and the preovulatory LH surge would not happen. The animals were 25 days old and puberty in rats occurs when they are around 42 days old (Sengupta, 2014). Without a preovulatory LH surge, the follicles recruited by eCG would undergo atresia. Luo and Zhu (2000) documented apoptotic cells from preovulatory follicles 120 h after eCG stimulation. In the present study, we did not observe any preovulatory follicle 120 h after eCG treatment and the ovaries exhibited corpora lutea at this moment, indicating that the preovulatory follicles had ovulated. The absence of atretic preovulatory follicles might also be related to the high levels of ovarian E₂ prior to gonadotropin surge. It is well known that oestrogen is associated with good follicular growth and exhibits anti-atresia effects (Revelli *et al.*, 2009). E₂ acts by binding to two nuclear receptors (ER α and ER β) and a recently discovered G protein-coupled oestrogen receptor 1 (GPER), all expressed in GCs (Pavlik *et al.*, 2011). These receptors stimulate the phosphoinositide 3-kinase/protein kinase B (IP3K-Akt) pathway that is linked to cellular survive (Heublein *et al.*, 2014). PI3K-Akt, which inactivates the protein Bad, a member of the family pro-apoptotic Bcl-2 (Quirk *et al.*, 2004), and caspase-9 (Johnson and Bridgham, 2002), prevents apoptosis (Franke *et al.*, 1997; Quirk *et al.*, 2004).

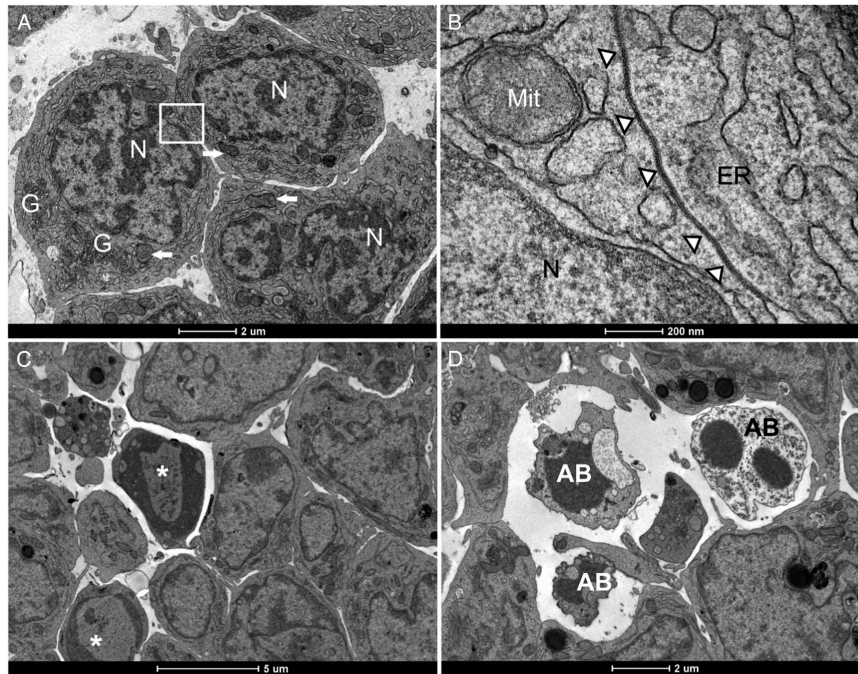


Figure 4. Transmission electron microscopy. (A) Healthy granulosa cells. (B) High magnification of selected area from (A) showing tight junctions (arrowheads). (C) Nucleus with compacted chromatin underling the nuclear envelope (asterisk), indicating apoptosis. (D) Apoptotic bodies (AB) of granulosa cells in apoptosis. ER, endoplasmic reticulum; G, Golgi apparatus; Mit, mitochondria; N, nucleus.

In summary, the present study reported the spontaneous ovulation in a well established rat model of atresia in immature eCG-treated rats and GCs apoptosis in early follicles. We also recommend that researchers should take on board the possibility of an endogenous gonadotropin surge in this model that culminates in ovulation that might prevent studies of atresia in pre-ovulatory follicles.

Acknowledgements. We are grateful to the Center of Microscopy at the Universidade Federal de Minas Gerais (<http://www.microscopia.ufmg.br>) for providing the equipment and technical support for experiments involving electron microscopy and Dr Cleida A. Oliveira for generous donation of the anti-cleaved caspase-3 antibody.

Financial support. This study was supported by Fundação de Amparo a Pesquisa do Estado de Minas Gerais FAPEMIG/Brazil (protocol APQ-00743–14) and Pró-Reitoria de Pós-Graduação e Pesquisa/UFVJM (grant to PIBIC scholarship to F. S. B.).

Conflict of interest. The authors declare no conflicts of interest.

Ethical standards. The experimental procedures were approved by the institutional Ethics Committee on Animal Use (Process no. 40/2016 CEUA/UFVJM).

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