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Atresia and Apoptosis in Preovulatory Follicles in the Ovary of *Danio rerio* (Zebrafish)

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Abstract

Although apoptosis, the programmed cell death, is the main mechanism in follicular atresia and especially postovulatory regression in mammals, its role in fish ovary is still controversial. In order to discuss the possible functions of atresia as a context of apoptosis, the preovulatory follicles of the freshwater teleost, *Danio rerio* (zebrafish) were investigated by light microscopy. The main characteristics of atresia of vitellogenic oocytes of zebrafish were determined as a significant hypertrophy and hyperplasia of the granulosa cells. The theca cells also became slightly hyperplasic while the zona radiata was degenerated and the basal membrane located between granulosa and theca was also disintegrated. The aggregation of compacted chromatin in a crescent pattern, and cell fragmentation that indicates to formation of apoptotic bodies were evaluated as the morphological evidences for apoptosis in granulosa cells of the oocytes of zebrafish. Towards the end of atresia, the vitellus is fully degenerated and absorbed, some apoptotic bodies were also observed between the translucent clusters of theca cells. Apoptotic process was still continued when an atrium had been formed.

Keywords: Atretic follicle, teleost, *Danio rerio,* apoptosis.

Introduction

Atresia is a term used to describe the breakdown and resorbtion of gametes (Kennedy, 2002) and follicular atresia is a degenerative process by which vertebrate ovarian follicles lose their integrity and are eliminated prior to ovulation (Santos *et al*., 2008). Although it is defined as a normal and physiological processes underlieing homeostasis in vertebrate ovaries (Hsueh *et al*., 1994; Imsland *et al*., 1997; Wood and van Der Kraak, 2001; Hussein, 2005), it has been also reported that the mechanisms of atresia might be triggered by variable factors such as hypophysectomy, photoperiodism and salinity changes, antigonadotropic agents, stress and pollution (Zelennikov, 1997; Bromley *et al*., 2000; Wood and van Der Kraak, 2002).

Ovarian atresia in non-mammalian vertebrates has received less attention than it has in mammals; however, biochemical and morphological characteristics and the functions of atresia in the teleost ovaries are widely discussed in last years (Wood and van Der Kraak, 2001, 2003; Kennedy, 2002; Carnevali *et al*., 2006; Santos *et al*., 2008).

Atresia was recently been emphasized together with the programmed cell death, apoptosis, which is recognized as being a golden key to maintain the tissue-level homeostasis in multicellular organisms (Hacker, 2000; Kennedy, 2002). It was also reported that, like atresia, apoptosis can also be induced by factors such as cytokines, hormones, viruses, xenobiotics, radiation, oxidative stress and hpoxia (Webb *et al*., 1997).

Since apoptosis in postovulatory follicles of the teleost, *Astyanax bimaculatus lacustris* was firstly reported by Drummond *et al.* (2000), many teleostean species were used for investigation of the characteristics of programmed cell death (Witthames *et al*., 2003; Santos *et al*., 2005; Thomé *et al*., 2006). By the way, the enzymatic activities and in apoptosis were especially discussed in recent years in some teleosts such as *Morone saxatilis* (Kennedy, 2002), *Danio rerio* (Carnevali *et al*., 2006), *Prochilodus argenteus* and *Leporinus taeniatus* (Santos *et al*., 2008).

As being a well-known tropical freshwater species, *Danio rerio* is a very popular aquarium fish and the mostly used vertebrate model organism in biological researches. The ovary of *Danio rerio* is asynchronous; oocytes develop in four main stages called primary oocyte growth, cortical alveolus stage, vitellogenesis and maturation, respectively (Çakıcı and İşisağ Üçüncü, 2007).

The present study was conducted to identify the main histological properties of the processes of atresia in prespawning vitellogenic oocytes in zebrafish. Surely, a variety of biochemical markers have been widely used as indicators of the onset and progress of atresia, but this is not to diminish the value of the basic histological evidence.

Materials and Methods

Fish Maintenance

Zebrafish (20 females, total weight 0.21±0.06 g and total lenght, 3.28±0.50 cm) were purchased from commercial dealers and acclimated to filtered and well aerated tap water in 20 L aquarium for 30 days. The water temperature was maintained at 27±3ºC, pH was measured at 7.5 and the photoperiod was set at 14L/10D. The water quality parameters were noted as 30±1 for hardness (French degree), 18±1 mg/L for sodium, 3.0±0.1 mg/L for potassium, 0.6±0.1 for free chlorine, 15±1 for chloride, 0 for nitrite and 5±1.5 mg/L for nitrate. Water chemical analyses were conducted biweekly.

Fish Feedings

The fishes were fed once daily by adding enough commercial fish food (Sera-San) to aquaria so that food would be available to all fish and nearly all food is eaten within 10 minutes. Every other day, one course of *Tubifex* sp. and *Daphnia* sp. (about 4 g) were also added. The filter was controlled daily and changed as needed.

Histological Analyzes

After the acclimation period, fishes were sacrificed with MS222 and the ovaries were removed and fixed in the Bouin's fixative for 48 hours. After dehydration accomplished in a graded ethanol series (50%, 75%, 95% and 95%) for 15 min. at each grade followed by two 30-min. washes in 100% ethanol., tissue samples were infiltrated and embedded in paraffin. Serial sections at 6-7 µm were stained with routine methods of Hematoxylin-Eosin (HE) and Paraldehyde Fuchsine (PAF) and examined by light microscope. In order to capture images, Jena NF binocular microscope and MF photomicrography attachment were used.

Results

Based on the morphological properties, four main steps of oocyte development (primary, cortical-alveolar, vitellogenic and mature) were identified and the vitellogenic oocytes were more often observed to undergo the process of atresia. These kinds of oocytes were easily characterized with their enlarged size. When stained with PAF, these oocytes were filled by yellowish yolk vesicles. The oocyte membranes; zona radiata, granulosa and theca were also distinguished clearly with both of the HE and PAF staining methods (Figure 1). The oocyte is bounded inmostly by a thick zona radiata, which is typically striated in appearance. The follicle layers overlying the zona radiata are consisted of a monolayer of basophilic granulosa cells and the multi-cellular theca. The layers of granulosa and theca were separated by a distinct basement membrane.

While the onset of atresia was determined by striking hypertrophy and hyperplasia in granulosa cells (Figure 2), a slight expansion for theca was also noted. Initally, some morphological differences were observed in granulosa cells: some of them were small and cuboidal in shape, while the enlarged ones were irregularly shaped phagocytes that breached zona radiata. Thus, the integrity of zona radiata was breached locally. As an evidence for disruption; the characteristic, transversely striated structure of the zona radiata had disappeared and the layer was expressed in heterogenous, disarranged and dissolved in appearance. The basement membrane located between granulosa and theca was also disrupted and became locally invisivible.

By the time, the follicles, which are observed to be non-spherical in shape, had progressed to atresia and the components of zona radiata had largely disintegrated. The apoptotic bodies were observed among the highly hypertrophic granulosa cells as a determining feature (Figure 3). Moreover, granulosa layer was completely disordered due to vacuolar formation and observed separately because of the edema. Remarkably enlarged granulosa cells were strikingly showed the characteristics of phagocytic cells that engulfed vitellus. Some of their nuclei were picnotic: a crescent-shaped chromatin material was distinguished as being of a characteristic feature of apoptosis (Figure 4). The vesicular and globular appearance of the vitellus was brokendown and the basement membrane was completely invisible.

Towards the end of atresia, due to the invasion and consumption of granulosa cells the vitellus was seen as a partly fractured mass (Figure 5). Engulfing cells completely filled the space which was formerly occupied by ooplasm containing yolk components, thereby the vitellus was completely absorbed and left a large cavity behind called atrium (Figure 6). Presence of apoptotic bodies in newly forming atrium (Figure 5) and among scattered clusters of granulosa cells (Figure 6) indicates that the apoptotic processes were still continued. The theca layer was also thickened considerably (Figure 6), apparently due to hyperplasia. Theca cells are observed in irregularly shaped clusters with large vacuoles and apoptotic bodies.

Discussion

As noted by Kennedy (2002), atresia has received only basic characterization in a variety of fish species; since its four basic stages (α, β, γ and δ) that comprised this morphological characterization in nonmammalian vertebrates were summarized by Saidapur (1978). Therefore, when compared with mammals, the information about the histological and histochemical properties of atresia in non-mammalian vertebrates is still limited. The reports generally focused on conditions known to induce massive ovarian atresia in farmed fishes, including handling, nutritional and temperature or other environmental stresses (Tyler *et al*., 1996). However, as noted by Janz and van der Kraak (1997), atresia of a limited

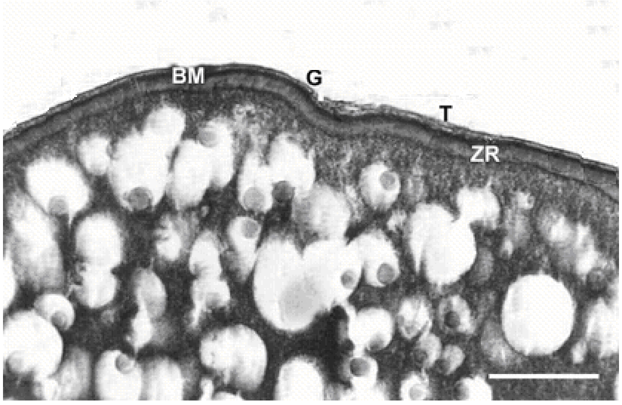

Figure 1. Follicle layers in a vitellogenic oocyte. Zona radiata (ZR), granulosa (G), and theca (T), note also the basal membrane (BM) between granulosa and theca. PAF. Bar represents 30 µm.

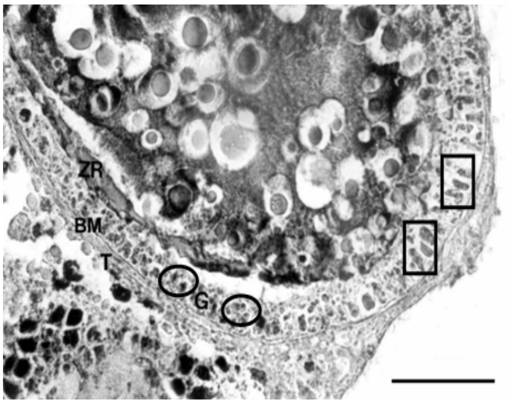

Figure 2. Distinctive hyperplasia and hypertrophy in granulosa cells (G), irregular appearance and breakdowns of zona radiata (ZR), slight enlargement in theca (T), and disintegration in basal membrane (BM). Note also the small, cuboidal (encircled), and large, irregulary shaped granulosa cell (rectangulated). PAF. Bar represents 50 µm.

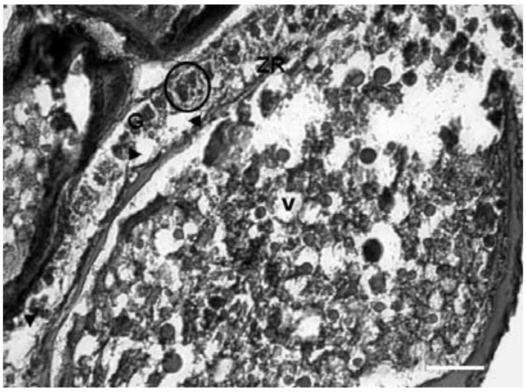

Figure 3. Apoptotic bodies (encircled) and large vacuoles (arrowheads) between hypertrophic granulosa cells (G), note the disintegrated zona radiata (ZR) and disruptured vitellus (V). PAF. Bar represents 30 µm.

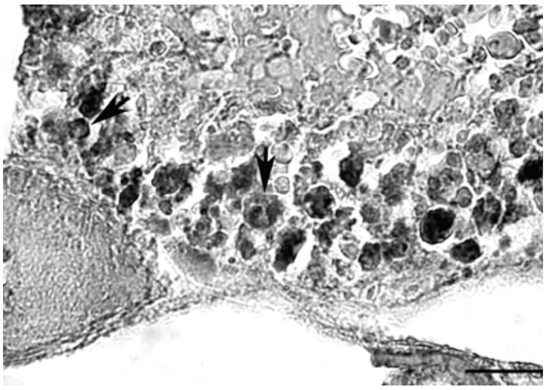

Figure 4. The picnotic nuclei (arrows) in apoptotic granulosa cells. PAF. Bar represents 20 µm.

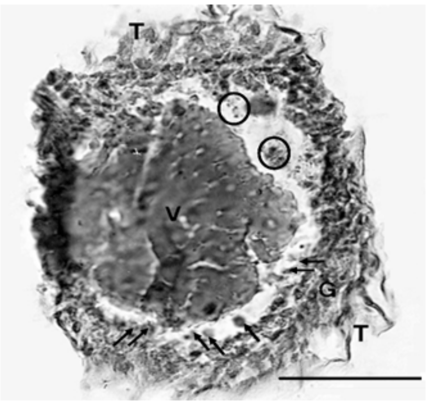

Figure 5. Hypertrophic theca cells (T), invading granulosa (G) cells (arrows), note the amorphous vitellus (V) during engulfed, and apoptotic bodies (encircled). HE. Bar represents 50 µm.

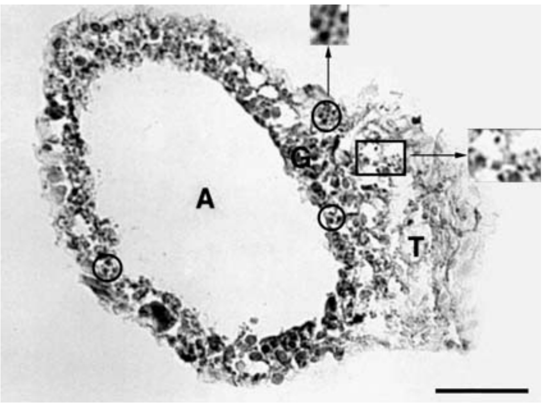

Figure 6. Apoptotic bodies encircled in granulosa (G) and rectangulated in theca (T) cells; A, atrium. HE. Bar represents 50 µm.

proportion of follicles early in the gametogenic cycle may help determine fish fecundity and massive ovarian atresia might be evaluated as an anomaly in nature. Miranda *et al.* (1999) reported that duration of atresia for fish kept under captivity conditions is shorter in one-time spawners than partial spawners. Thus, although atretic follicles are important indicators of environmental impact on fish ovaries, the mechanisms of follicular atresia are not yet fully understood (Santos *et al*., 2008).

The reproductive strategy of zebrafish is multiple spawning, like the most teleost species with asynchronous ovary. Although it is difficult to determine the rate of atresia in developmental stages in asynchronous ovaries, increased number of atresia were observed in vitellogenic oocytes in *Carassius auratus* (Wood and van Der Kraak, 2001); *Seriola dumerilii* (Kagawa, 1991) and *Brycon orthotaenia* (Gonçalves *et al*., 2006). The results of the present study were in consistence with these findings. However, atresia was also observed more often in vitellogenic oocytes in some single-clutch group-synchronous spawners such as rainbow trout, *Oncorhynchus mykiss* (Tyler *et al*., 1996), or synchronously hatched fishes like the striped-bass, *Morone saxatilis* (Mylonas *et al*., 1998; Kennedy, 2002).

As reported by Rizzo and Bazzoli (1995) and Miranda *et al.* (1999), the higher rate of absorbtion of vitellogenic oocytes led to a proportional decrease in reproductive potential, which should obviously be overviewed in terms of the functions of atresia. As it is known, both the number of eggs and the amount of vitellus are much higher in oviparous organisms, when compared to viviparous. Furthermore, the number of eggs to be spawned is significantly different among the oviparous species. For instance, in domestic hens, an average of one out of 20 follicles can grow into an egg (Johnson *et al*., 1996), while the remaining ones are atretic, whereas in a fish ovary, thousands of follicles are produced and under normal conditions, most of them grow to be spawned. For instance, *Oncorhynchus mykiss* ovary which makes up 25% of the total body weight in prespawning period contains 2,000-3,000 oocytes per kilogram. As is already known, in the high vitellus content of each oocyte in teleosts, high energy is stored, which is assumed to be essentially used for embryonic development. Thus, in *Oncorhynchus mykiss*, 80% of the total oocyte volume consists of vitellus (Tyler *et al*., 1996). From this point of view, teleost ovarian atresia has to be evaluated in a special manner.

In order to give an explanation to the reason why the higher incidence of atresia was occured in vitellogenic oocytes, Wood and van Der Kraak (2001) has brought a novel perspective by using biochemical methods and DNA techniques in their *in vivo* and *in vitro* investigations conducted with *Carassius auratus* and *Oncorhynchus mykiss* spawners. Their results were indicated that apoptosis is involved in normal ovarian growth and postovulatory regression in teleosts, but that it does not appear to be an early event in teleost follicular atresia. In the subsequent report (Wood and van Der Kraak, 2003) on *Oncorhynchus mykiss*, the authors noted that atresia in many oviparous vertebrates is an energy-conversion process involved proteolytic degradation of the energy-rich yolk storage proteins within the oocyte. Thus, more energy conversion would be maintananed with vitellogenic oocytes contained more vitellus. Such an explication is strongly seemed to be advisable.

Therefore, atresia in oviparous teleosts is really just a process involved energy conversion, it is easy to explain why the vitellogenic oocytes which contained higher amount of vitellus often became atretic when compared to developing ones. At this point, it is possible to make a simple conclusion about the sophisticated relationships between atresia and apoptosis: newly-developing oocytes are more resistant to apoptosis than vitellogenic oocytes due to the differences in their own program. This suggests that apoptotic mechanisms are related to the oocyte growth cycle. In “dormant” or primary oocytes, the incidence of atresia is lower, because their genetic programming towards tissue modelling is less active. The fact that the proteins stored in the final maturation stage are immediately hydrolized in order to achive free-swimming in many pelagic egg-laying teleost species (Wallace and Selman, 1985; Matsubara *et al*., 1999) confirms this opinion.

The initial stage of follicular atresia in teleosts and other oviparous vertebrates is characterized by a striking hypertrophy and hyperplasia observed in the granulosa cells, in parallel with disintegration of zona radiata. These changes are followed by the invasion of the hypertrophied granulosa cells towards vitellus and absorbtion of vitellus through phagocytotic activities. Once the absorption is over, follicular cells degenerate (Saidapur, 1978). Our findings are in parallel with this information; however, an indistinct hypertrophy in theca cells was also identifed at the beginning.

The morphological differences identified in granulosa cells were noted by Kasuya (1997) as being two kinds: one was intact, ingesting neighboring dead granulosa cells and the other was large and round cell identified as macrophages. Kennedy (2002) was noted a profound metamorphosis of the granulosa from endocrine cells to phagocytes and concluded that it would be accompanied by a loss of steroidogenic capacity. Histologically, the granulosa cells of *Danio rerio* are also conluded as two types, in present study. The remarkable metamorphosis of the granulosa cells was also known as an argument for their high lysosomic activity by secreting proteolytic enzymes (Kagawa, 1991). Furthermore, it is suggested that immune system cells also participated in to the activation of granulosa cells in order to absorbe the vitellus and to achive a synergistic cooperation (Besseau and Faliex, 1994). The formation of large vacuoles indicated to edema was demostrated in granulosa layer of *Danio rerio and* it is evaluated as an evidence for inflammation.

As the follicular atresia is being to taken into consideration with apoptosis, programmed cell death in the ovaries of birds and mammals has been discussed from different point of views by using advanced techniques in last decades (Johnson *et al*., 1996; Hall, 1999; Andreu-Vieyra and Habibi, 2000; Counis and Torruglia, 2000; Zwain and Amato, 2001; Markström *et al*., 2002; Hussein, 2005; Sundaresan *et al*., 2007)

As formerly noted by Kerr *et al.* (1972), programmed cell death is a genetically regulated and hormonally controlled active process essential for maintenance of tissue homeostasis in all multicellular organisms, where cells that are no longer needed to undergo self-destruction by activation of an intrinsic cell-suicide program.

Apoptosis has been known for years in the embryologic development process, as well as in the ovaries, branchiae, lymphoid tissues and cerebella of adult teleosts. However, Drummond *et al.* (2000) suggested that, in postovulatory follicles of *Astyanax bimaculatus lacustris*, apoptosis plays a critical role in resorbtion process by acting to eliminate the wall of follicular cells that remain after spawning. This kind of suggestion was widely investigated: Choi *et al.* (2000) reported that sex hormones might be involved in regulating atresia in striped bass, as they are in controlling atresia and apoptosis of follicles. Uchida *et al.* (2002) analyzed sexual differentiation-apoptosis relationship in juvenile *Danio rerio,* while Witthames *et al.* (2003) and Santos *et al.* (2005) revealed apoptosis in regressing follicles of post- and pre-spawning duration of *Solea solea*, *Gadus morhua and Leporinus taeniatus*, respectively. Thomé *et al.* (2006) also investigated apoptosis in the ovary of *Prochilodotus costatus* during postspawning.

MacKenzie *et al.* (2006) examined the in vivo and in vitro effects of lipopolysaccharide on the reproductive function of sexually mature female trout, *Salvelinus fontinalis*. Eimon *et al.* (2006) defined the extrinsic apoptosis pathway in zebrafish and showed that it resembles its mammalian counterpart. As being a subject of conservation program conducted in Rhone catchments area, both female and male *Zingel asper* was used in order to determine the occurrence of apoptosis in the sexual development by Chevalier *at al.* (2008). More recently, Santos *et al.* (2008) revealed the relationship among follicular apoptosis, integrin beta1 and collagen type IV during early ovarian regression in *Prochilodus argenteus* after induced spawning. Despite these kinds of approaches, the apoptotic mechanisms in teleost ovary are still not fully understood.

According to Santos *et al.* (2008), when the cell death program is "on", target cells are immediately affected and separated from neighbouring cells and the basement membrane. Cell integrity is maintained by the shattering of the dying cell into apoptotic cell bodies, which are then engulfed by healthy adjacent cells or phagocytes. An inflammatory reaction is avoided at last, surely.

As noted by the pioneer reports of Kerr *et al.* (1972), apoptosis of the follicular cells was detected histologically by the following features: chromatin condensation in a crescent pattern underlying the nuclear envelope, cell shrinkage, loss of cell–cell interactions detachment from basement membrane and cellular fragmentation in apoptotic bodies. The main characteristics of apoptosis are plasma membrane swelling, cell deformation, the crescent-shaped accumulation of chromosomal material just beneath the nuclear membrane (picnotic nucleus), formation of apoptotic bodies by dissolved cell particles (Matsuda-Minehata *et al*., 2006). Our results that contained the typical hypertrophy and hyperplasia in granulosa cells, the picnotic appearance of nucleus and the formation of apoptotic bodies, are generally in accordance with these argument. On the other hand, a slight hyperplasia and the presence of apoptotic bodies in theca cells are also demonstrated.

Based on their greater size and more amount, the granulosa cells were suggested as more active components for prespawning follicular structure formation (Khoo, 1975) and this argument had generally been accepted for more years until Wood and van Der Kraak (2003) have offered a new approach. The last authors started a discussion about the *sine qua non* roles of granulosa cells in the atresia and suggested that theca cells might play more active roles. From only histological point of view, we subscribe to this opinion. It is no doubt that the main cells responsible for vitellus absorbtion are the granulosa cells; however, the expansion in theca layer was observed at the initial phases of atresia and then gradually became evident. Therefore, it was seemed that at least the number of theca cells are increased. Moreover, theca cells have turned into completely amorphous translucent clusters with large cavities while the granulosa were observed with their distinct cell membrane. According to these findings, it is possible to say that the cell cycle in theca is much faster. By other means, the process of programmed death of theca cells may be working faster than that of absorbtion. According to Kennedy (2002), both theca and granulosa have ceased steroidogenic processes and were destined to one of three fates: apoptosis, necrosis, or phagocytosis. Surely, no absolute conclusions could be drawn without any available data concerning the time-dependent enzyme activity of granulosa and theca cells. More recently, Carnevali *et al.* (2006) noted that the lysosomal enzymes cathepsin B, D and L, which were played a key role in the oogenesis of oviparous vertebrates, have been found to be involved in viable egg production. The authors also reported that the activation of these enzymes is time-specific; furthermore, they work in a different manner than lysosomal enzymes in any other organ/tissue. According to the same investigators, atresia is under the control of cathepsins and affects the potential fecundity of the organism. The activation of these enzymes in the ovary appears very complex and they are regulated by many different factors including sexual hormones such as estradiol.

In conlusion, terminal-deoxynucleotidyl transpherase and Biotin-16-Dutp (TUNEL) enzyme and fluorescence methods are absolutely used to determine which protease group really accounts for the digestion of vitellus (Uchida *et al.*, 2002; Wood and van Der Kraak, 2001, 2003; MacKenzie *et al.*, 2006; Santos *et al.*, 2008). At this point, we once more emphasize that no enzymatic analysis was performed for the present study, our investigation is only based the histological findings. However, in order to distinguish the role of granulosa and theca cells in atresia and apoptosis exactly, it is histologically clear that, further studies would certainly be performed for determination of time-dependent enzymatic activities.

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