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Apoptosis in Granulosa cells during follicular atresia: relationship with steroids and insulin-like growth factors

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ABSTRACT

It is well known that during mammalian ovarian follicular development, the majority of follicles undergo atresia at various stages of their development. However, the mechanisms controlling this selection process remain unknown. In this study, we investigated apoptosis in granulosa cells during goat follicular atresia by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). The changes in the levels of steroids, insulin-like growth factors (IGFs) and IGF receptors were studied by radioimmunoassay (RIA) and semi-quantitative reverse transcription-PCR. We found that the percentage of apoptotic granulosa cells in the atretic (A) follicles was significantly higher than that in the slightly atretic (SA) and healthy (H) follicles. The level of estradiol and the ratio of estradiol to progesterone in H follicles were significantly higher than those in A follicles. On the other hand, the level of progesterone was not significantly different among these follicle types. We also found that the level of IGF-I in H follicles was higher than in SA and A follicles, whereas the amount of IGF-II did not vary significantly. The expression of IGF receptor also decreased in A follicles as compared to that in H and SA follicles. These results suggested that estradiol and IGF-I might be involved in controlling apoptosis in granulosa cells during follicular atresia.

***Keywords*:** apoptosis, ovarian follicle, steroids, IGFs, atresia.

INTRODUCTION

During mammalian ovarian follicular development, only limited numbers of follicles are selected for ovulation, whereas the rest undergo atresia at various stages of development [1]. Despite the overwhelming evidence for follicular atresia in the ovary, the cellular and molecular mechanisms underlying this phenomenon remain poorly understood. Previous studies indicate that the death of granulosa cells (GC) triggers atresia of the follicles [2-3], and death of the GC itself by apoptosis was found to occur in chicken, mouse [2], cow [4], ewe [5] and pig [6-7]. Although it has been suggested that alterations in steroidogenesis might be involved in the initiation of follicular atresia [4, 8-9], the exact pattern of steroid hormone during atresia has yet to be established. It has been shown that Insulin-like growth factors (IGFs) could block apoptosis induced by dexamethasone in various cell types. It is thus possible that IGFs play an important role in regulating follicular development and granulosa cell apoptosis [10-11]. Available data related to the expression of IGF-I, IGF-II (rat [12], mouse [13], human [14]) and type-1 IGFR (rat [10]) in the ovary are controversial. Very little is known about the expression of IGFs and their receptors in ruminant species. Therefore, the regulating mechanism of follicular atresia may be different among different species. In this study we systematically studied the relationship between the levels of IGFs and steroids and granulosa cell apoptosis in goat ovary. Our results indicate that significant apoptosis occurs in atretic, but not healthy follicles. Apoptosis of granulosa cells is related to the imbalance between estradiol and progesterone in the follicular fluid. The level of IGF-I, but not IGF-II, is the crucial factor in deciding whether a follicle will mature or undergo atresia.

MATERIALS AND METHODS

Isolation of follicles and granulosa cells

Goat ovaries were collected from a local abattoir and brought to the laboratory in saline at 30-35 ºC within 2 h after euthanasia. Follicles with diameters of 2-5 mm were excised from the ovary and were classified using a stereomicroscope into healthy (H), slightly

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atretic (SA) and atretic (A) groups according to a previous report [15]. Briefly, the H follicles had vascularized (pink or red) theca interna and clear amber follicular fluid (FF) with no debris; the SA follicles had rough and off-white theca interna with fewer but larger blood vessels and thick FF with much debris; and the A follicles had gray theca interna with no blood vessels and flocculent FF with many dark gobbets.

Follicles were cut in half and the interior walls were gently scraped with an inoculating loop to remove GC. The GC were harvested by centrifuging the FF at 400 g for 10 min. Cells were then washed three times with PBS. The GC pellets were re-suspended in PBS and smeared for detection of apoptosis by TUNEL. FF Supernatant was centrifuged at 2000 g to remove debris and stored at -20ºC before RIA for steroids and IGFs.

Preparation of smears and detection of apoptotic cells

Granulosa cells were fixed by combining equal volumes of granulosa-cell suspension and 4% paraformaldehyde; after 20 min, cells were washed. A small drop of the suspension was then smeared on a slide and allowed to dry. Apoptosis in granulosa cells was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using a cell apoptosis detection kit (Boehringer Mannheim, Indianapolis, IN USA). The procedures were as follows: cells were 1) fixed in 4 % paraformaldehyde in PBS for 1 h at room temperature; 2) rinsed 3 times with PBS and incubated with 0.3 % H_2O_2 in methanol for 1 h to quench endogenous peroxidase activity; 3) rinsed 3 times in PBS and treated with 0.1 % Triton X-100 at 4 °C for 2 min; 4) rinsed 3 times in PBS and incubated with TUNEL reaction mixture (50 μl) in a humidified chamber at 37 °C for 1 h; 5) washed 3 times in PBS and incubated with 50 μl Converter-POD (Peroxidase, Boehringer Mannheim) in a humidified chamber at 37°C for 30 min; 6) rinsed 3 times with PBS and exposed to the DAB (3, 3'-diaminobezidine tetrahydrochloride) -substrate solution for color development in a dark chamber at room temperature for 3 to 5 min; and 7) washed in PBS, dehydrated in ethanol, cleared in xylene, and mounted with balsam. For negative controls, slides were incubated with 50 μl label solution (without terminal transferase) instead of TUNEL reaction mixture. Smears were observed under a light microscope and percentages of apoptotic cells were calculated from 500 cells on each smear.

Reverse transcription-PCR

Twenty follicles from each category were homogenized and treated with Trizol (Gibco BRL, Gaithersbrug, MD) and total RNA was extracted according to the manufacturer's instructions. The RNA was re-suspended in DEPC-dH_2O and digested with PQ1 DNase I (Promega Corp., Madison, WI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for the quality of cDNA preparations. Since DNA or cDNA sequences for goat GAPDH, IGF-I, IGF-II were not available from GenBank, we identified fully conserved sequences among human, mouse and ovine. The sense and antisense primers used for RT-PCR were: GAPDH, 5'-ACCACAGTCCATGCCATCAC-3'and 5'-TCCACCACCCTGTTGCTGTA-3' for amplification of a 452-bp fragment; IGF-I, 5'-GACAGGAATCGTGGATGAGTG-3' and 5'-AACAGGTAACTCGTGACGAGC-3' for amplification of a 277bp fragment; IGF-II, 5'-CGTGGCATCGTGGAAGAGTGT-3' and 5-'GGTGACTCTTGGCCTCTCTGA-3' for amplification of a 270bp fragment; IGFR, 5'-ACTGACCTCATGCGCATGTGCTGG-3' and 5'CTCGTTCTTGCGGCCCCCGTTCAT-3' for amplification of a 345bp fragment, respectively. The RNA samples were reverse-transcribed and PCR-amplified using Takara BcaBEST RNA PCR kit (Takara Biotechniques, Dalian, China) according to the manufacturer's protocol. The RT reaction was performed at 65°C for 1 min, 30°C for 5 min, 65°C for 30 min, 98°C for 5 min and 5°C for 5 min. The amplification of IGF-I and IGFR cDNA was done for 35 cycles at 94°C for 2.5 min, 59°C for 30 sec and 72°C for 1min. IGF-II and GAPDH cDNA amplification was performed for 28 cycles at 94°C for 2.5 min, 60°C for 30 sec and 72°C for 1 min. The accuracy of this method was checked by regression analysis and the amplification coefficient was determined. The relative expression of mRNA for each gene of interest was calculated using a formula devised by Prelle *et al* [20]. To check for the specificity of RT-PCR, three controls were tested for genomic DNA contamination: 1) RNA samples directly amplified without reverse transcription, 2) reverse transcription done without adding reverse transcriptase, followed by PCR amplification. 3) RNA samples replaced by DEPC-dH_2O in RT-PCR. RT-PCR reactions were carried out only after ascertaining that all 3 controls were free of DNA contamination.

Radioimmunoassay

Concentrations of progesterone (P4) and estradiol (E2) in follicular fluid in unextracted samples were determined by direct radioimmunoassay (RIA) using a commercial kit (Jiuding Biotechniques Ltd, Tianjin, China), which has been validated for goat samples [17]. A blank culture medium assay showed no cross-reactivity and values below the limit of sensitivity (0.1ng/ml for P4 and 1pg/ml for E2). The intra- and inter-assay CVs were 7.2% and 6.9%, 8.0% and 7.7%, respectively. IGF-I and IGF-II were also detected by RIA. Samples were acid-ethanol extract-concentrated [18-19] and their concentrations were determined using a commercial kit (Jiuding biotechniques Ltd). The limits of sensitivity were 0.1ng/ml and 0.1ng/ml, respectively, and the intra- and inter-assay CVs were <10% and <15%, and <10% and <15%, respectively.

Statistical Analysis

For each treatment, at least three replicates were run. Statistical analyses were carried out by ANOVA. Differences between treatment groups were evaluated by the Duncan multiple comparison test. Data are expressed as mean ± SE and $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Apoptosis in granulosa cells of follicles at different stages of atresia

During mammalian follicle development, most follicles undergo atresia at different stages, a process critical to allow only a specific number of follicles to mature in each mammalian species. It is believed that, during atresia, cell death occurs in granulosa cells. Due to the difficulties in the isolation of granulosa cells, the nature of this cell death still is not clearly defined. Hughes and Gorospe [2] reported an oligonucleosomal ladder pattern of genomic DNA

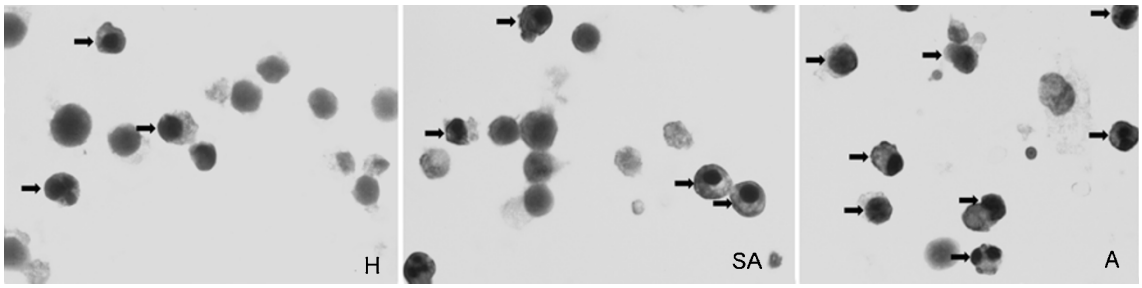

Fig. 1 Photomicrographs (×1000) of granulosa cell smears from healthy (H), slightly atretic (SA) and atretic (A) follicles after TUNEL staining. TUNEL positive cells (arrows) are sparse in H follicles but numerous in A follicles.

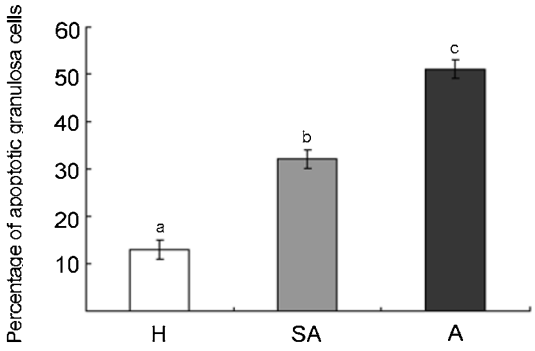

Fig. 2 Percentages of apoptotic granulosa cells in atretic (A), slightly atretic (SA) and healthy (H) follicles. Differences between values marked with different letters are significant (P<0.05).

fragments in atretic rat follicles. However, a similar DNA fragment pattern was also shown in the healthy follicles, although the ladder was fainter. On the other hand, Palumbo and Yeh [20] were unable to confirm apoptotic granulosa cells in the healthy follicles of the same species. Other studies in sheep [4, 21] and bovine [22] also demonstrated granulosa cell apoptosis in the healthy follicles. To verify the difference in apoptosis between atretic and healthy follicles, we employed a more definitive protocol to determine genomic DNA fragmentation using the TUNEL assay. We isolated granulosa cells from freshly procured goat follicles and staged them according to [15]. Granulosa cells were collected and cell smears were prepared on glass slides processed for TUNEL staining. We found that TUNEL positive apoptotic cells were sparse (13 ± 2%) in granulosa cells of H follicles (Fig. 1H). The number of TUNEL positive cells was significantly higher in SA follicles (32 ± 2%) (Fig.1SA). On the other hand, around half of granulosa cells (51 ± 2%) from A follicles underwent apoptosis as defined by the TUNEL assay (Fig. 1A). Forty-five follicles from 9 goats were tested and comparisons by ANOVA revealed significant difference among the three groups (P<0.01, Fig. 2). Therefore, our TUNEL assay showed clear differences in apoptotic cells during follicular atresia.

Changes in P4 and E2 levels in follicular fluid during atresia

Studies showed that follicular atresia was related to the production of steroids. It has been suggested that the balance between estradiol and progesterone may be a good predictor of whether a follicle will proceed to maturity or to undergo atresia [21, 23-24]. We, therefore, measured the concentration of E2 and P4 in FF and found that the level of estradiol is significantly lower in atretic follicles than that in healthy follicles. The level of progesterone, on the other hand, was higher in astretic follicles, which resulted in a significant change in the ratio of E2/P4: >1 in healthy follicles and <1 in atretic follicles (Tab. 1). These results suggest that an imbalance between E2 and P4 in follicular fluid is involved in the initiation of granulosa cell apoptosis and hence follicular atresia.

Changes in IGF expression during follicle atresia

IGFs are some of the most prominent apoptosis inhibitory cytokines and their regulated expression in various tissues play a pivotal role in regulating cellular homeostasis [25]. Expression of IGF-I and IGF-II in the ovary is different among different species. In the rat, ovarian IGF-I gene expression was restricted to granulosa cells of the healthy, but not atretic or luteinized, antral follicles [12]. In the mouse, IGF-I transcripts increased in healthy follicles, but decreased as a function of atresia [13]. In human, however, the level of IGF-I was similar in both the estrogen and androgen dominant follicles, while the level of IGF-II was higher in the estrogen dominant follicles [14]. Reports on the expression pattern of IGFs in the ruminant ovary are controversial. Yuan *et al* [26]

Tab. 1 Concentrations of E2 and P4 and the E2/P4 ratio in the follicular fluid from follicles with different degrees of atresia

Type of follicles	Concentration of E2 (ng/ml)	Concentration of P4 (ng/ml)	E2/P4
Healthy	0.2 ± 0.04 [a]	0.17 ± 0.06 [a]	3.9 ± 2.5 [a]
Slightly atretic	0.07 ± 0.01 [a]	1.2 ± 0.6 [a]	0.2 ± 0.2 [b]
Atretic	0.05 ± 0.01 [b]	1.7 ± 1.1 [a]	0.08 ± 0.06 [b]

Differences between values marked with different superscript letters in the same column are significant ($p<0.05$).

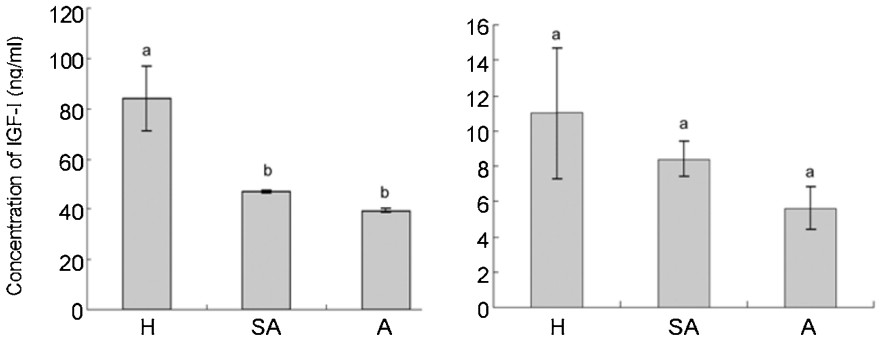

Fig. 3 Concentrations of IGF-I (A) and IGF-II (B) in the follicular fluid from healthy (H), slightly atretic (SA) and atretic (A) follicles. Differences between values marked with different letters are significant ($P<0.05$).

proved mRNA expression of both IGF-I and IGF-II in bovine granulosa cells. Ginther *et al* [27] reported an increased level of IGF-I in the largest follicles of bovine. However, Armstrong *et al* [28] were unable to confirm the expression of IGF-I mRNA in bovine granulosa cells, but they found IGF-II expression in the theca cells instead.

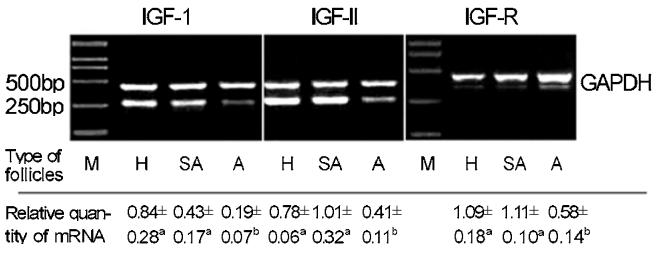

Fig. 4 Expression of IGF-I, IGF-II and IGFR mRNAs in healthy (H), slightly atretic (SA) and atretic (A) follicles. Standard marker (M) shows 500 and 250bp bands. In other lanes, the bands below GAPDH are mRNAs of IGFs or IGFRs. The mRNA levels were normalized to the GAPDH signals, and the relative quantity of mRNA expression is expressed as a fraction of Max OD. Differences between values marked with different superscript letters are significant ($P<0.05$).

We examined the levels of IGF-I and IGF-II by RIA and found that the level of IGF-I was significantly higher than that of IGF-II in all three follicle types. The concentration of IGF-I in H follicles (83.5 ± 12.9 ng/ml) was significantly higher ($P<0.05$) than that in SA (46.9 ± 0.3 ng/ml) and A follicles (39.3 ± 0.9ng/ml) (Fig. 3A). However, the concentration of IGF-II did not change significantly ($P>0.05$) during follicle atresia (Fig. 3B). To further verify this result, we examined the level of IGF messages in granulosa cells by semi-quantitative RT-PCR. Our results demonstrate that the relative quantity (Max OD) of IGF-I mRNA expression decreased with follicular atresia (Fig. 4). Besides, our previous study showed that IGF-I significantly reduced the percentage of apoptotic granulosa cells cultured *in vitro* [17].

The action of IGFs was mainly mediated via the type-1 IGF receptor [29]. In bovine, the level of type-1 IGFR was related to the degree of follicular atresia [14], but in the rat, type-1 IGFR was consistently expressed in follicles at different stages and degrees of atresia [10]. We showed in this study that, as in bovine, the expression of type-1 IGFR mRNA decreased with follicular atresia (Fig. 4). Therefore, it seems that, in the goat, it is IGF-I

that plays a more important role in the control of follicular atresia.

In conclusion, our results indicate that (i) significant apoptosis occurs during follicle atresia; (ii) apoptosis of granulosa cells is related to the imbalance between estradiol and progesterone in the follicular fluid; and (iii) the level of IGF-I, but not IGF-II, is the crucial factor in deciding whether a follicle matures or undergoes atresia in the goat ovary.

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