Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology)
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

Morphologic observation and classification criteria of atretic follicles in guinea pigs*

Wei WANG, Hong-lin LIU, Wei TIAN, Fen-fen ZHANG, Yan GONG, Jin-wei CHEN, Da-gan MAO, Fang-xiong SHI†‡

(*College of Animal Science and Technology, Nanjing Agricultural University, Nanjing 210095, China*)

†E-mail: fxshi@njau.edu.cn

Received Dec. 3, 2009; Revision accepted Mar. 4, 2010; Crosschecked Apr. 7, 2010

Abstract: There is a lack of appropriate classification criteria for the determination of atretic follicles in guinea pigs. In the present study, new criteria were established based on the latest morphologic criteria for cell death proposed by the Nomenclature Committee on Cell Death (NCCD) in 2009. Ovaries of guinea pigs were sampled on different stages of estrous cycle, and the morphologic observations of atretic follicles were investigated in serial sections. The results showed that the process of follicular atresia could be classified into four continuous stages: (1) the granulosa layer became loose, and some apoptotic bodies began to appear; (2) the granulosa cells were massively eliminated; (3) the theca interna cells differentiated; and (4) the residual follicular cells degenerated. In addition, the examination revealed that these morphologic criteria were accurate and feasible. In conclusion, this study provides new criteria for the classification of atretic follicles in guinea pigs, and this knowledge can inform future research in the area.

Key words: Guinea pig, Follicular atresia, Classification criteria
doi:10.1631/jzus.B0900391 **Document code:** A **CLC number:** Q24

1 Introduction

The guinea pig is an excellent animal model for studies of the reproductive system in humans and most domestic animals (Suzuki *et al*., 2003). Unlike other laboratorial rodents, guinea pigs have an extended estrous cycle of 16 to 18 d (Joshi *et al*., 1973; Trewin *et al*., 1998), cannot be superovulated by follicle-stimulating hormone (FSH) or equine chorionic gonadotropin (eCG) but rather with inhibin immunizations (Shi *et al*., 2000a), and have a full gestation of 68 d and a small litter size of 3.8 on average (van Kan *et al*., 2009). Owing to these special biological characteristics, the process of follicular atresia in guinea pigs also has unique features.

To date, many studies of follicular atresia have been done, and the classification criteria for atresia have been mainly based on morphologic studies. For example, follicular atresia in mice (Byskov, 1974), rats (Osman, 1985; Hirshfield, 1988), pigs (Sugimoto *et al*., 1998), goats (Garcia *et al*., 1997), and yaks (Cui and Yu, 1999) was always divided into two or three stages. For guinea pigs, however, it has been difficult to provide uniform classification criteria because there are unique morphologic changes in the atretic process. The differentiation of theca cells (Logothetopoulos *et al*., 1995; Kasuya, 1997) is an example of one unique change. Fortunately, the process of follicular atresia was described in detail and divided into seven stages for the first time in 1995 (Logothetopoulos *et al*., 1995). This produced criteria for atresia that were, however, too complicated to observe and apply.

‡ Corresponding author
* Project supported by the National Basic Research Program (973) of China (No. 2007CB947403) and the Open-Ended Fund for the Youth of China Agricultural University—Nanjing Agricultural University (No. NC2008005)

In 2005, the Nomenclature Committee on Cell Death (NCCD) was established by the editors of the journal of *Cell Death and Differentiation*. In the absence of some clearly defined biochemical indices for cell death, the NCCD suggested that the official classification of cell death had to be entirely dependent upon morphologic changes (Kroemer *et al*., 2005; 2009). Therefore, in the present study, the morphologic changes of atretic follicles are fully described in order to establish new classification criteria for follicular atresia.

2 Materials and methods

2.1 Animals and treatments

Adult female Hartley guinea pigs (*Cavia porcellus*), with an initial weight of 400–700 g (5 months old), were used. They were housed at four animals per cage under a controlled temperature at (23±2) °C, and fed commercial food and tap water ad libitum. Estrous cycles were recorded by daily examination of vaginal smears whenever the vagina was open. The day of ovulation was estimated as the day when the maximal cornification was seen in the smear (Norris and Adams, 1979), and was designated as Day 0 of the cycle. Days of the estrous cycle followed from this point. Animals with at least 2 consecutive regular cycles of 16 d were sacrificed on Days 1, 4, 8, 12, and 16 (5 animals per day) (Garris and Mitchell, 1979; Logothetopoulos *et al*., 1995).

Ovaries were then fixed in 4% (w/v) paraformaldehyde at room temperature for 24 h, dehydrated through increasing alcohol concentrations, embedded in paraffin, sectioned serially at 10 μm, and stained with hematoxylin and eosin (HE).

2.2 Classification criteria for follicular atresia

In 2005 and 2009, the NCCD proposed that a cell should be considered dead when any one of the following morphologic criteria is met: the cell has lost the integrity of its plasma membrane, the cell has undergone complete fragmentation into discrete bodies, or its corpse has been engulfed by an adjacent cell in vivo (Kerr *et al*., 1994; Kroemer *et al*., 2005; 2009). Currently, cell death modes generally include apoptosis, autophagy, cornification, and necrosis, but apoptosis is the main concern in follicular atresia. Generally, apoptosis is accompanied by rounding-up of the cell, reduction in cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), plasma membrane blebbing and engulfment by resident phagocytes (in vivo) (Galluzzi *et al*., 2007; Kroemer *et al*., 2005; 2009). In addition, the NCCD suggested that morphologic changes had to be the basis for the classification of cell death (Kroemer *et al*., 2005; 2009). Therefore, our study was based on these morphologic criteria, and the granulosa cells, theca cells, and oocytes were investigated.

It has been widely accepted that the follicular growth of guinea pigs is biphasic in the whole estrous cycle (Bland, 1980; Hutz *et al*., 1990; Fortune, 1994). During the first 12 d of the estrous cycle, unovulated follicles in the former estrous cycle degenerated and the first wave of follicular growth culminated on Days 10–11 (Bland, 1980). But it is difficult to illustrate the follicular atresia commensurate with the second follicular wave growth, and the follicular growth rate was indistinct in this period (Bland, 1980). Therefore, the Days of 1, 4, 8, 12 and 16 were selected for evaluation of the classification criteria.

For assessing the accuracy and specificity of the new classification criteria for follicular atresia, the follicular diameter and thicknesses of follicular wall, granulosa layer and theca layer were measured. Each index was measured among the different stages of atretic follicles. Only the follicles that had the largest cross-sectioned surface area in the serial sections were selected, and 0 to 5 follicles were selected for each individual. The diameter was taken as the mean of two diameters measured at right-angles (Bland, 1980). The thicknesses of follicular wall, granulosa layer and theca layer were measured at six different positions, and the mean was used for statistical analyses.

2.3 Statistics

All statistical analyses were performed using the general linear model of SAS 6.12. One-way analysis of variance (ANOVA) was used to analyze the data. Differences among means were tested using Tukey's new multiple range test. All data were expressed as the mean±standard error of the mean (SEM). A value of $P<0.05$ was considered to be statistically significant.

3 Results

3.1 Morphologic changes of atretic follicles in estrous cycle

On Day 1, after ovulation occurred, the fresh corpora lutea were observed. The process of atresia was acute, and a mass of dead granulosa cells was scattered in the antrum, or the granulosa layers were cleared away. Furthermore, the theca layer had thickened, and the theca interna cells differentiated to a kind of fibroblast-like cell and invaded the antrum.

On Day 4, many atretic follicles had shrunk, and manifested a smaller volume and irregular shape, though follicles with fibroblast-like cells still existed. At this time, the atretic follicles always contained a degenerate oocyte.

On Day 8, with the rapid wave of follicular growth, a group of antral follicles appeared. The process of atresia occurred concomitantly. The granulosa layer became loose, and apoptotic bodies were observed.

The characteristics on Day 12 were similar to Day 8, but the process of follicular atresia was accelerated, and showed more atretic follicles with fibroblast-like cells.

Day 16 was identified by maximal cornification in the vaginal smear. Many antral follicles with thick granulosa layers were observed; however, few were found to be healthy. Ovaries at this time contained 0–3 unruptured preovulatory follicles. Morphologic analysis revealed that the preovulatory follicles had large average diameters of more than 700 μm, with smooth outlines, and the periphery of the follicle and the ovarian surface epithelium were juxtaposed.

3.2 Establishment of new classification criteria for follicular atresia

Based on the above results, the remarkable morphologic changes in atretic follicles were depicted as follows: loose connections in granulosa layers, rapid elimination of granulosa cells, differentiation of theca interna cells, and degeneration of residual follicular cells. Atresia appeared to be a continual process, and the atresia of follicles was divided into four continual stages I, II, III and IV (Table 1).

Table 1 Classification criteria of follicular atresia

Stage	Morphologic characteristics of atresia
I	The connections in granulosa layers became loose. Pyknotic granulosa cells and apoptotic bodies were observed.
II	Few healthy granulosa cells were observed, and a mass of dead granulosa cells and their corpses was scattered throughout the antrum.
III	The dead granulosa cells were eliminated entirely, and the follicular antrum was occupied by differentiated cells from the theca layer.
IV	The differentiated cells disappeared, and the oocyte degenerated entirely. The atrophic follicles were mainly composed of hypertrophic theca cells.

3.3 Morphologic changes of atretic follicles at different stages

3.3.1 Stage I: loose connections in granulosa layers

This is an early stage of follicular atresia (Figs. 1a and 1b). The granulosa layer became loose, there were some deeply stained pyknotic granulosa cells and apoptotic bodies were observed in the granulosa layer. There was evidence that these deeply stained pyknotic cells were dying or had died by apoptosis. The outline of the antrum was ragged. Only a small number of dead cells or apoptotic bodies, however, were observed, and they may have been phagocytosed by neighboring intact granulosa cells, which was confirmed by electron microscopic observation (Logothetopoulos *et al*., 1995; Kasuya, 1997). Moreover, the follicular size and theca cells did not change in an obvious way, and the oocytes still seemed to be healthy.

3.3.2 Stage II: rapid elimination of granulosa cells

At this stage, the healthy granulosa cells were rarely seen (Fig. 1c). A number of dead cells and apoptotic bodies were found in the antrum, and it was obvious that the granulosa cells degenerated rapidly. The dead granulosa cells or apoptotic bodies floated along the border of the antrum, and the closer they were to the basal lamina, the more deeply they were stained. It is suggested that the granulosa cells close to the basal lamina were affected last. The follicular antrum center, however, was eosinophilic, and few dead granulosa cells or apoptotic bodies were observed.

Close to the basal lamina, the columnar granulosa cells appeared to be desquamated (Fig. 1d). The

basal lamina which separated the granulosa layers from the theca layers became indistinguishable. In addition to the dead granulosa cells and apoptotic bodies, some large cells appeared (Fig. 1e). The large cells had diameters of 20–60 μm and included many deeply stained dead cells. It is evident that these large cells play an important role in the elimination of dead granulosa cells.

In addition, the degenerated oocytes were surrounded by a small number of pyknotic cumulus cells, and floated in the antrum.

3.3.3 Stage III: differentiation of theca interna cells

The most complicated cellular morphologies appeared at this stage. After the dead granulosa cells were cleared away, the atrophic follicular cavity was occupied by a loose network of slender cells which had a fibroblast-like, polyhedral and amoeboid appearance (Fig. 1f). Meanwhile, a visible change was observed in the theca layer. The hypertrophic theca interna cells appeared, which developed from slender shapes into spherical or spindle-shaped. In addition, the alignment of the theca interna cells changed abruptly, with the longitudinal axis rotated in a centripetal orientation. This strongly suggests that the fibroblast-like cells differentiated from the inner layer of theca interna.

Interestingly, based on the strong eosinophilic stain, it seemed that a new tissue was formed in the atretic follicles, and that there was abundant extracellular matrix secreted from this new issue (Figs. 1g and 1h). The oocytes had further degenerated, and the corona radiate cells disappeared. The zona pellucida, however, did not rupture or collapse and was still notable. In addition, the cells around the zona pellucida also differentiated, and they had a larger volume and dense extracellular matrix (Figs. 1i and 1j).

3.3.4 Stage IV: degeneration of residual follicular cells

The final stage of follicular atresia is the elimination of the residual follicular cells. The atrophic follicles had irregular shapes and a smaller volume. At this stage, the fibroblast-like cells disappeared, and the main component was the differentiated theca cells (Fig. 1k).

As the atresia progressed, the follicle volume became smaller and smaller (Fig. 1l). It seemed that these final structures shrank further by continuous loss of cellular elements, and eventually lost their identity by blending with interstitial tissue.

3.4 Abnormal manner of follicular atresia

The process of follicular atresia was not exclusive, as described previously. In some small antral follicles, the cumulus granulosa cells were eliminated first, and the rest seemed relatively intact. Moreover, some Call-Exner bodies were usually found in the granulosa layer (Figs. 1m and 1n). They might have degenerated in an abnormal manner of atresia. As a result of their scant numbers, further investigation was not warranted. This was a relatively simple mode for preantral follicular atresia. There was no massive elimination of cells, and the granulosa cells were lost gradually (Figs. 1o and 1p).

3.5 Degeneration of oocytes

Although it was evident that the granulosa cells degenerated by apoptosis, this was not the case for oocytes. The oocytes underwent a long process of degeneration in antral follicles, but chromatin condensation, nuclear fragmentation, and plasma membrane blebbing were not found (Fig. 2). Furthermore, the oocyte was enveloped by the zona pellucida, and it appeared to be impervious to the phagocytic cells.

Moreover, the morphologic characteristics of oocyte degeneration are in line with the autophagic cell death to some extent (Kroemer *et al.*, 2005; 2009). In the early stage, some cavities appeared in the oocyte (Figs. 2a and 2b), and the cumulus granulosa cells were shed from the zona pellucida gradually (Fig. 2c). Then, the enlarged cavities formed a loose network in the oocyte (Fig. 2d). With the process of degeneration, the oocytes degenerated to a small volume, and the shape of the zona pellucida became irregular (Fig. 2e). When the oocytes degenerated completely, the zona pellucida collapsed and had a compressed shape (Fig. 2f). It is evident that the oocyte degeneration is different from typical apoptosis, but similar to autophagic cell death.

3.6 Accurate and specific tests for the new classification criteria

Measurements of follicular diameter and thicknesses of the follicular wall, granulosa layer, and theca layer were made to test the new classification criteria for follicular atresia. The results are shown in Fig. 3.

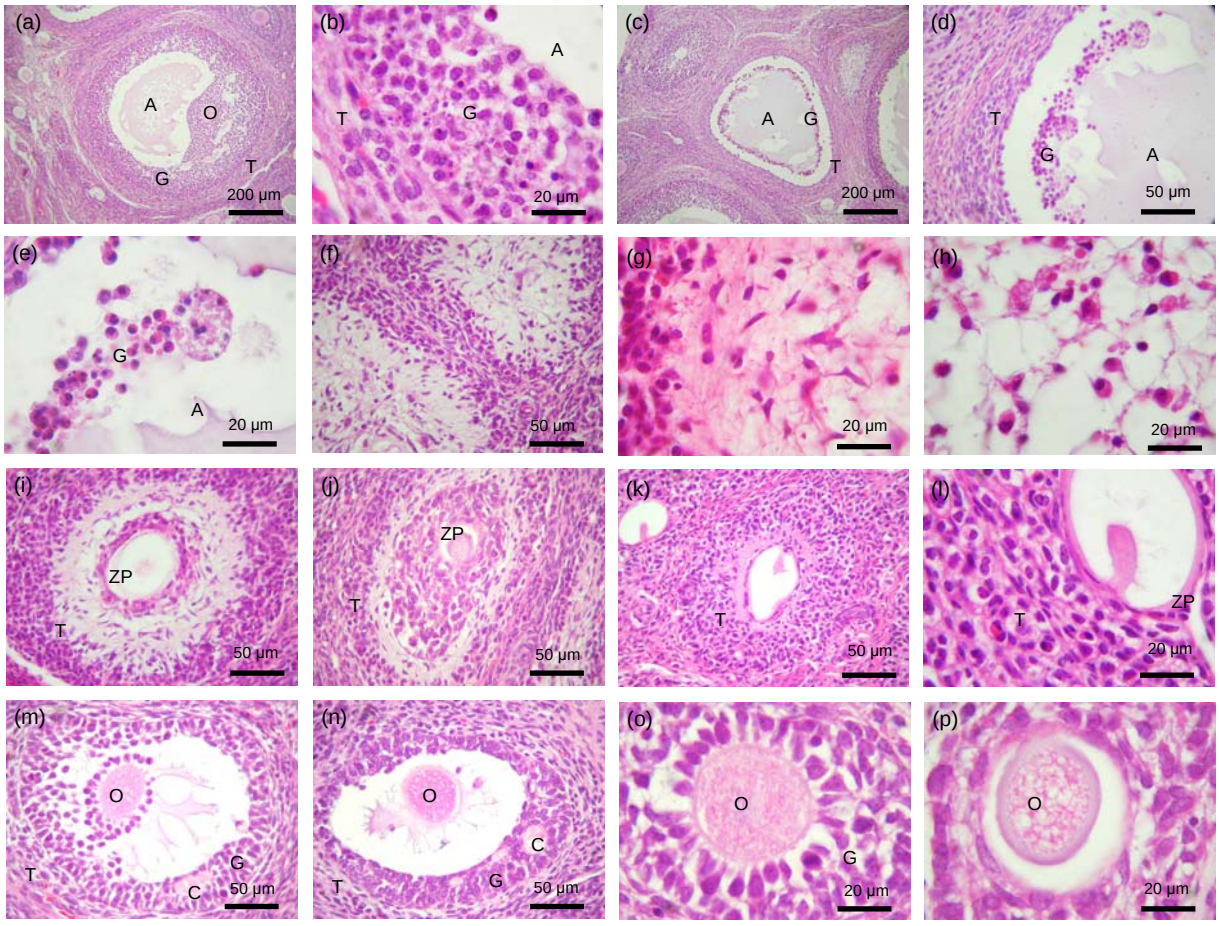

Fig. 1 Morphologic observation of follicular atresia

(a) & (b) Stage I: granulosa layer became loose and the pyknotic cells appeared; (c), (d) & (e) Stage II: granulosa cells were eliminated massively and floated in the antrum; (f), (g) & (h) Early phase of Stage III: atrophic follicular cavity was occupied by fibroblast-like cells with slender shapes (g) and round shapes (h); (i) & (j) Late phase of Stage III: at first, only a small quantity of differentiated cells appeared around the zona pellucida (i), however, it increased greatly and formed a new tissue subsequently (j); (k) & (l) Stage IV: follicle volume became smaller and smaller; (m) & (n) Abnormal manner of follicular atresia: in some small antral follicles (m), the cumulus granulosa cells were eliminated first and the granulosa layers were relatively intact (n); (o) & (p) Preantral follicular atresia: there was no massive elimination of cells (o), preantral follicles degenerated gradually (p). O: oocyte; G: granulosa layer; T: theca layer; A: follicular antrum; C: Call-Exner body; ZP: zona pellucida

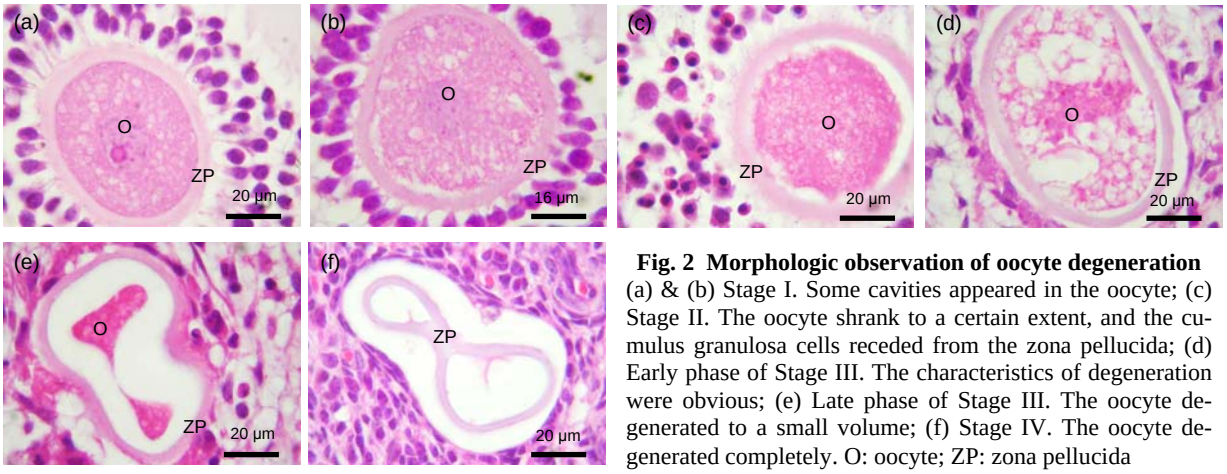

Fig. 2 Morphologic observation of oocyte degeneration

(a) & (b) Stage I. Some cavities appeared in the oocyte; (c) Stage II. The oocyte shrank to a certain extent, and the cumulus granulosa cells receded from the zona pellucida; (d) Early phase of Stage III. The characteristics of degeneration were obvious; (e) Late phase of Stage III. The oocyte degenerated to a small volume; (f) Stage IV. The oocyte degenerated completely. O: oocyte; ZP: zona pellucida

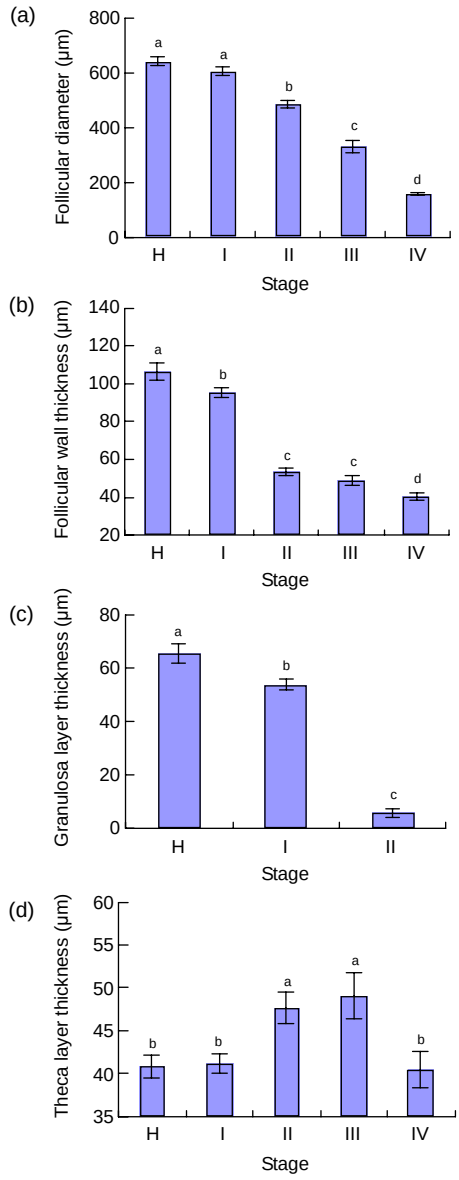

Fig. 3 Measurements of the follicular diameter (a) and thicknesses of follicular wall (b), granulosa layer (c) and theca layer (d)

Measurements were taken of healthy follicles (H, n=28) and atresia follicles in Stages I (n=46), II (n=35), III (n=37) and IV (n=33). Each value represents the mean±SEM. One-way analysis of variance (ANOVA) was used to analyze the data. $^{a, b, c, d}$ Significant values (P<0.05) by Tukey's multiple-range test

Generally, the measurements of the follicular diameter and thicknesses of follicular wall and granulosa layer showed the same trends. Along with the process of the atresia, these measurements decreased. The thickness of the theca layer, however, was different. As a result of differentiation of the theca cells, the measurements of Stages II and III were increased. Generally speaking, each of the atretic indices was significantly different from the others (P<0.05). Therefore, we can draw the conclusion that the new classification criteria were specific and feasible.

4 Discussion

In the present study, we demonstrated the process of follicular atresia in the estrous cycle of guinea pigs. According to the criteria of cell death proposed by NCCD in 2009 (Kroemer *et al*., 2009), the continuous morphologic changes of the atretic process were investigated. Based on obvious morphologic changes, new classification criteria for follicular atresia are provided in this study. These include the loose connections in the granulosa layer (Stage I), the rapid elimination of dead granulosa cells (Stage II), the differentiation of inner theca layer (Stage III), and the degeneration of residual follicular cells (Stage IV).

Compared with seven-stage criteria (Logothetopoulos *et al*., 1995), it is evident that the new criteria are very dissimilar. The proposed new criteria are compact and easy to apply because they rely on only the most obvious morphologic changes. For example, the morphologic changes at the differentiation stage were too complex and were not included. Furthermore, the seven-stage criteria ignored the invasion of differentiated cells (Fig. 1f) and the morphologic changes of the cells surrounded the zona pellucida (Figs. 1i–1k), and thus could disorder the stages readily. For example, Stages IV, V, and VI might be arranged as Stages VI, IV, and V, respectively. No such problems exist in the new proposed criteria, because these changes are included in Stage III.

After ovulation, a large number of Stages II and III follicles appeared on Day 1, and the granulosa cells degenerated and were eliminated in an apoptotic manner in a short time. Subsequently, the fibroblast-like cells filled the antrum, and these were differentiated from the theca interna cells. Although Stages II and III follicles also appeared on Days 8, 12, and 16, it was mainly on Day 1 that they appeared. This may have been caused by the dramatic changes of gonadotropin hormones around the time of ovulation (Shi *et*

al., 1999; 2000b; Trewin *et al*., 1998). Along with the process of atresia, the follicles that appeared on Day 4 were mainly at Stages III and IV. With the new follicular wave, the healthy, Stages I and II follicles appeared together on Days 8, 12 and 16. Based on these morphologic changes in the granulosa and theca layers, it can be concluded that the sequences of Stages I, II, III and IV are a continuous process, with indices representing characteristics of atresia. Although the main route of cell death for follicular cells was by apoptosis, this was not to the case with oocytes, which seemed to degenerate in an autophagic manner.

In addition, apoptosis was present in all atretic follicles, though the intensity and extension were variable. In Stage I, some pyknotic cells and apoptotic bodies were interspersed among the healthy cells. It was accepted that these dead cells or apoptotic bodies were eliminated by macrophages or neighboring intact cells (Kasuya, 1997; Wu *et al*., 2004). Stages III and IV showed the course of apoptosis in its mildest form. Stage II, however, was different: when the granulosa layer collapsed, a mass of dead cells and apoptotic bodies scattered throughout the antrum, and there were few healthy granulosa cells remaining. It is obvious that these dead cells could not be cleared away by the above-noted mechanism. Then, some large macrophages appeared, having been demonstrated to be large phagocytes (Kasuya, 1997). The large macrophages had diameters of 20–60 μm, and contained many dead cells and apoptotic bodies. This illustrates that the macrophages are important regulators in the ovaries, and that they can regulate cellular proliferation, differentiation and apoptosis (van der Hoek *et al*., 2000; Wu *et al*., 2004). Therefore, the large phagocytes are important regulators in Stage II atretic follicles, and play an important role in the elimination of dead cells.

The follicular cells were gradually eliminated in the process of atresia, but this was not the only characteristic of atresia. Atresia is a complex and orderly process, and cellular differentiation also occurs. It is difficult to explain the functions of the new tissue that appear in the atretic follicle, yet its existence was confirmed. In Stage III, the theca interna cells differentiated first, and then the fibroblast-like cells appeared and invaded the antrum. Subsequently, a type of hyperplastic cell started to surround the zona pellucida. In Stage IV, flat epithelial-like cells appeared and enveloped the cavity in the atretic follicle. Therefore, the process of atresia was not as simple as we had previously thought. Although the current study cannot explain why these new cells appeared, the cells appear to be indispensable in the atretic process.

In conclusion, follicular atresia in guinea pigs might not be a simple process of cell apoptosis and elimination; rather, it is highly complex. Based on obvious morphologic changes, the process of follicular atresia can be divided into four stages: loose connections between granulosa cells, massive elimination of granulosa cells, differentiation of theca cells, and degeneration of the residual follicular cells. This classification scheme is both specific and feasible.

5 Acknowledgement

We express our gratitude to Dr. Reinhold J. HUTZ of the Department of Biological Sciences, University of Wisconsin-Milwaukee, Wisconsin, USA for reading the original manuscript and offering valuable suggestions.

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