

## Atresia of large ovarian follicles of the rat

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**Abstract:** In the rat, at the beginning of pregnancy a cohort of antral follicles develops until the preovulatory stage. However, these follicles, differentiating in the hyperprolactinemic milieu, produce only small amount of estradiol, do not ovulate and undergo rapid degeneration. They constitute an interesting physiological model of atresia. In the present study, we analysed the development and subsequent degeneration of such follicles. The study was performed on Wistar female rats killed in succession between days 1-9 of pregnancy. Excised ovaries were submitted to a routine histological procedure. Paraffin sections were subjected to hematoxylin and eosin staining or *in situ* DNA labelling. Histological and TUNEL staining revealed that the investigated group of follicles grew slower than that on the corresponding days of the estrous cycle and reached a preovulatory size and morphological appearance on day 5 of pregnancy. They did not ovulate and between days 6 and 9 of pregnancy an increasing number of apoptotic cells appeared within these follicles. They were localized predominantly in the antral granulosa layer, especially near the cumulus oophorus complex (COC) and in the region linking the COC with the follicular wall. The COC and the theca layer were much less affected. In late stages of atresia, also cumulus cells became apoptotic but degenerating oocytes did not exhibit positive TUNEL staining. Only limited number of the theca cells have undergone apoptosis and generally they were not hypertrophied. Our findings indicate that much smaller than normal amount of intrafollicular estradiol was sufficient to support a normal, according to the morphological criteria, although slower development of antral follicles to the late preovulatory stage.

**Key words:** Apoptosis - Preovulatory follicles - Hyperprolactinemia - Rat

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### Introduction

The vast majority of ovarian follicles is destined to undergo atresia at various stages of their development. Many aspects of this degenerative process, mediated by apoptosis, have already been investigated including studies on survival and atretogenic factors as well as apoptotic and proliferative changes accompanying atresia [4, 5, 8, 14, 17]. In these studies, experimental models of induced atresia have been used, many of which were based on the deprivation of gonadotropins, regarded as key survival factors for antral follicles. Such experimental models provided a cohort of antral follicles undergoing atresia synchronously, in contrast to ovaries of intact animals, including cycling ones, containing atretic follicles at various stages of growth and degeneration. However, studies on the ovaries of cycling animals, especially rats [e.g. 11, 20] have also been performed in the past, and provided detailed histological descriptions of the particu-

lar stages and rates of atresia of various types of follicles. It is rather difficult to find a group of follicles undergoing synchronous atresia in physiological conditions. Antral follicles, degenerating during pregnancy in the rat constitute such a cohort. In this species, the recruitment of a group of small antral follicles to a rapid preovulatory development occurs at proestrus and estrus [3, 7]. If the estrous cycle continues, they differentiate and ovulate after the next preovulatory gonadotropin surge. However, if pregnancy occurs, this recruited cohort, corresponding to the estrous cycle one, also differentiates up to the preovulatory stage during the first few days of pregnancy but the follicles do not ovulate and start to degenerate. Their atresia results presumably from a hostile hormonal environment created above all by high concentrations of prolactin, characteristic for pregnancy in the rat [2, 10, 18]. There are reports on hormonal function of these follicles, indicating too small amount of estradiol produced [24, 27], but their morphological development and the following atretic changes have not been investigated. Histological analysis and localization of apoptotic cells in this group of follicles was the main objective of the present study.

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## Materials and methods

**Animals and tissue preparation.** The animals were handled according to the approved national guidelines for animal care. They were kept under controlled light conditions (LD 12:12 h, L 08.00 to 20.00 h) and fed an ordinary laboratory diet. Mature female Wistar rats, 2 to 3 months of age, exhibiting a regular 4-day estrous cycle, were used in the present study. The estrous cycle was followed by daily vaginal smears. On the day of proestrus, females were placed with males and the next day, on which spermatozoa were found in the smears, was designated day 1 of pregnancy. The rats were killed in groups on the consecutive days of pregnancy (day 1 to 9;  $n=3$  or 4, and  $n=1$  on day 9). Dissected ovaries, destined for histology and *in-situ* DNA labelling, were fixed in 10% buffered formalin for 24 h and, after routine histological procedure, embedded in Paraplast (Monoject Scientific Division of Sherwood Medical, St Louis, MO, USA). Sections (7  $\mu\text{m}$ ) were mounted on slides coated with 3-amino-isopropyl-triethoxysilane (Sigma Chemical Co. Ltd, Poole, Dorset, UK).

**Histology and morphometry.** The sections were stained with hematoxylin and eosin (H-E). The aim of the morphometric studies was to establish whether and when the investigated follicles reached the preovulatory size. In the ovarian sections from days 4 and 5 of pregnancy, two perpendicular diameters, the longest and the shortest, were measured in the investigated follicles in which the nucleus of the oocyte was visible. Similar sections containing proestrous follicles of cycling rats, used in our previous studies [28], served for the assessment of the preovulatory follicular size.

***In situ* DNA labelling by TUNEL assay.** Since histological analysis revealed that the investigated follicles did not exhibit any signs of atresia until day 5, for *in situ* DNA labelling only ovarian sections from days 5-8 of pregnancy, containing investigated follicles with cumuli oophorii were chosen. Apoptotic cells were identified with the APOPTAG kit (Roche Diagnostics GmbH Roche Applied Science, Mannheim). The assay is designed to label the free 3'-OH DNA termini with fluorescein-labelled nucleotides. In order to expose the 3'-OH ends of DNA, sections were incubated with proteinase K and then the nucleotides were added enzymatically to the DNA by terminal deoxynucleotidyl transferase (TdT). Slides were analysed with fluorescence microscopy. For negative control, incubation with TdT was omitted.

## Results

### ***Histological evaluation of atresia and morphometric analysis***

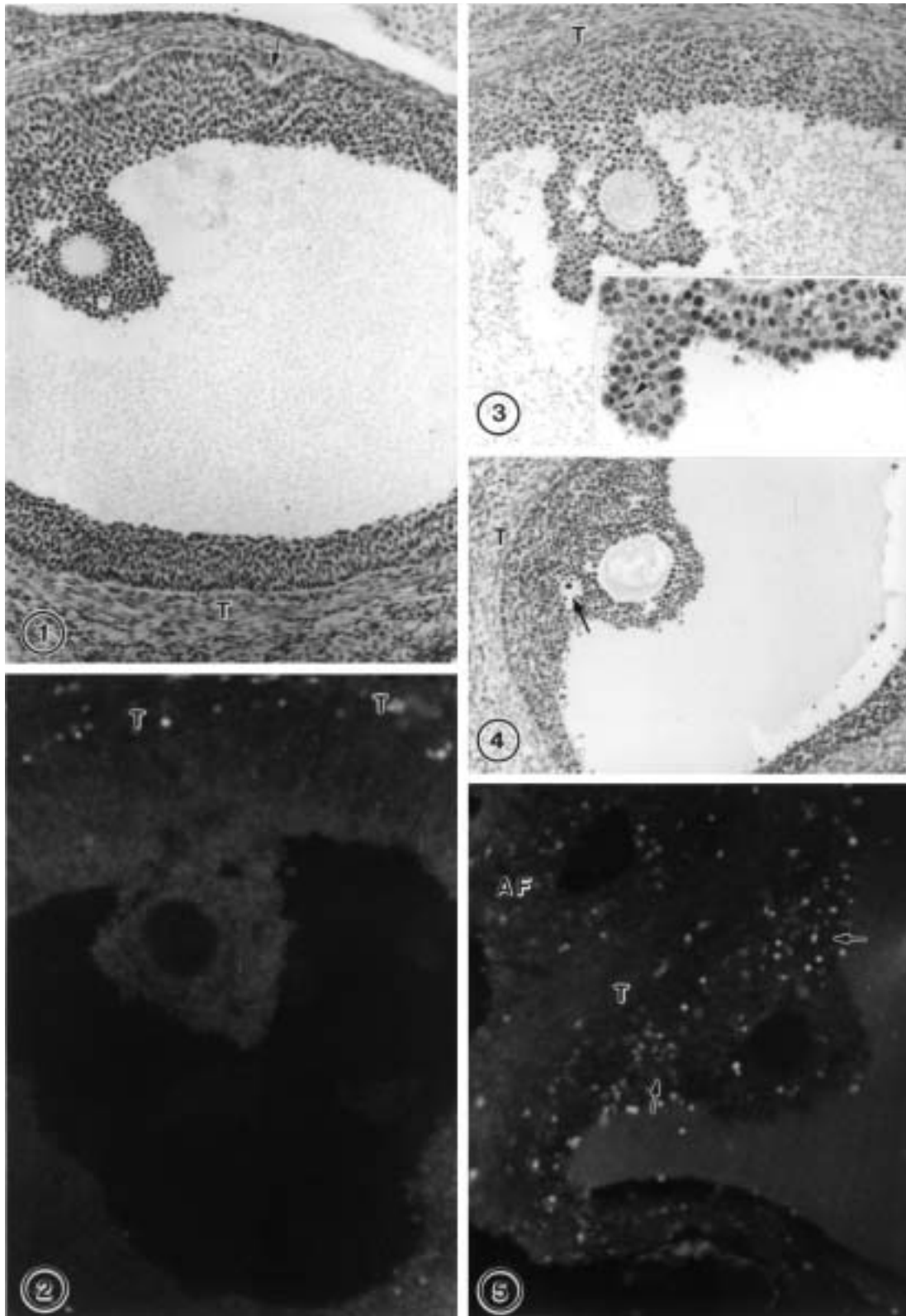
Histological analysis revealed that between days 1-5 of pregnancy the investigated follicles looked normal and did not show any signs of atresia. They grew, differentiated and reached the preovulatory size on day 5 of pregnancy, that is one day later than their counterparts during the 4-day estrous cycle. Morphometric analysis revealed that the average size of preovulatory follicles was  $844 \mu\text{m} \times 662 \mu\text{m}$  in the pregnant rats and  $838 \mu\text{m} \times 710 \mu\text{m}$  in the cycling rats. On day 5 of pregnancy, the investigated follicles exhibited also morphological characteristics of preovulatory cycling ones, including a thick granulosa layer, pseudostratified in the region adjacent to the basement membrane. In some follicles, this pseudostratified layer showed a characteristic undulation caused by

invaginations of the theca interna cells (Fig. 1), a feature typical of the late preovulatory stage [28]. Apoptotic bodies were only sporadically observed at the periphery of the antral cavity and/or in the region linking cumulus oophorus complex (COC) with the follicular wall. However, the oocytes neither resumed meiosis nor ovulated. The expansion of the COC was not observed, either.

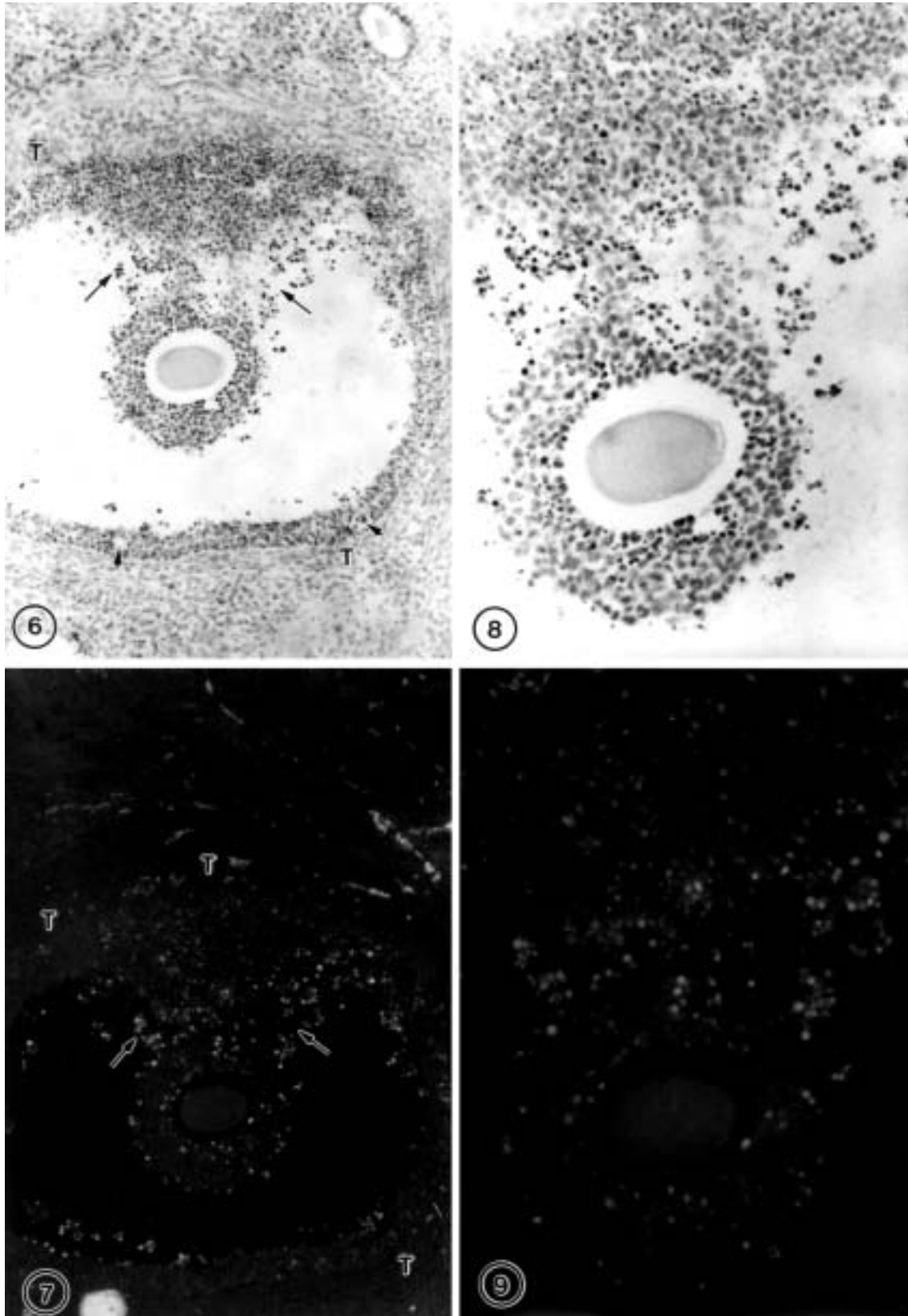
On day 6 of pregnancy, atretic changes appeared in this unovulated group of follicles. Previously observed undulation and pseudostratification disappeared (Figs 3, 4). The granulosa layer was thinner, especially in the region opposite to the COC. Relatively numerous apoptotic bodies were often gathered near the periphery of the antrum, particularly in the vicinity of the COC, and were also present in the region of granulosa layer linking the COC with the follicular wall (Fig. 4). The COCs looked relatively normal, and even mitotic figures were sporadically observed in the cumulus granulosa cells (Fig. 3). The theca layer seemed unaffected (Figs 3, 4). The range and intensity of the described changes varied between particular follicles but generally the process of atresia, although not yet advanced, was clearly visible.

On day 7 of pregnancy, the investigated follicles were smaller in size and the granulosa layer became thin. Translucent, most probably lytic cavities, filled with apoptotic bodies started to appear in this layer. In many follicles, pyknotic cells and apoptotic bodies were especially numerous in the granulosa region forming the base of the COC, and in the follicular fluid in the vicinity of the COC (Figs 6, 8). Some oocytes looked still healthy but many showed distorted shapes and in some of them the process of fragmentation became visible. Also the cumulus granulosa cells exhibited degenerative changes. The theca cells were slightly distorted and apart from the apical region, more loosely packed. However, apoptotic bodies were only occasionally visible there (Fig. 6).

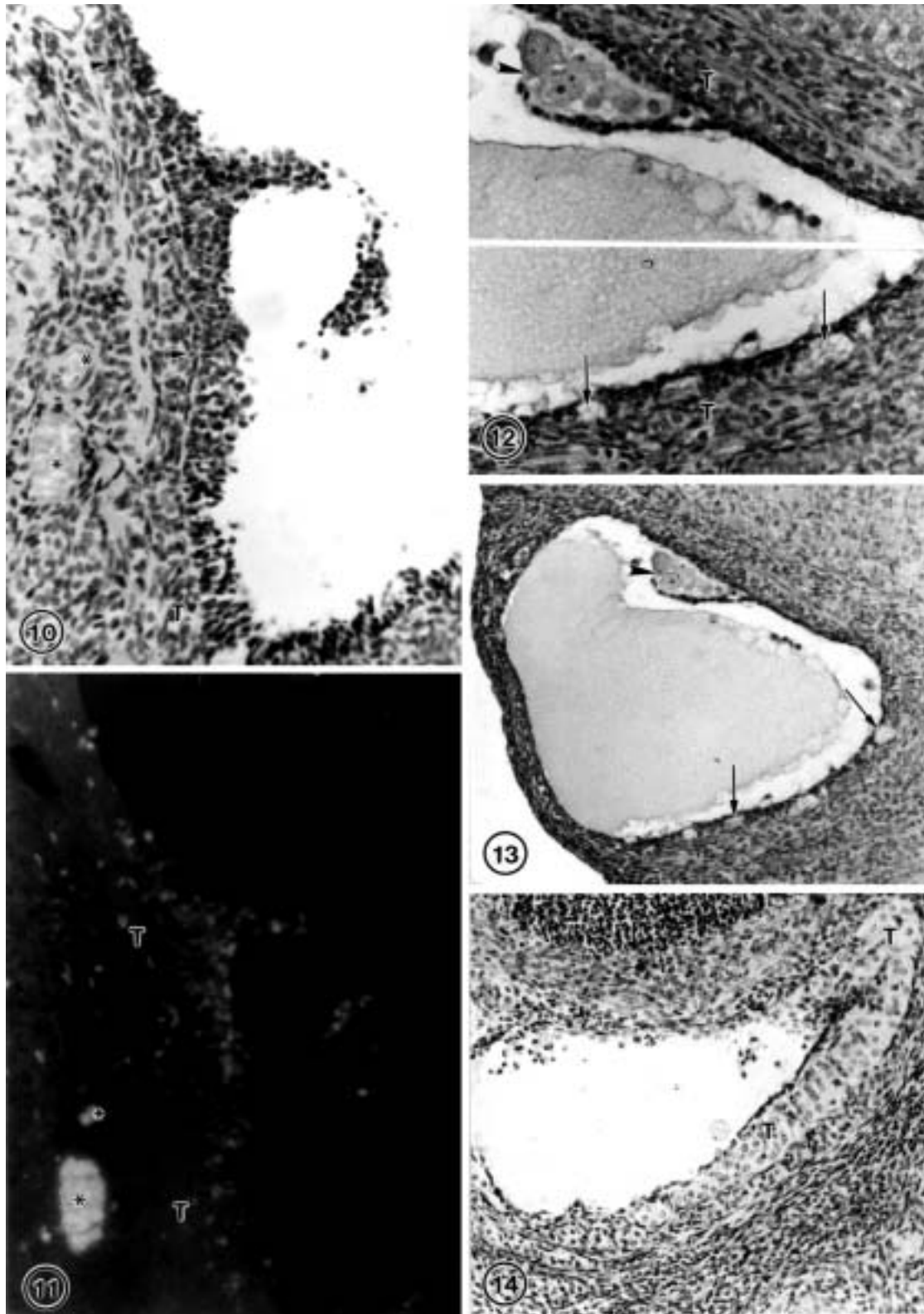
On days 8 and 9 of pregnancy, the investigated follicles were increasingly smaller and possessed very thin and often deformed wall. The granulosa layer contained numerous lytic cavities, which were empty or filled with remnants of apoptotic bodies (Figs 12, 13). In the COCs, the degenerating or fragmented oocytes were surrounded by a small number of pyknotic cumulus cells. In fragmented oocytes a few nucleoli were frequently present (Figs 12, 13). In many regions, especially on day 9, the boundary between the granulosa and theca cells became indistinguishable and the degeneration of the follicular wall was very advanced. However, only a single theca cells, or a very small group of them were slightly hypertrophied (Figs 10, 12, 13), in contrast to a strong hypertrophy observed in advanced stages of atresia in many antral follicles not belonging to the investigated group (Fig. 14).



**Figs 1, 2.** Day 5 of pregnancy. **Fig. 1.** Cross-section through a healthy preovulatory follicle showing thick granulosa layer, pseudostratified in the region adjacent to the basement membrane; note invagination of the theca interna (arrow), characteristic for the periovarian rat follicles. T - theca layer. H-E. **Fig. 2.** TUNEL staining. The granulosa layer with COC, and the theca layer exhibit almost negative staining for apoptosis. Nonspecific fluorescence is seen in numerous blood vessels present in the theca layer (T). **Figs 3-5.** Day 6 of pregnancy. **Fig. 3.** Fragment of a relatively healthy follicle; inset shows mitotic figures in COC (arrowheads). H-E. **Fig. 4.** Cross-section through follicle exhibiting more numerous apoptotic bodies in the granulosa layer, especially near COC (arrow), while theca cells (T) look unaffected. H-E. **Fig. 5.** TUNEL staining. Fragment of an atretic follicle with particularly numerous fluorescent apoptotic cells in the vicinity of COC (arrows). T - theca layer; AF - preantral atretic follicle. Figs 1 - 5:  $\times 120$ ; inset in Fig. 3:  $\times 500$ .



**Figs 6-9.** Day 7 of pregnancy. **Fig. 6** shows the same section of investigated follicle as that visible in Fig. 7. After TUNEL assay, the section was stained with H-E. Many apoptotic cells and bodies are seen at the periphery of the antral cavity and in the region linking COC with the follicular wall (arrows). Arrowheads point to appearing lytic cavities.  $\times 120$ . **Fig. 7.** TUNEL staining. The pattern of fluorescent cells and bodies is similar to that revealed by H-E staining in Fig. 6. Positive staining in the theca layer (T) results predominantly from the presence of blood vessels.  $\times 120$ . **Figs 8 and 9** show higher magnifications of COC regions present, respectively, in Figs 6 and 7. Note that H-E staining reveals even more apoptotic cells and bodies than TUNEL assay.  $\times 500$ .



**Figs. 10, 11.** Day 8 of pregnancy. **Fig. 10.** The same section of investigated follicle as in Fig. 11. After TUNEL assay the section was stained with H-E. Pyknotic cumulus cells form a thin and incomplete ring around the oocyte, while in the theca layer (T) only occasional apoptotic bodies (arrowheads) are visible. Asterisks mark blood vessels.  $\times 500$ . **Fig. 11.** In TUNEL stained section, a similar pattern of apoptotic cells as in Fig. 10 is visible apart from the theca layer (T), which also contains fluorescent erythrocytes. Asterisks mark blood vessels.  $\times 500$ . **Figs 12, 13.** Day 9 of pregnancy. **Fig. 12** shows two magnified fragments of the degenerated follicle shown in Fig. 13. Fragmented oocyte (arrowheads) contains several nucleoli. Arrows point to some of numerous lytic cavities. H-E. Fig. 12:  $\times 500$ , Fig. 13:  $\times 120$ . **Fig. 14.** Different type of late atresia, characteristic for some antral follicles not belonging to the investigated group of follicles. Note strongly hypertrophied theca cells (T) contrasting with theca cells visible in Figs 12 and 13. H-E.  $\times 120$ .

### *In situ* DNA labelling by TUNEL assay

On day 5 of pregnancy, the majority of the investigated follicles was almost negative for apoptotic immunostaining and showed only single cells exhibiting a positive TUNEL staining (Fig. 2). Only in some follicles a few apoptotic cells were visible in the granulosa layer adjacent to the COC. However, on day 6 of pregnancy many of the investigated follicles already contained numerous apoptotic granulosa cells. They were located predominantly in the region adjacent to the COC (Fig. 5). On day 7 of pregnancy, the number of TUNEL-stained cells was much higher than on day 6. Numerous granulosa cells linking the COC with the follicular wall and remnants of cells surrounding the antrum, especially in the vicinity of the COC, were brightly fluorescent, forming an area separating less labelled COC and the mural granulosa layer (Figs 7, 9).

On day 8 of pregnancy, the fluorescent structures were predominantly remnants of the COC and granulosa layer, while the theca cells showed much less fluorescence (Fig. 11). A positive TUNEL staining has never been observed in the oocytes. Negative controls, in which TdT was omitted, were negative, except for the erythrocytes, which revealed autofluorescence [30].

### Discussion

In the present study we investigated the effect of the hyperprolactinemic microenvironment on the development and consecutive atresia of antral follicles in the ovaries of pregnant rats. The obtained results revealed that the cohort of investigated follicles grew and differentiated slower, reaching the preovulatory size on day 5 of pregnancy, that is one day later than the equivalent follicles during the 4-day estrous cycle. Sporadic appearance of apoptotic cells and some morphological features, such as pseudostratification and undulation of the granulosa layer, observed on day 5 of pregnancy, suggested that the follicles were healthy and reached a late preovulatory stage characteristic of cycling rats at proestrus [28].

It is well established that prolactin inhibits follicular estradiol production [*e.g.* 12, 13, 31, 32]. Indeed, as previously stated, the investigated follicles contained much less estradiol than their cyclic counterparts [26, 27]. In the rodents, estrogens are essential for follicular growth and differentiation. They are also known to prevent apoptosis of rat preantral and early antral follicles [1]. The present findings indicate that much lower than physiological amount of follicular estradiol was sufficient to support the development of antral follicles to the late preovulatory stage, and this development, although lasting longer, was morphologically similar to that occurring during the estrous cycle [28]. As proved by Richards and Kersey [24], 100% of rats ovulated in

response to 20 IU of hCG when injected on day 5 of pregnancy. However, their studies, although very valuable, did not include histological analysis of the investigated follicles and they did not demonstrate that the follicles were healthy. It has already been established that preovulatory follicles showing even advanced atretic changes are able to ovulate in response to hCG treatment [28]. As suggested by Richards and Kersey [24], the lack of ovulation of the investigated follicles of pregnancy resulted most probably from a very low level of circulating estradiol, too low to trigger the preovulatory surge of gonadotropins.

In the present study, these unovulated, gradually degenerating follicles were thoroughly investigated during the subsequent days of pregnancy. Atretic changes, although not yet advanced, were detected on day 6 of pregnancy and concerned mainly the granulosa layer. They included the appearance of apoptotic cells and bodies in the antral region, especially near the COC and the bridge linking the COC with the follicular wall, while the COC region was only slightly affected. The number of apoptotic granulosa cells increased on day 7. Apoptosis detected by DNA fragmentation paralleled morphological changes visualised by the H-E staining. As demonstrated in the same follicular section (Figs 6-9) H-E staining revealed even more pyknotic cells and apoptotic bodies in the granulosa layer than the TUNEL staining, since the latter marks only late stages of cellular apoptosis. However, it was mainly due to the positive TUNEL staining that an area, formed by the fluorescent apoptotic bodies and granulosa cells adjacent to the antral cavity and linking the COC with the follicular wall, was so well visible on day 7. Such an area was much less distinguishable in the sections stained with H-E. The pattern of apoptosis found in the present study seems to reflect the existence and differences between the three various cell populations within the granulosa layer, with the antral granulosa cells, comprising also the cells located at the base of the COC, programmed to undergo apoptosis as the first ones, before the mural and cumulus granulosa cells.

Apoptosis in the rat preovulatory follicles has already been investigated by several authors. In the experimental models used, follicular atresia was induced by blocking the preovulatory luteinizing hormone surge [8, 19] or by injection of immature rats with eCG without subsequent treatment with hCG [21, 34]. However, in the majority of the mentioned studies the authors focused mainly on the time of the onset and course of atretic changes, paying less attention to the thorough analysis of apoptosis in the particular follicular compartments. More detailed analysis was provided by studies of Durlinger *et al.* [8] in which the authors followed histological and DNA apoptotic changes in the preovulatory rat follicles, ovulation of which was blocked by administration of an GnRH-antagonist. They described atretic changes of the

granulosa layer, which resembled those found in the present study, and stated that the cumulus cells were the last to become pyknotic. However, together with the other authors, they did not mention the presence of characteristic numerous apoptotic cells near the COCs. It is difficult to conclude whether the pattern of apoptosis found in the present study is exceptional for the preovulatory follicles degenerating in the hyperprolactinemic conditions or is it a more general feature.

In the majority of studies devoted to the follicular atresia, the emphasis was put on the apoptosis of the granulosa layer and the oocyte, while relatively little is known about apoptosis of the theca cells. However, there are reports confirming that apoptotic cell death occurs also, although to much less extent, in the theca cells of pig [22] rabbit [16] guinea-pig [15] and rat [21] follicles. The results of the present study are in agreement with those data, as the investigated cohort of follicles undergoing atresia showed only a small number of apoptotic cells in the theca layer in late stages of atresia. It is well recognised that the theca cells which undergo hypertrophy are predestined to survive and to transform into the secondary interstitial cells but the fate of the theca cells which neither become hypertrophied nor undergo apoptosis is unknown. Most likely they are also incorporated into the interstitium. Interestingly, a marked increase in apoptosis, and caspase-3 and -7 activity in the theca interstitial cells was observed in the studies of Yacobi *et al.* [34], in which the rat preovulatory follicles of eCG primed immature rats were cultured in the medium containing either LH or FSH. One of the possible explanations proposed by the authors was the facilitation of stigma formation and the subsequent rupture of the follicle.

As discussed in detail by Devine *et al.* [6], it remains still controversial whether the oocyte undergoes apoptosis. The authors conclude that oocyte death does not involve classically described apoptosis. However, Fujino *et al.* [9] and Perez *et al.* [23] observed a positive TUNEL reaction in all ovulated mouse oocytes exhibiting fragmentation and they regarded the process of fragmentation as a result of apoptosis. Fluorescent oocytes have not been observed in our present and previous studies concerning postovulatory degeneration of unfertilized rat oocytes (unpublished data). Our findings are more in agreement with those of Van Blerkom and Davis [33], who only rarely observed TUNEL fluorescence in ovulated (including fragmented) mouse oocytes. Nucleolar segmentation, accompanying oocyte fragmentation, shown in our present and previous studies [29], was also observed by Shinohara and Matsuda [25].

In conclusion, the present study provides new information on the development and subsequent apoptosis of antral follicles, growing in the hyperprolactinemic microenvironment and synthesising much lower than

physiological amount of estradiol. The obtained results indicate that such small amount of intrafollicular estradiol is sufficient to support a normal, according to morphological criteria, though slower, development of antral follicles up to the late preovulatory stage. A subsequent atresia is characterised by apoptosis of the granulosa cells, being the most pronounced in the vicinity of the COC, and by the lack of hypertrophy of the theca layer.

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