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PCNA and Ki-67 Expression in the Rabbit Ovary during Pregnancy.

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ABSTRACT

The cell proliferation markers PCNA (Proliferating Cell Nuclear Antigen) and Ki-67 (Proliferating Cell Antigen) were studied in the ovarian follicles and corpora lutea of rabbit ovary during pregnancy. Ki-67 is expressed in the nucleus of the proliferating cells during the active phases of the cell cycle. It cannot be identified in silent or resting cells (G_0). In contrast, PCNA expression was homogeneously dense, with nuclei showing variability in staining intensity. Some proportion of cells displaying the immunoreactivity in the cytoplasm as well. The quantity of PCNA-positive cells was higher than that of Ki-67, possibly due to their higher sensitivity. Both antibodies showed similar patterns in the different follicular categories. Granulosa cells of tertiary follicles showed a higher proliferation index. The theca interna presented lower proliferation index when compared with tertiary follicles. In the wall of secondary and tertiary follicles the majority of granulosa and theca cells were positively stained. Express sign of PCNA in granulosa cells begins upon the formation of a primary follicle, and its level of expression appeared to increase during the stages of follicular development. Cells of newly formed corpus luteum were PCNA positive. A higher number of Ki-67 positively stained cells in the CL were much greater in mid-pregnancy than that in late and early pregnancy. Ki-67 staining reached its maximum expression at the late stage of pregnancy**.** No obvious Ki-67 staining was detected in the early pregnancy**.** The highest percentage of positive cells was found in granulosa cells of atretic follicles. Tertiary follicles presented higher staining marked cells.

Keywords: Ovary, Pregnancy, Proliferation, PCNA, Ki-67

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INTRODUCTION

In the ovary, apoptosis occurs during both antral follicular atresia and luteal regression [1-3]. These normal cyclic processes have been attributed to decreasing gonadotrophic support [4].

The proliferation index was evaluated by PCNA and Ki-67. Both cellular proliferation markers showed strong staining of the cell nuclei. PCNA is required for DNA synthesis and appears to be involved in follicular growth. The Ki-67 protein is a cellular marker for proliferation. It is strictly associated with cell proliferation. It is present at low levels in acquiescent cells but is increased in proliferating cells. During interphase, the Ki-67 antigen can be exclusively detected within the cell nucleus.

The increase expression of PCNA in oocyte around the initiation of primordial follicle formation, and the observation that many of the oocytes die during primordial follicle assembly [5,6] suggested a role of PCNA in the regulation of oocyte fate; apoptosis or survival to form primordial and then growing follicles. PCNA has been reported to participate actively in regulation of apoptosis, either by promoting pro-apoptotic protein, or suppressing anti-apoptotic proteins [7, 8, 9]. This study aim to describe immunoreactivity localization of the cell proliferation markers (PCNA and Ki-67) in the rabbit ovary during pregnancy.

MATERIAL AND METHODS

Eighteen adult female healthy rabbits (Oryctolagus cuniculus f. Domestica), aged 14 to 20 weeks and weighing 2.2 to 3.5 Kg, were used in this study. They were obtained from a local farm of Faculty of Veterinary Medicine - Cairo University. The rabbits were housed in separate cages with daily illumination of 16 hours of light and allowed free access to water and rations. The female rabbits were mated with bucks of proven fertility. The day of mating was the 0-Day of pregnancy. The ovaries were then removed from 3 does in each of the following stages during pregnancy (Table-1).

Table-1: Selected stages for samples collection.

Early-Pregnancy	3^{rd} - 7^{th} Day
Mid-Pregnancy	14^{th}-21^{th} Day
Late Pregnancy	25^{st}-28^{th} Day

In each stage, the does were anaesthetized with an overdose of Nembutal sodium solution (Pentobarbital sodium injection, Abbott, USA) and killed. The ovaries were removed, fixed in 10% neutral buffered formalin and embedded in paraffin. Streptavidin − biotin method was used for detection of PCNA and Ki-67. After deparaffinization and rehydration in 96% ethanol, endogenous peroxide activity was blocked with 3% hydrogen peroxide in tris buffered saline (TBS pH 7.6) for 10 min and then rinsed with TBS. For antigen retrieval, the sections were boiled in citrate buffer for 15 min and left to cool at room temperature. After washing with TBS, sections were incubated for 5 min to block non-specific back ground staining. Primary antibodies to anti Ki-67 were added and the samples were incubated for 2 h at room temperature. After rinsing thoroughly with TBS, the slides were overlaid with secondary antibody for 10 min. The immunoperoxidase labeling was performed using streptavidin peroxidase for 10 min. Then aminoethylcarbazole was used as a chromogen for visualization of antibody binding. Finally, the sections were counterstained with Mayer's hematoxylin, cleared, and mounted for examination. Cells exhibiting the brown nuclear or cytoplasmic staining are considered positive for PCNA or Ki-67 respectively [10].

RESULTS

PCNA Expression

In Follicles:

The results demonstrated intense nuclear expression for PCNA in the oocytes of most follicles (Fig.1). In most primordial follicles, PCNA immuno-stain was present only in the nuclei of the oocytes. The surrounding squamous cells appeared stain-free (Fig.2). Intense PCNA staining was observed in the cell nuclei of small and medium follicles throughout the ovary. In secondary and tertiary follicles the majority of granulosa and theca

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cells appeared positive (Fig.3&4). PCNA staining was also observed in the granulosa cells of growing follicles. In antral follicles, the granulosa cells of the cumulus oophorus stained for PCNA (Fig.5) more than those near the basement membrane. During mid and late pregnancy, occasionally, the nuclei of oocytes found in medium and large follicles stained for PCNA, as did a few of the thecal cells surrounding the follicles. In late pregnancy, multiple growing follicles showed PCNA immunostaining. The large antral follicles revealed some apoptotic negatively stained granulose cells. Little or no staining was detected in the granulosa cells of the atretic follicles (Fig.6).

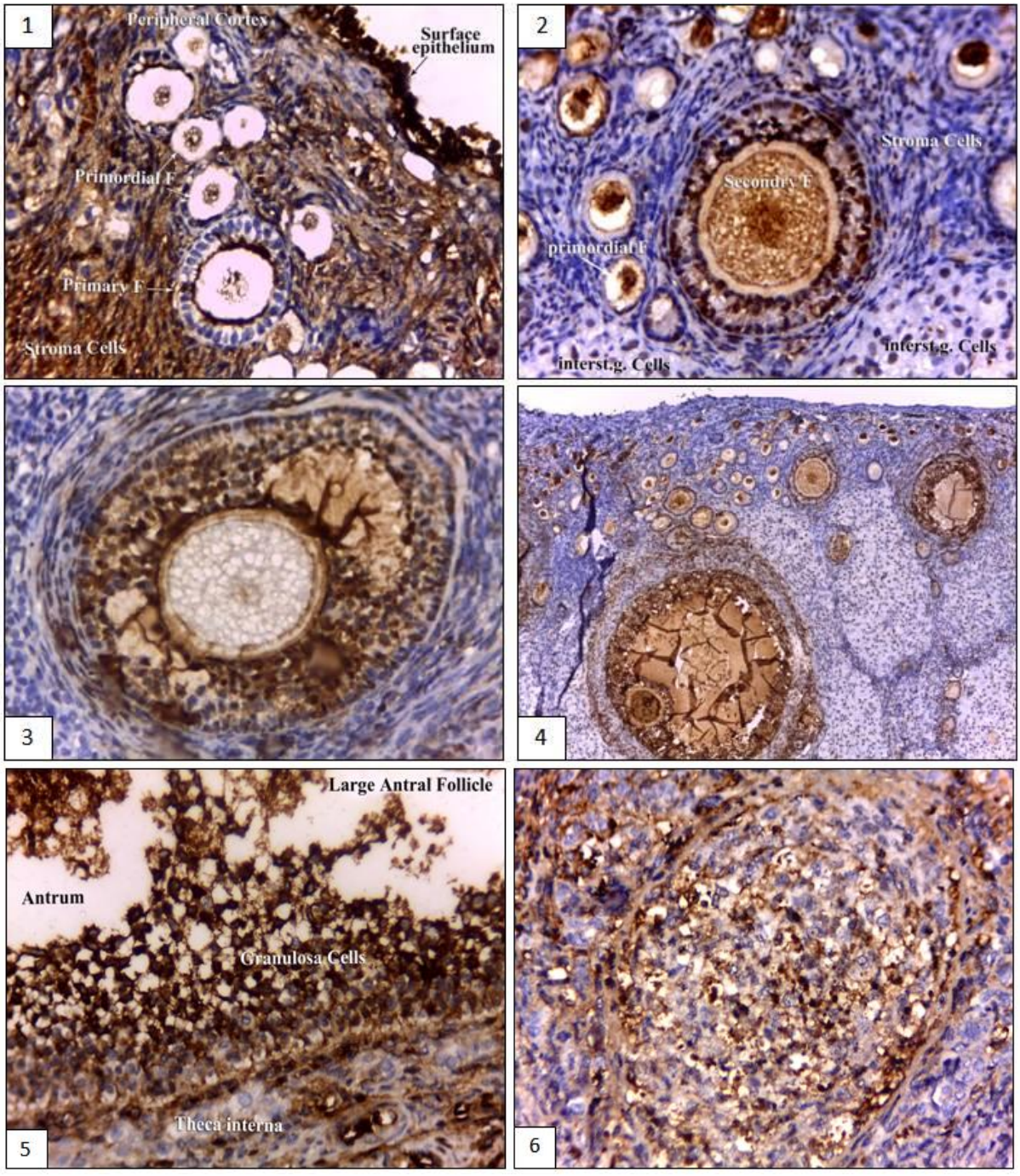

Fig 1: Intense nuclear immunostaining for PCNA in the oocytes of primordial and primary follicles. The squamous pregranulosa cells appeared stain-free X10

Fig 2: Primordial and secondary follicles showing PCNA expression in the oocytes and the pregranulosa cells.Both the zona pellucida and the surrounding stroma are unreactive. X16

Fig 3: PCNA immunostained expression in the wall of growing or tertiary follicle. X16

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Fig 4: PCNA staining is clear in the granulosa cells of follicles follicle. X16
Fig 5: In antral follicle, during mid and late pregnancy, the granulosa cells close to the oocyte and the cells of the cumulus oophorus stain for PCNA. Notice the negative nuclei of the cells near the basement membrane. PCNA expression is seen in a few of the thecal cells surrounding the follicle. X25.
Fig 6: Large atretic follicle showing PCNA expression. X25.

Corpora lutea:

In early pregnancy newly formed corpora lutea were recognized. They were composed of large lutein cells. Most of these cells were negatively stained (Fig.7). Few scattered lutein cells, positive for PCNA, were radiating from the center of the CL. They showed faint brown reaction (Fig.8). They were distinguished in-between the unstained cells. During mid and late-pregnancy most lutein cells were PCNA negative. However, intense PCNA expression was found in the endothelium of the blood capillaries in-between these lutein cells (Fig.9).

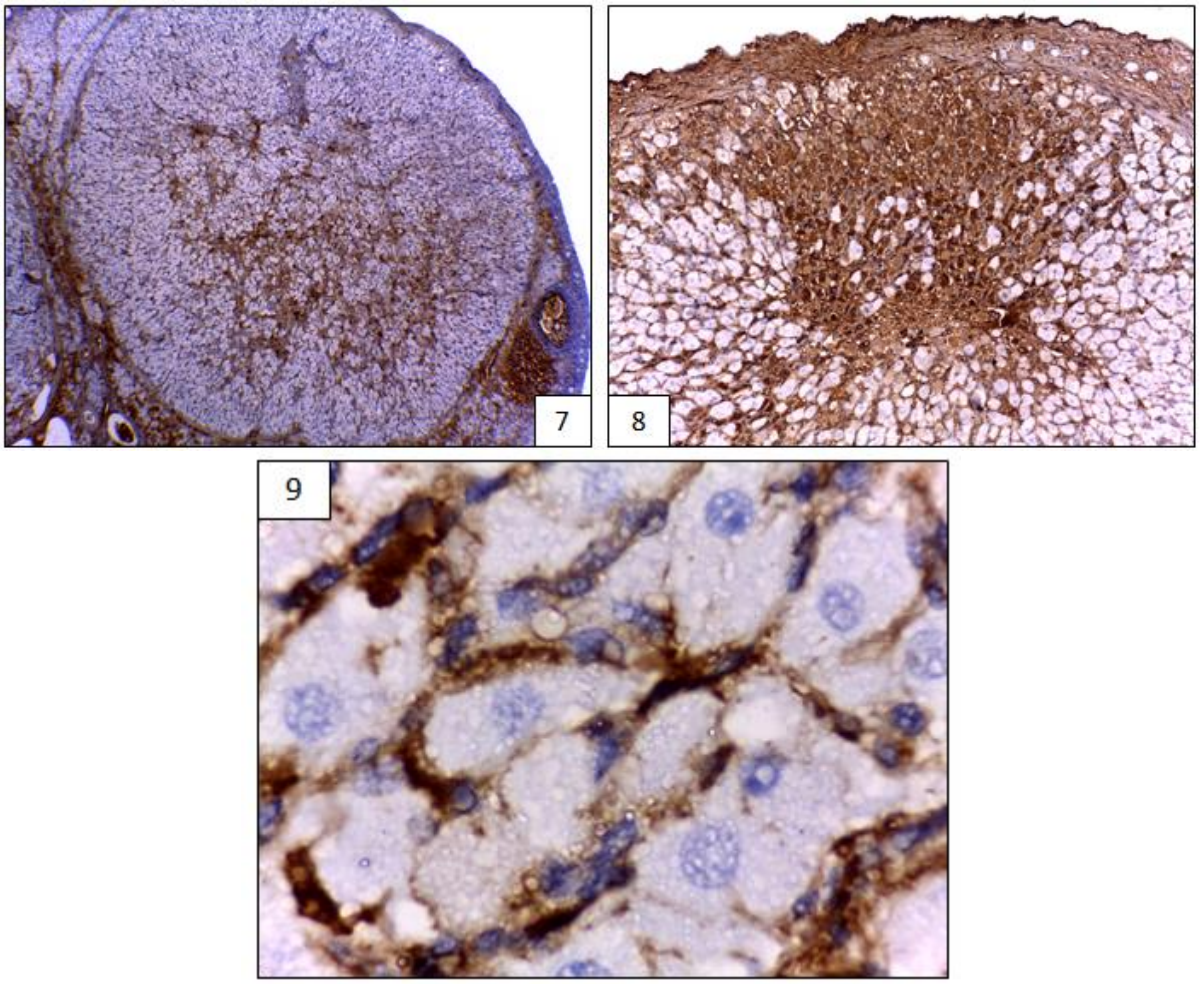

Fig 7: In early pregnancy newly formed corpus luteum is recognized. X4
Fig 8: Section in the corpus luteum in early pregnancy composed of large lutein cells. Most of these cells are negatively stained. Few scattered lutein cells are positive for PCNA antibody binding. X10
Fig 9: Section in the CL during mid-pregnancy showing intense PCNA expression in the endothelium of the blood capillaries in between the lutein cells. X100

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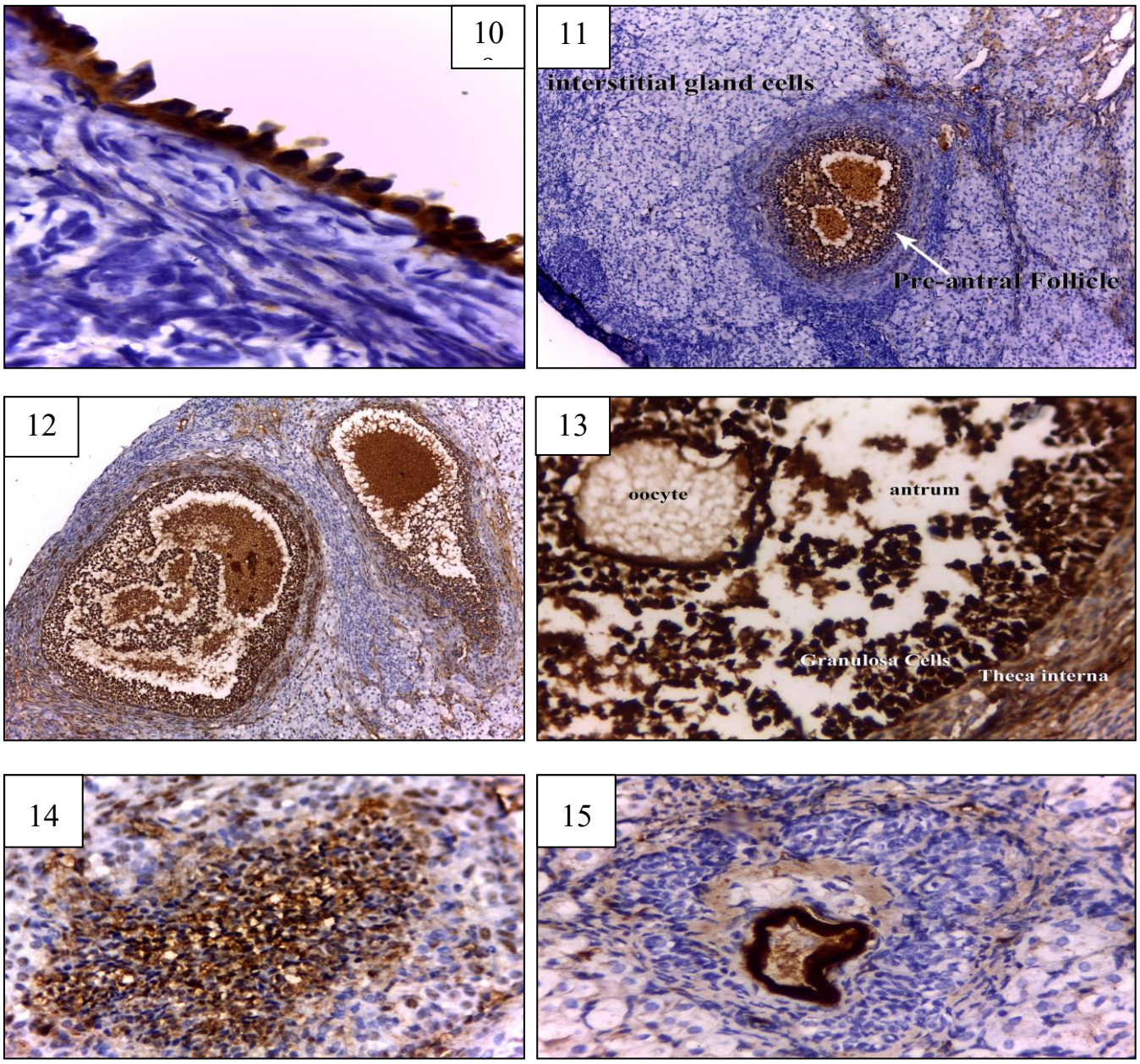

Fig. 10: Ki-67 antigen is predominantly localized in the ovarian surface epithelium. An intense expression is observed in the cytoplasm and nuclei of the surface epithelial cells. X25
Fig. 11, 12: Tertiary follicles showing higher staining marked cells. X10
Fig. 13: In antral follicles the theca interna have a few positive cells. X10&X16
Fig.14: Atretic follicles express high Ki-67 expression. Notice the intense expression in the corrugated zona pellucid. X16
Fig.15: Stromal cells react negatively with Ki-67 which shows no cell proliferation in the ovarian stroma of rabbit ovary during pregnancy. X16

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Ki-67 Expression

In early pregnancy, Ki-67 antigen was predominantly localized in the ovarian surface epithelium (Fig. 10). An intense positive Ki-67 expression was observed in the nuclei and cytoplasm of the surface epithelial cells.

In Follicles:

Ki-67 marker reached its maximum expression toward the end of pregnancy**.** During mid- and late-pregnancy tertiary follicles presented higher staining marked cells (Fig. 11, 12). An intense expression of Ki-67 was observed in granulosa cells of the antral follicles during the second half of pregnancy. Also in the antral follicles the theca interna had a few positive cells in late-pregnancy (Fig. 13). No differences were found in the theca externa between follicular categories throughout pregnancy. Atretic follicles presented high levels of Ki-67 (Fig.14) especially in granulosa cells in late-pregnancy.

No obvious Ki-67 staining was detected in the early pregnancy**.**

Stromal cells reacted negatively with Ki-67 (Fig. 15), which showed no cell proliferation in the ovarian stroma of rabbit ovary during pregnancy.

The number of cells which showed signs of apoptosis in the ovarian follicles was low on early and mid-pregnancy.

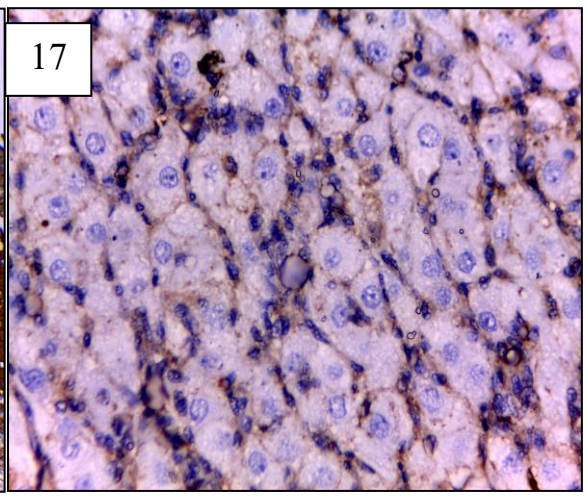
Corpora lutea:

Ki-67 expression in the CL was observed in the proliferative phase. It was suppressed in the secretory phase. Little expression was observed throughout pregnancy and was suppressed during postpartum.

In the first week of pregnancy, most of the lutein cells were Ki-67-negative (Fig.16).

During mid-pregnancy there were a few positive cells in the CL (Fig. 17). The blood capillary's endothelium, which were scattered in-between the lutein cells, were the only structure which showed positive Ki-67 expression (Fig.18). A high number of the interstitial gland cells in the examined ovary appeared Ki-67-negative) Fig. 19). The staining expression was mainly localized in the spaces in-between the lutein cells (Fig. 20).

A higher number of Ki-67-positive lutein cells were observed during mid-pregnancy than in late pregnancy (Fig. 21). The staining expression was mainly found in the cytoplasm of a few scattered individual cells**.**



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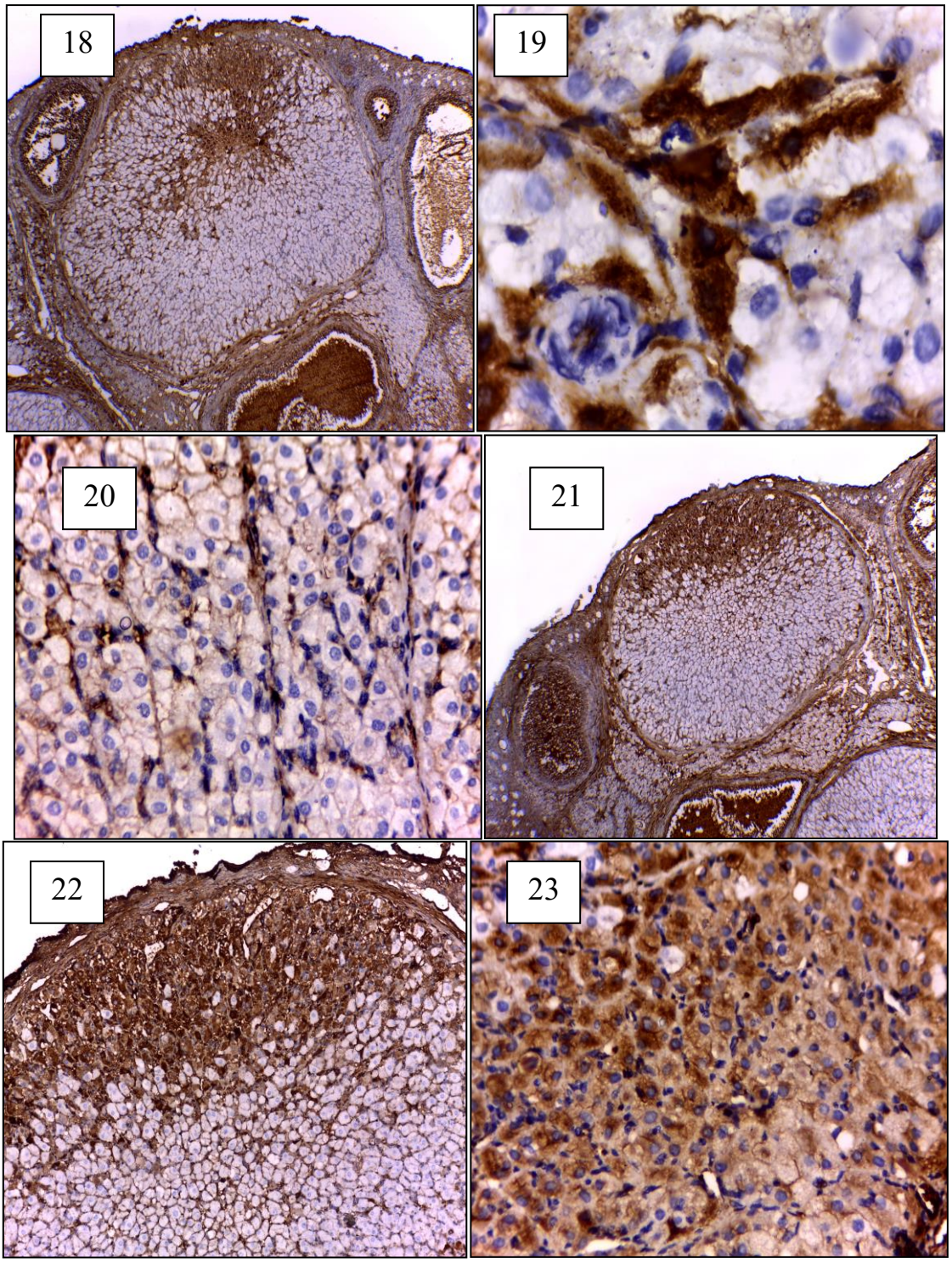

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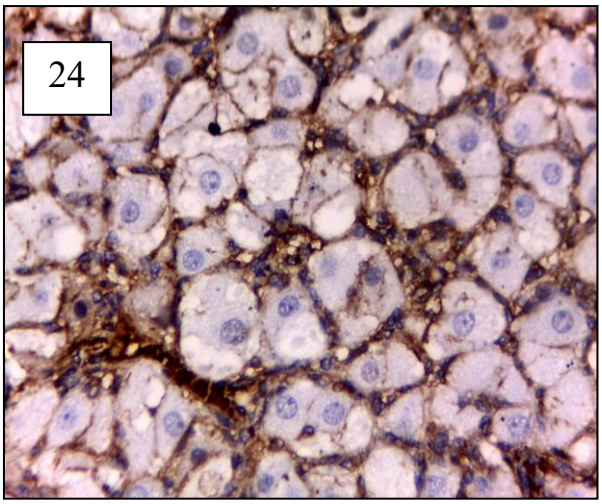

Fig 16: In early pregnancy the majority of the lutein cells are Ki-67 negative. Only a few cells are showing Ki-67 expression. X4
Fig 17: Most of the lutein cells of the corpus luteum during early pregnancy are Ki-67-negative cells. Notice the faint expression in the cytoplasm of the endothelial capillaries. X100
Fig 18: relatively moderate cells exhibiting Ki-67 expression. These stained cells are scattered in the CL during mid-pregnancy. The remaining lutein cells are unstained. X4
Fig 19: Higher magnification of a portion of the CL showing the brown Ki-67-expression. It is mainly localized in the capillary endothelium found in the tiny spaces in-between the lutein cells of the CL during mid-pregnancy. X100
Fig 20: A high number of Ki-67 negative interstitial gland cells are seen in the ovary during mid-pregnancy. Notice the clear expression in the cytoplasm of the endothelial capillaries found in-between. X25
Fig 21: Obvious Ki-67 positive expression scattered in the cells of the peripheral zone of the CL towards the ovarian surface during late pregnancy. X4
Fig 22: Section in the CL during late- pregnancy exhibiting an intense Ki-67 expression in peripheral portion. The expressing lutein cells showed deep brown color in their cytoplasm. X16
Fig 23: An intense Ki-67-expression in the CL during late- pregnancy. The expressing lutein cells showed deep to faint brown color in their cytoplasm but not in their nuclei. X25
Fig 24: Section in the CL during late-pregnancy. The Ki-67 staining expression is mainly localized in the spaces in-between the lutein cells where the blood capillaries and fine delicate fibrous tissue are found. X100

DISCUSSION

In the present study we observed significant differences between apoptosis and proliferation rate in rabbit's ovarian follicles. Cellular proliferation quantified by means of the immunodetection of PCNA and Ki-67 was higher in the granulosa cells of the healthy follicles. In theca cells, proliferation was lower in antral follicles than in tertiary follicles. Atretic follicles of all categories presented scarce proliferation indeces with Ki-67. The highest level in proliferation was found in atretic follicles with PCNA. This is probably because these follicles still express this protein although some of their cells are already in elimination through the programmed cell death. Similar findings were described by [11].

As seen presently, during the luteal phase, the central and peripheral region of the corpus luteum of the rabbit showed scattered Ki-67 positive cells. A higher number of Ki-67-positive lutein cells were observed during mid-pregnancy than in late pregnancy. Contrary to this in the middle and late luteal phases, no cells containing Ki-67 were identified in the corpus luteum of human and in the interstitial glands [11]. However, the stroma still expressed Ki-67.

Recently, it was reported that bitches luteal cells at different stages of pregnancy did not express the Ki-67 factor but was present in all ovarian follicular cells [12]. The absence of Ki-67 antigen points to the absence of growth signals.

Moreover, proliferation index in luteal cells (reflected as mitotic cells) was increased at late pregnancy that could be related with a mechanism of self-renewal and attempting to survive. This means that the gland stimulates a survive mechanism trying to maintain their lifespan. This result suggests that luteal cells at late gestation may have higher capacity of hormonal synthesis. The Ki-67 is a cell proliferation indicator, so mitosis figures observed in H&E stained sections (unpublished study) can be related to this marker. In this view, signs of apoptosis were also observed during the morphological examination and

were defined by chromatin condensation and enhanced cytoplasmic eosinophilia, as well as karyolysis and karyokinesis. We did not measure the expression of the Ki-67 marker for apoptosis. Yet, the morphological signs of apoptosis observed in luteal cells suggest that there is perhaps a dynamic mechanism of cellular proliferation and degeneration during CL's survival period.

Moreover, the difference between interstitial gland cells and ovarian stroma during the secretion and the proliferation phases in pregnancy may represent the difference in receptors of sex hormones. In this respect, the expression of receptors of estrogen and progesterone in glandular cells is regulated negatively during the secretion phase, whereas the expression of progesterone receptors in stromal cells continues positively during the secretion phase [13, 14].

PCNA is an auxiliary protein of DNA polymerases necessary for DNA synthesis and is thus a marker of proliferation [15&16]. However, PCNA expression in oocytes cannot be attributed to cell proliferation since the oocyte is arrested meiotic ally [17].

The significance of the PCNA expression in the oocyte is unknown and needs to be elucidated. It has been demonstrated that PCNA is involved in DNA repair [16, 18] which suggests that PCNA may be expressed by cells that are not proliferating. Correspondingly, it has been hypothesized that although no new DNA synthesis takes place in the growing oocyte, it is possible that DNA polymerases are activated to repair potential damage to the genetic material in the oocytes selected to grow [19].

The presence of PCNA in oocytes of primordial follicles observed in this study suggests a role for this protein in earlier stages of folliculogenesis.

It appears that in oocytes of primordial follicles, PCNA is expressed at lower levels compared to the more mature follicles and was not detected in the study by [19], because ovaries were fixed in Bouin's solution.

In agreement with [20, 21] strong PCNA expression of oocyte nuclei in rabbit ovary makes oocytes and primordial follicles easy to distinguish from other labeled cells. Thus, in PCNA-stained sections, follicles of all degrees of maturity are much easier to distinguish from ovarian background. These results demonstrate that PCNA is a useful marker for ovarian follicle counts in rabbits.

In the present study the granulosa cells of the small follicles do not stain for PCNA. Granulosa cells in large follicles stained for PCNA.

According to [22], the granulosa cells in antral follicles can be divided into two groups, the cumulus and mural granulosa cells. Correspondingly, the present study with PCNA localization revealed more cumulus granulose cells staining for PCNA as compared to the mural granulosa cells. Strong PCNA expression of oocyte nuclei in combination with their large size makes oocytes easy to distinguish from other labeled cells. Also the ability to detect and identify primordial follicles is particularly enhanced. Thus, in PCNA-stained sections, follicles of all degrees of maturity are much easier to distinguish from ovarian background than in H&E-stained sections.

Our results demonstrated that PCNA is a useful marker for ovarian follicle counts in rabbits. The ability to mark oocyte nuclei distinctly with PCNA antibody significantly increases speed and accuracy of counting.

CONCLUSION

The large antral follicles revealed some apoptotic negatively stained granulose cells. Little or no staining was detected in the granulosa cells of the atretic follicles. During mid and late-pregnancy most lutein cells were PCNA negative. The number of the Ki-67-positive cells in the CL was much greater in the mid-Pregnancy phase than in the late and early pregnancy. The capillary's endothelium scattered in-between the lutein cells showed positive Ki-67 expression.

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