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Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the pig ovary

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Abstract: The aim of the study was to determine the expression of proliferating cell nuclear antigen protein (PCNA) in the pig ovary. The localization of PCNA was demonstrated in paraffin sections of pig ovarian tissue using primary mouse monoclonal anti-PCNA antibody. In primordial follicles, no remarkable staining for PCNA either in granulosa cells or in the oocytes was observed. In primary to secondary follicles, positive staining in oocytes and in some granulosa cells was detected. The advanced preantral and particularly actively growing small to large antral follicles showed extensive PCNA labeling in the layers of granulosa and theca cells and in the cumulus cells encircling the oocyte. PCNA labeling was expressed in nuclei of oocytes in preantral and small antral follicles. In atretic follicles, the level of PCNA protein expression was dependent on the stage of atresia. Follicles demonstrating advanced atresia showed only limited or no PCNA labeled granulosa and theca cells. The results of the study demonstrate that follicular growth and development in pig ovary may be effectively monitored by determining the granulosa cell expression of PCNA.

Key words: PCNA - Ovary - Granulosa cells - Oocyte - Pig

Introduction

Proliferating cell nuclear antigen (PCNA), an essential regulator of the cell cycle, is 36 kDa molecule which is highly conserved among species. It has been shown that PCNA serves as a co-factor for DNA polymerase delta in S-phase and is involved in DNA damage repair during DNA synthesis [1, 19]. The temporal pattern of PCNA expression makes it a useful tool to study cell proliferation. It starts to accumulate in G1 phase of the cell cycle, reaches the highest level during the S phase and decreases during G2/M phase [11].

Growth and development of ovarian follicles is characterized by marked processes of granulosa cell proliferation and differentiation. The earliest stage of follicular growth is marked by very slow proliferation of granulosa cells [9]. Granulosa cells of growing follicles start to be responsive to gonadotrophin stimulation and secrete increasing amounts of estradiol [16]. Exposure to gonadotrophins and estradiol initiate the phase of rapid granulosa cells proliferation [2, 15]. It has been shown in mouse and rat studies that the most rapid granulosa cell proliferation takes place at the time of antrum formation. The final phase of follicular growth accompanied by maximal production of estradiol is characterized by significantly diminished cell proliferation [8]. However, the vast majority of follicles which enter the growing pool do not reach preovulatory stage and undergo the process of atresia which is characterized by suppressed DNA synthesis and granulosa cell proliferation [6, 7, 12].

PCNA expression has been localized in ovary in association with the studies of initiation and early events of follicular growth *in vivo* in rat [14] and *in vitro* in cow [21] and baboon [20]. To our knowledge, there is no study of proliferation process throughout the follicular development in pig ovary. Determination of PCNA expression was used as a marker of proliferation in the studies of GnRH agonist action on pig granulosa cells *in vitro* [17, 18] and in the study of cell proliferation and apoptosis in pig follicles xenotransplanted to immunodeficient mice [10].

The aim of our study was to determine by immunohistochemistry the PCNA expression pattern throughout the follicular development in pig ovary in order to show the rate of granulosa cell proliferation and atresia at different follicular stages.

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Materials and methods

Animals and tissue handling. Porcine ovaries from prepubertal gilts were obtained from a local abbatoir and transported to laboratory in a thermo-container filled with PBS. Ovarian tissue was cut into small pieces and fixed in Bouin's solution for 6-8 h. After the fixation, the specimens were dehydrated by increasing concentrations of ethanol (70-90-95-100%) and following the xylen bath they were embedded in paraffin (Merck, Germany). The pieces of ovarian tissue were serially sectioned at 7 μm and sections were mounted on silan-coated slides.

Immunohistochemistry. Prior to PCNA localization, sections were deparaffinized by washing with xylene and rehydrated in decreasing concentrations of ethanol and PBS. In order to block endogenous peroxidase activity, the sections were incubated for 10 min in 0.5% hydrogen peroxide. After washing with PBS, sections were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Nonspecific binding was blocked by incubating the sections with 10% inactivated goat serum. Monoclonal mouse anti-PCNA, clone PC 10 (Dako, Denmark) diluted 1:100 in PBS+0.2% BSA was used as the first antibody. To eliminate nonspecific binding, non-immune goat serum was added to the primary antibody. Sections were incubated overnight at 4°C. The next day, sections were washed with PBS and DAKO LSAB R+ peroxidase kit (DAKO, Denmark) was used to detect the labeling. After washing with PBS, the second LINK antibody was applied for 30 min at room temperature followed by streptavidin-peroxidase for 30 min. The binding of primary antibody was visualized using diaminobenzidine (DAB) for 3-5 min. After washing with distilled water, sections were counterstained with hematoxylin (Vector Laboratories). Subsequently sections were washed with tap water, acetic acid and redistilled water. Finally, sections were plunged in 1.5% NH_4OH in ethanol solution, rinsed with water, dehydrated in ethanol and toluene and mounted in the Depex medium (Agar Scientific Ltd). PCNA labeling was examined using Leica DMLB microscope and the images were recorded by Leica DC 200 digital camera.

Results

Using immunohistochemical labeling we have assayed expression of proliferating cell nuclear antigen (PCNA) in the ovarian follicles in pig. At the earliest follicular developmental stages, the primordial follicles, no remarkable PCNA staining was found in oocytes or individual granulosa cells (Fig. 1A). In primary to secondary follicles (Fig. 1B) PCNA immunoreactivity was detected in few granulosa cells as well as in the nuclei of the oocytes. Small number of PCNA labeled cells may suggest that proliferation activity is still low in this category of follicles in pig. A remarkable increase in the number of immunoreactive granulosa cells was found in more advanced preantral (Fig. 1C) and particularly small antral (Fig. 1D) follicles. Theca interna was already morphologically differentiated in small antral follicles and also showed PCNA cell immunoreactivity (Fig. 1D). Oocyte nuclei in these categories of follicles were PCNA-positive. Further follicle enlargement was correlated with enhanced PCNA immunoreactivity in granulosa cell layers. Theca interna was morphologically differentiated and showed increased number of PCNA labeled cells (Fig. 1E,F).

In the large, preovulatory follicles (Fig. 2A), granulosa cell layers still showed intense PCNA immunopositivity. There was no marked PCNA staining in oocytes but PCNA-positive cells were observed in differentiated cumulus cells (Fig. 2B).

The progress of atretic changes in the follicles is accompanied by decrease in the number of PCNA labeled granulosa and theca cells. In comparison with healthy follicle (Fig 2C), markedly decreased number of PCNA immunoreactive cells in granulosa cell layers was observed in early atretic follicles. Accumulation of granulosa cells with pycnotic nuclei was observed in the follicular antrum (Fig. 2D). Only few PCNA stained cells were found in theca. The progress in atretic changes in the follicles was accompanied by further decrease in PCNA immunoreactivity in granulosa and theca cells (Fig. 2E). Theca interna was morphologically less distinguishable and showed no PCNA labeled cells. The terminal atresia was characterized by disruption of basal lamina and disappearace of theca layers. Only a few faintly PCNA immunostained cells were seen in such follicle (Fig. 2F).

Discussion

The results of the present study support the previous suggestions that PCNA may serve as a reliable marker of proliferation processes occuring in the ovary. To study the earliest events in folliculogenesis, the presence of PCNA was detected by immunohistochemistry in the rat [14] and mouse [2] ovary. As a marker of proliferation PCNA expression was analyzed in studies aimed at initiation of primordial follicle growth *in vitro* in cattle and baboon [20, 21]. More recently, PCNA expression was proposed as a marker for ovarian follicle counts in toxicological studies [13] and bioassays [17, 18] and in studies focused on intercellular communication and impaired folliculogenesis in mouse ovary [5].

Herein the PCNA expression carried out by immunohistochemisty on pig ovarian tissue sections is demonstrated. In our study, we did not observe marked PCNA expression in granulosa cells in primordial follicles. In rat [14], PCNA immunoreactivity was not demonstrated in primordial follicles but in case when at least one enlarged granulosa cell was observed in follicle cross-section, the PCNA immunoreactivity was detected. Additionally, the authors observed that PCNA positive granulosa cells of early primary follicles were not always enlarged which indicated that PCNA was expressed at the earliest stages of follicular growth and was sometimes present in granulosa cells before they committed enlarging. This observations differs from the results obtained by Wandji *et al.* [21] in bovine primordial follicles cultured *in vitro*. The

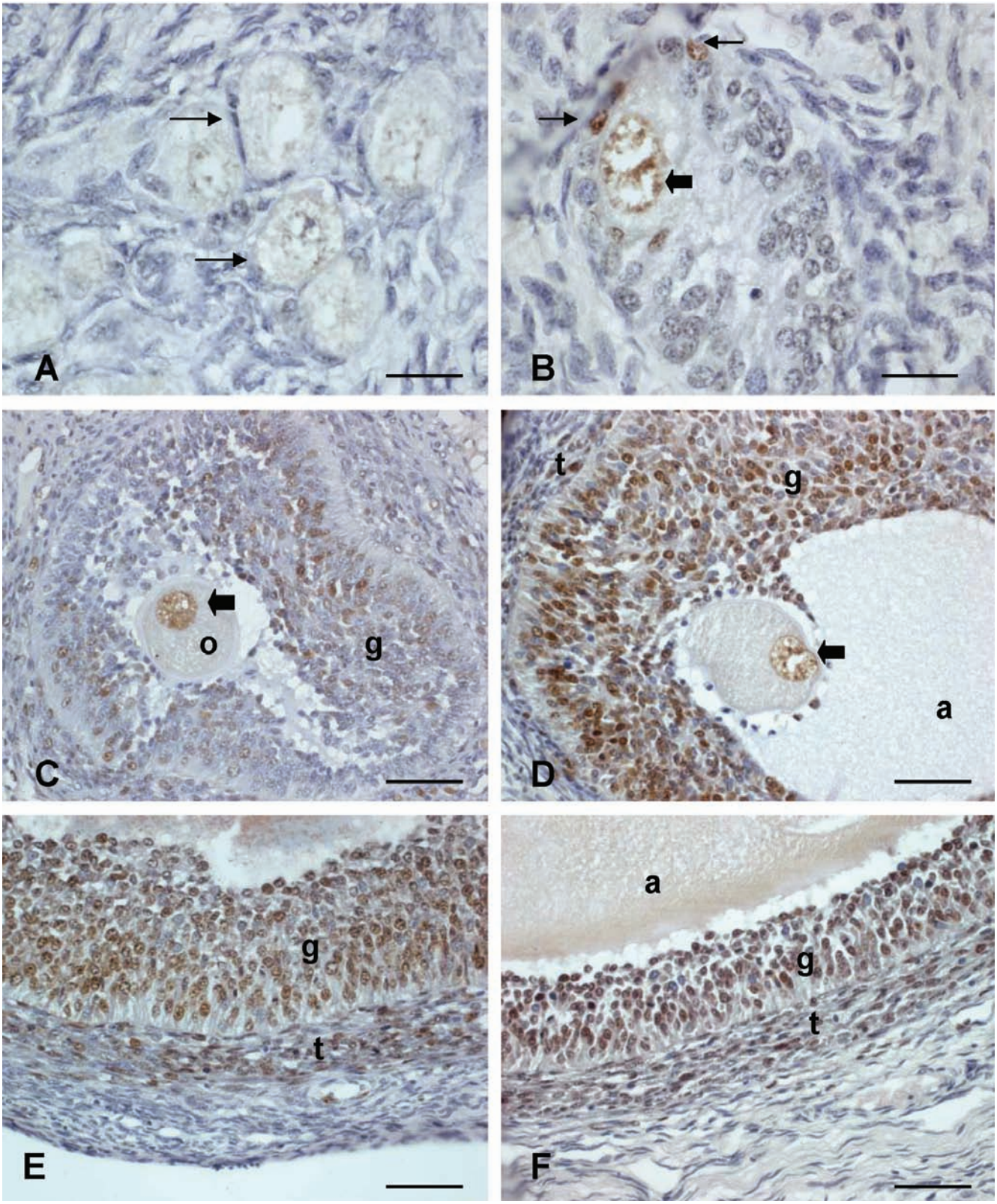

Fig. 1. Immunohistochemical detection of PCNA in pig ovary. No remarkable staining was localized in primordial follicles (arrows), (**A**). In primary to secondary follicles (**B**), PCNA expression was localized in nuclei of oocytes (thick arrow) and in some granulosa cells (arrows). In advanced preantral (**C**) and particularly actively growing small to large antral follicles (**D-F**) extensive PCNA labeling was localized in the layers of granulosa and theca cells. PCNA staining was expressed in nuclei of oocytes (thick arrows) in preantral and small antral follicles. Scale bars: 20 µm (A, B); 50 µm (C-F). Symbols: g - granulosa, t - theca, o - oocyte, a - antrum.

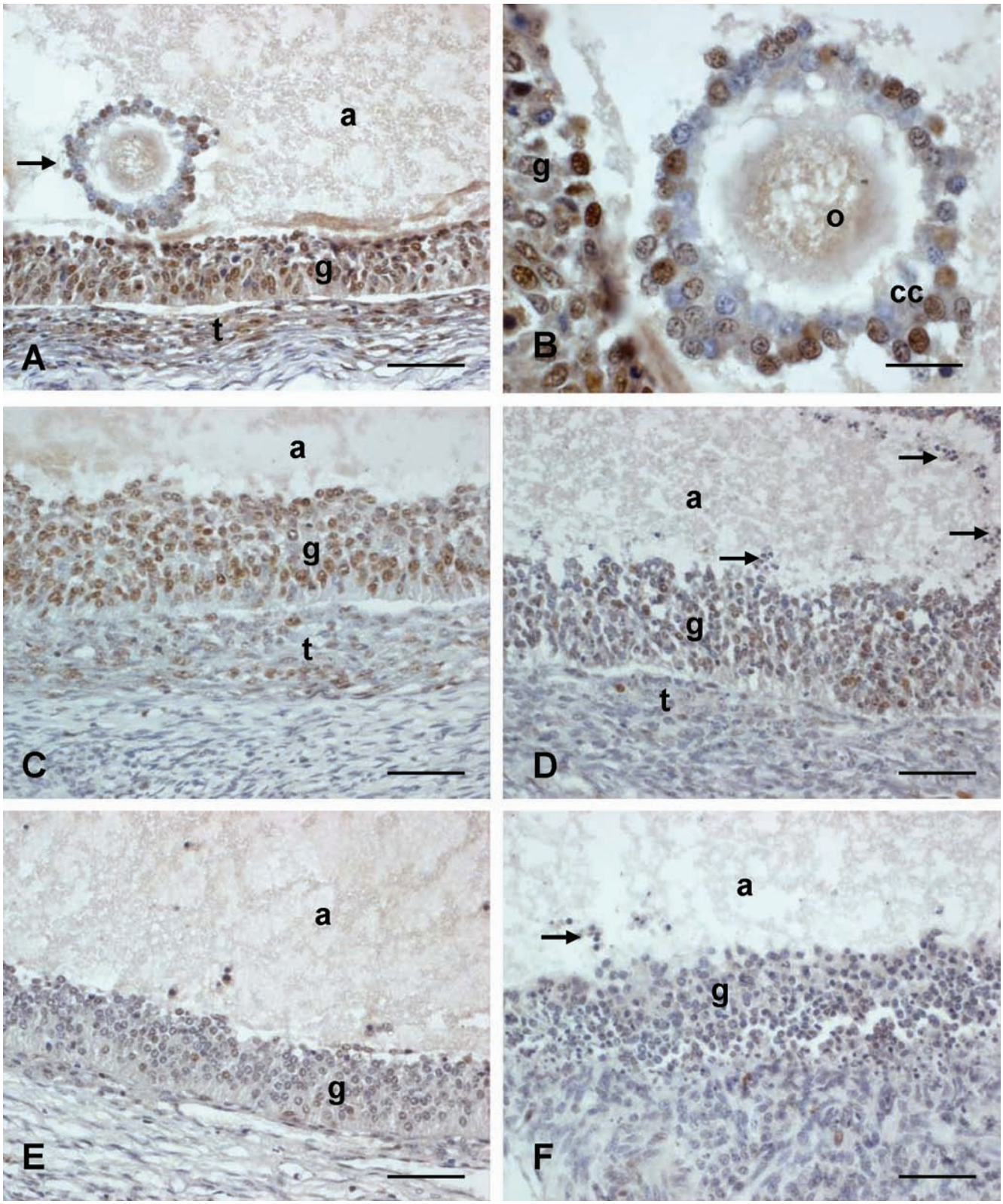

Fig. 2. Patterns of PCNA expression in large healthy and atretic follicles. Figures **A-C** demonstrate enhanced number of PCNA labeled cells in granulosa, theca and cumulus cells (arrow) in healthy follicles. Early atretic follicles (**D**) were characterized by significantly decreased number of PCNA labeled granulosa cells, disappearance of PCNA labeling in theca cell and accumulation of granulosa cells with pycnotic nuclei (arrows) in the follicular antrum. In follicles with advanced atresia (**E, F**) only few or no PCNA labeled cells were seen in granulosa layers. No PCNA labeling was seen in theca layer which was gradually degraded and became morphologically indistinguishable (F). Scale bars: 20 µm (B); 50 µm (A, C-F). Symbols: g - granulosa, t - theca, o - oocyte, cc - cumulus cells, a - antrum.

authors noted PCNA expression in some of granulosa cells in primordial follicles before they started to become cuboidal.

In the present study, we did not observe marked PCNA staining in the oocytes of pig primordial follicles. Similar results were obtained in other investigated species. In primordial follicles the oocytes are quiescent, do not replicate genomic DNA and metabolic activity has not been yet initiated. Interestingly, we have detected PCNA expression in nuclei of pig oocytes of primary/secondary to early antral follicles. This is consistent with observations previously achieved in rat and cow [14, 21]. In primary follicles, oocytes commence growing and intensive metabolic processes occur. According to Wandji *et al.* [21], the PCNA presence in growing oocytes is not related to cell proliferation as oocytes are meiotically arrested and do not replicate nuclear DNA. However, its appereance in oocytes may be related to specific role of PCNA being the auxiliary protein of DNA polymerase delta which is involved in the mechanisms of DNA repair [20, 21]. The authors suggest that DNA polymerase may be activated in growing oocytes to repair the genetic material during transcription process.

Likewise in rat studies, in pig preantral follicles PCNA positive staining was observed in a number of granulosa cells. According to Hirshfield [9], the most rapid granulosa cell proliferation occurs in large preantral follicles just before the antrum formation. At that stage of development, follicles start to enlarge rapidly and extensive PCNA immunoreactivity reflects the high proliferation rates in granulosa and theca cells of healthy antral follicles. Parallelly, as a result of response to gonadotrophin stimulation, granulosa and theca cells develop steroidogenic functions and increasing amounts of steroid hormones are produced. The high rate of steroidogenesis in large follicles is accompanied by decrease in cell proliferation rate.

During the follicular development, more than 99% of follicles undergo the proccess of atresia while only a few reach the final size and ovulate. Using the PCNA as a marker, Feranil *et al.* [3, 4] found in buffalo and cattle ovary significantly higher frequency of PCNA labeled cells in healthy follicles than in the early and advanced atretic follicles. Reduced number of PCNA immunoreactive cells during atresia was also found in theca.

In our study in pig ovary we observed that PCNA expression negatively correlated with the stage of follicular atresia. In follicles displaying the signs of early atresia, decreased number of PCNA labeled granulosa cells and disappearance of PCNA labeling in theca cells were seen. Granulosa cells with pycnotic nuclei appeared in the follicular antrum. The observation of diminished proliferative activity in apparently morphologically homogeneous layers of granulosa cells in early atretic follicles may be important from the point of view of *in vitro* granulosa cell studies. In our experiments *in vitro,* we often encountered a low viability of granulosa cells in suspensions obtained from seemingly healthy follicles. This might be caused by increased number of granulosa cells being out of the cell cycle. In advanced atretic follicles, extensive pycnotic changes and only few or no PCNA labeled cells were observed in granulosa layers. No PCNA labeling either, was seen in theca which was gradually degraded and became morphologically indistinguishable.

In summary, the expression of PCNA detected by immunohistochemistry may serve as a reliable tool to detect the proliferative activity of granulosa and theca cells in the pig ovary.

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References

[1] Bravo R, McDonald-Bravo H (1987) Existence of two populations of cyclin proliferating cell nuclear antigen during the cell-cycle-association with DNA-replication sites. J Cell Biol 105: 1549-1554

[2] Britt KL, Sauders PK, McPherson SJ, Misso ML, Simpson ER and Findlay JK (2004) Estrogen actions on follicle formation and early follicle development. Biol Reprod 71: 1712-1723

[3] Feranil JB, Isobe N, Nakao T (2004) Cell proliferation in the atretic follicles of buffalo and cattle ovary. Reprod Domest Anim 39: 405-409

[4] Feranil JB, Isobe N, Nakao T (2005) Apoptosis in the antral follicles of swamp buffalo and cattle ovary: TUNEL and caspase-3 histochemistry. Reprod Domest Anim 40: 111-116

[5] Gittens JEI, Barr KJ, Vanderhyden BC and Kidder GM (2005) Interplay between paracrine signaling and gap junctional communication in ovarian follicle. J Cell Sci 118: 113-122

[6] Greenwald GS (1989) Temporal and topographic changes in DNA synthesis after induced follicular atresia. Biol Reprod 40: 175-181

[7] Guthrie HD, Grimes RW, Cooper BS, Hammond JM (1995) Follicular atresia in pigs: measurement and physiology. J Anim Sci 73: 2834-2844

[8] Hirshfield AN (1986) Effect of a low- dose of pregnant mares serum gonadotropin on follicular recruitment and atresia in cycling rats. Biol Reprod 35: 113-118

[9] Hirshfield AN (1981) Development of follicles in the mammalian ovary. Int Rev Cytol 124: 43-101

[10] Kagawa N, Sakurai Y, Miyano T , Manabe N (2005) Effect of long-term grafting on follicular growth in porcine ovarian cortical grafts xenotransplanted to severe combined immunodeficient (SCID) mice. J Reprod Dev 51: 77-85

[11] Kurki P, Ogata K, Tan EM (1988) Monoclonal-antibodies to proliferating cell nuclear antigen (PCNA) cyclin as probes for proliferating cells by immunofluorescence microscopy and flow-cytometry. J Immunol Methods 109: 49-59

[12] Manabe N, Goto Y, Matsuda-Minehata F, Inoue N, Maeda A, Sakamaki K, Miyano T (2004) Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cells apoptosis during atresia. J Reprod Dev 50: 493-514

[13] Muskhelishvili L, Wingard SK, Latendresse JR (2005) Proliferating cell nuclear antigen - a marker for ovarian follicle counts. Toxicol Pathol 33: 365-368
[14] Oktay K, Schenken RS, Nelson JF (1995) Proliferating cell nuclear antigen marks the initiation of follicular growth in the rat. Biol Reprod 53: 295-301
[15] Rao MC, Modgley AR, Richards JS (1978) Hormonal regulation of ovarian cellular proliferation. Cell 14: 71-78
[16] Richards JS (1975) Estradiol receptor content in rat granulosa cells during follicular development. Endocrinology 97: 1174-1184
[17] Takekida S, Matsuo H, Maruo T (2003) GnRH agonist action on granulosa cells at varying follicular stages. Mol Cell Endocrinol 202: 155-164
[18] Takekida S, Deguchi J, Samoto T, Matsuo N, Maruo T (2000) Comparative analysis of the effect of gonadotropin-releasing hormone agonist on the proliferative activity, apoptosis and steroidogenesis in cultured porcine granulosa cells at varying stages of follicular growth. Endocrine 12: 61-67
[19] Tan CK, Sullivan K, Li XY, Tan EM, Downey KM, So AG (1987) Autoantibody to the proliferating cell nuclear antigen neutralizes the activity of the auxiliary protein for DNA - polymerase delta. Nucl Acid Res 15: 9299-9308
[20] Wandji SA, Sršeň V, Nathanielsz PW, Eppig JJ, Fortune JE (1997) Initiation of growth of baboon primordial follicles. Hum Reprod 12: 1993-2001
[21] Wandji SA, Sršeň V, Voss AK, Eppig JJ, Fortune JE (1996) Initiation *in vitro* of growth of bovine primordial follicles. Biol Reprod 55: 942-948

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