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Apoptosis in ovarian cells in postmenopausal women

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Abstract: Apoptosis is a natural process which accompanies human ovary from the moment of birth until old age. While it is a well-known process at the reproductive age, it still needs to be thoroughly examined when referring to the postmenopausal age. The study involved 30 postmenopausal women who had their ovaries removed by laparotomy due to non-neoplastic diseases of the uterus. The women were divided into 3 groups depending on the time that had passed since the last menstruation. Group A consisted of women who had their last menstruation no more than 5 years earlier. In group B menopause occurred 5 to 10 years earlier. Group C was composed of patients who had the last menstruation over 10 years earlier. In all the patients concentrations of follitropin (FSH) and estradiol (E2) in blood plasma were measured. Ovarian tissue was obtained during surgery. For morphological studies, ovaries were fixed in Bouin`s solution and 4% formalin and embedded in paraffin. Morphological analysis was carried out after hematoxylin-eosin (H-E) staining. For histochemical detection of apoptotic cells (in situ localization of fragment DNA), the TUNEL method was used. The expression of caspase-3 positive cells was determined immunohistochemically in paraffin-embedded specimens. Comparing to groups A and B, the ovaries in group C contained small number of corpora albicantia located in the medullary part as well as thinned blood vessels and few lymphatic vessels and nerves. In contrast to group A where the number of TUNEL-positive cells was high and caspase-3 expression was observed, no TUNEL-positive nuclei and caspase-3 expression were found in the examined ovaries of group C women.

Key words: Ovary - Apoptosis - Menopause - Women

Introduction

Apoptosis, or programmed cell death, accompanies human ovary from the moment of birth. While it is a quite well-known process at the reproductive age [1,2,10-13,15-17,22,30,41], there are very few reports on that question when referring to postmenopausal age [14,23,37].

At the reproductive age, ovaries are hormonally active glands, in which the process of apoptosis is very intensive and related mainly to granulosa and theca cells. Apoptosis restricts the number and development of ovarian follicles causing their atresia before they are capable of ovulation [26]. It also intensifies degenerative processes in already formed follicles, and causes germinal involution [9,11,27,38]. It is a natural, physiological process which provides organ homeostasis and protects cells from improper functioning [12,15,37,39].

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Gonadoliberin, gonadotrophin, estrogens and androgens [21,29] and also growth factors: insulin growth factor 1 (IGF-1) and its binding proteins, fibroblast growth factor β (FGF-β), epithelial growth factor (EGF), tumor growth factor-α (TGF-α) and tumor necrosis factor-α (TNF-α) [3,28,34] are considered as the most important factors regulating apoptosis in ovarian granulosa cells.

Commonly applied methods to demonstrate apoptosis in human ovary at the reproductive age include TUNEL method and detection of caspase-3 [1,7,10,12,20,22,27]. The TUNEL method is highly specific and allows identification of apoptotic cells even at early stages of apoptosis [1,12,20,22,33]. Subsequently, the method for detecting caspase-3, an executioner enzyme in the apoptosis, proves that the process of apoptosis in female ovarian granulosa cells is completed [7,10,27]. While these methods are also applied to assess effectiveness of chemotherapy in ovarian neoplasms, so far, they have not been used in research on postmenopausal ovary [20].

The structure of a human ovary changes with age. At the reproductive age, an ovary is distinctly divided

into cortex filled with follicles at various stages of development, and medulla composed of loose connective tissue with elastic fibres, single myocytes, blood and lymphatic vessels and nerves. After menopause the volume of an ovary is considerably reduced. Such an ovary has no longer follicles and its stroma is composed mainly of fibrous connective tissue, corpora albicantia, blood and lymphatic vessels, and nerves. Corpora albicantia are composed of collagen fibres and connective tissue cells, among them: fibroblasts, myofibroblasts and macrophages [14,32,35,40,41]. Early manifestations of the ovarian aging (also called perimenopause) are related to the disturbed function of the follicle apparatus [5,24,37]. Clinical symptoms are: diminished fertility, significant percentage of miscarriages and increased chromosomal aberrations. Perimenopausal women have fewer granulosa cells per one follicle, lowered production of steroid hormones, especially progesterone, and reduced production of inhibin. Granulosa cells in these women are exposed to oxygen deficiency [12,18,25,36]. We still do not know, however, if there are any differences in the structure and function of an ovary depending on the lapse of time from the last menstruation, and there are very few reports on that issue [5,14].

The aim of the present study was to investigate the role of apoptosis in ovarian cells in postmenopausal women. That is why we decided to demonstrate how intensity of apoptosis and structure of a female ovary change during the postmenopause period depending on the lapse of time from the last menstruation.

Materials and methods

Patients. The study involved 30 postmenopausal women who did not use substitutive hormone therapy before, and had their ovaries removed by laparotomy due to non-neoplastic diseases of the uterus. The Bioethical Commission gave its consent for this study. All cases were diagnosed at the Department of Reproduction and Gynecology, Pomeranian Medical University, Szczecin.

The women were divided into 3 groups depending on the time that had passed since the last menstruation. Group A (12 patients) consisted of women who had their last menstruation no more than 5 years earlier. In group B (10 patients) menopause occurred 5 to 10 years earlier. Group C (8 patients) was composed of patients who had the last menstruation over 10 years earlier.

Hormone measurements. In all the patients concentrations of follitropin (FSH) and estradiol (E2) in blood plasma were measured using ELISA method enzyme immunoassay kit (Spi-Bio France).

Morphology. Ovarian tissue (cortex and medulla) was obtained during surgery. For morphological studies, ovaries were fixed in Bouin`s solution and 4% formalin and embedded in paraffin. Sections were cut from the respective parts of ovaries. Morphological analysis was carried out after hematoxylin-eosin (H+E) staining.

Histochemistry. For histochemical detection of apoptotic cells (*in situ* localization of fragment DNA), the TUNEL method was used (Apo-DETEK, and horseradish peroxidase-DAB In situ detection system, Enzo-Diagnostics, USA). Following deparaffinization and rehydration, tissue sections were digested with proteinase K for 15 min at room temperature. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS. Sections were incubated for 1 h with TdT and Bio-16-dUTP in a humidified chamber at 37°C, followed by strepavidin-biotinylated horseradish peroxidase and diaminobenzidine (DAB) as chromogen. The sections were counterstained with hematoxylin, dehydrated, mounted, and coverslipped. For negative control, incubation with TdT was omitted.

Immunohistochemistry. The expression of caspase-3 positive cells was determined immunohistochemically in paraffin-embedded specimens fixed in 4% buffered formalin. The slides were heated in citrate buffer, pH=6.0 for 30 min. in a water bath at 96°C. The sections were next incubated for 1 h at room temperature in dark, humidified chamber with rabbit polyclonal anti-human caspase-3 antibody (Dako, Denmark). Detection of anti - caspase-3 antibody was performed using Dako EnVision System, Alkaline Phosphatase - Fast Red. Levamisole was used as an inhibitor of endogenous alkaline phosphatase (Sigma, USA). Fast Red was used to visualize the immunohistochemical reaction. Finally, the sections were counterstained with Mayer's hematoxylin. After each step, sections were rinsed with Tris-buffered saline (TBS). Control sections were incubated with TBS instead of the primary antibody.

Statistical analysis. The Kruskal-Wallis test was used for statistical analysis. The accepted significance level was $p<0.05$.

Results

Age of the examined patients

In group A average age of the patients was 47.1±1.8 years (M±SD), in group B: 54.6±2.1 (M±SD), and in group C: 59.9±1.2 (M±SD). Considerable age differences were found between women from groups: A and B, B and C, and A and C ($p<0.05$).

The levels of FSH and E2 in serum of the examined patients

In group A, the mean E2 concentration was 20.3 pg/mL ± 6.9 (M±SD), in group B - 21.6 pg/mL ± 8.3 (M±SD) and in group C - 13.8 pg/mL ± 3.2 (M±SD) (Fig. 1).

The mean E2 serum concentrations did not significantly differ from each other ($p>0.05$).

In group A, the mean FSH concentration was 51.1 mUI/mL ± 15.8 (M±SD), in group B - 55.8 mUI/mL ± 19.7 (M±SD), and in group C - 87.6 mUI/mL ± 11.0 (M±SD).

Considerable differences in the mean serum FSH values were found between the examined groups of women (between groups A and B, B and C, and A and C) (Fig. 2) ($p<0.05$).

Morphology (H+E staining)

In the ovaries of group A women, numerous corpora albicantia and well-developed fibrous connective tissue as well as abundant blood and lymphatic vessels

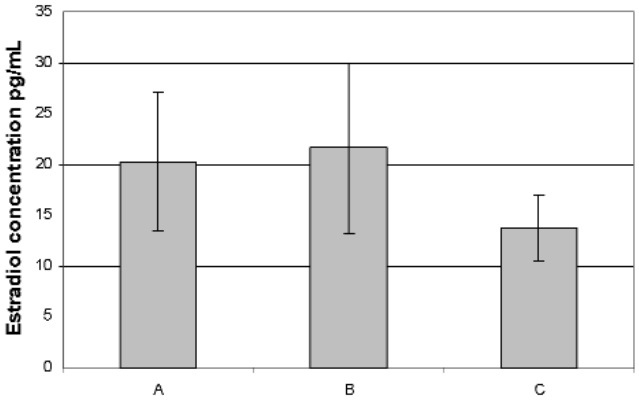

Fig. 1. The mean serum estradiol concentration (pg/mL) in the examined women. Significant differences were not observed in the analyzed groups of patients (mean ± SD), $p>0.05$.

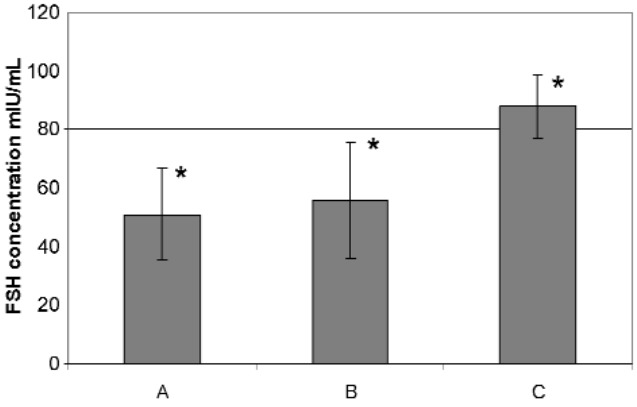

Fig. 2. The mean serum FSH concentration (mIU/mL) in the examined patients. Significant differences were noted between the examined groups of women (mean ± SD), *$p<0.05$.

and nerves were observed (Fig. 3). Group B women had only few, partially fragmentated corpora albicantia in their ovaries, and their blood vessels were partly hyalinizated (Fig. 4). Morphological picture of the ovaries in group C was significantly different from that of group A. The ovaries in group C contained only single corpora albicantia located in the medullary part as well as thinned blood vessels and few lymphatic vessels and nerves (Fig. 5).

Histochemical results (the TUNEL method)

In the women examined no more than 5 years after menopause (group A), the number of TUNEL-positive cells was high and concerned mainly to vascular endothelial cells, connective tissue cells and corpora albicantia. The highest expression of TUNEL-positive cells was observed in connective tissue cells of stroma and in connective tissue cells of corpus albicans. The expression of TUNEL-positive cells was observed in vascular myocytes and endothelial cells. (Fig. 6, 7 and 8). The more years passed from the last menstruation, the fewer TUNEL-positive cells were found. In group B, TUNEL-stained nuclei appear in connective tissue cells of stroma and in vascular endothelial cells. Only single TUNEL-positive nuclei were observed in vascular myocytes of the hyalinizated artery (Fig. 9) and in connective tissue cells of corpora albicantia. In group C, TUNEL-positive nuclei were not found in the examined ovaries at all (Fig. 10).

Immunohistochemical results (caspase-3 reaction)

In the ovaries of group A women, the highest level of caspase-3 expression was observed in cytoplasm of connective tissue cells (Fig. 11), in vascular myocytes and in corpora albicantia. With patients` age, expression of caspase-3 decreased. In group B, single caspase-3 positive cells were found among connective tissue cells, in vascular myocytes and corpora albicantia. No caspase-3-possitive cells were observed in group C (Fig. 12).

Discussion

Considering the discussions on the role of ovaries in postmenopausal women, we examined hormone concentration in blood serum. We also conducted morphological, histochemical and immunohistochemical analyses of ovaries in postmenopausal women depending on the lapse of time from their last menstruation.

Significant differences were found in serum FSH concentrations between the particular groups (A, B, C), whereas estradiol concentrations in serum did not considerably vary depending on the length of time from the last menstruation. Our results are consistent with those reported by other authors [5,6,31]. As we know from written sources, FSH concentration in women at the reproductive age is between 5 and 15 mIU/mL, and E2 concentrations between 50 and 520 pg/mL, unless the cycle is stimulated. As Buckler and Casella *et al.* [6,8] claim, FSH and E2 are very sensitive markers of endocrinological changes in the organism of perimenopausal woman. Concentration of FSH increases as first in perimenopausal period signalizing the onset of menopause; then it remains at maximum values until senium begins. Estradiol concentration practically does not change following the menopause. Numerous authors say that serum estradiol concentrations lower than 50 pg/mL cause disappearance of menstruation and appearance of menopause symptoms which vary individually [6,8,19].

Ovaries of postmenopausal women are known to vary in size and morphology [4,5,14,37]. Focchi *et al.* [14] state that the number and size of corpora albican-

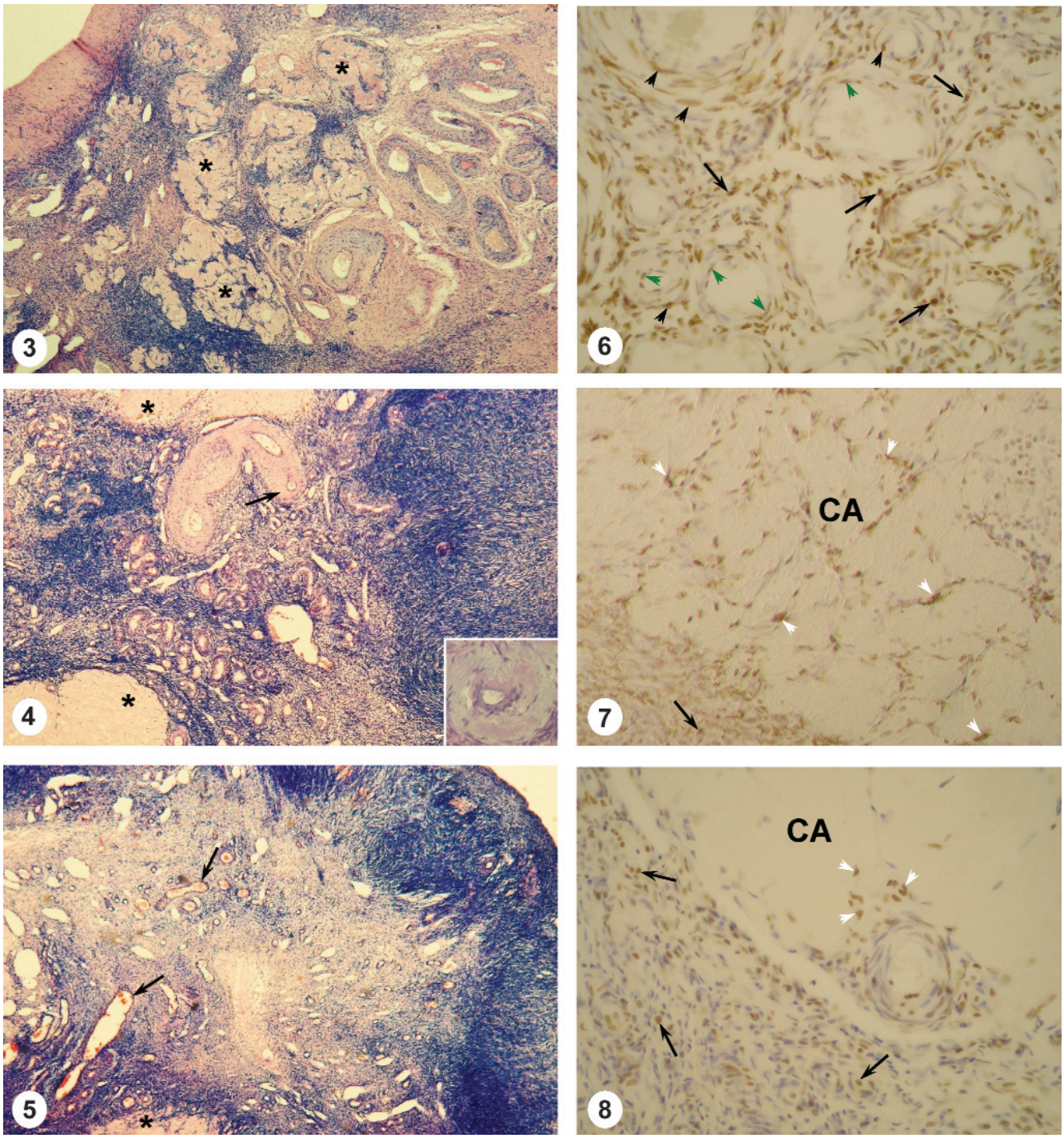

Fig. 3. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). Numerous corpora albicantia (asterisks), well developed connective tissue and abundant blood vessels. H-E staining (magnification ×160). **Fig. 4**. Ovary of group B postmenopausal women (examined from 5 to 10 years after menopause). Only few corpora albicantia (asterisks) and hyalinizated artery (arrow). H-E staining (magnification ×160). Insert represents the hyalinizated artery (magnification ×330). **Fig. 5**. Ovary of group C postmenopausal women (examined more than 10 years after menopause). Single corpora albicantia (asterisk) located in the medulla part and thinned blood vessels (arrows). H-E staining (magnification ×160). **Fig. 6**. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). TUNEL-stained nuclei appear mainly in connective tissue cells (arrows), vascular myocytes (black arrowheads) and endothelial cells (green arrowheads) (magnification ×330). **Fig. 7**. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). Corpus albicans (CA). TUNEL-stained nuclei appear mainly in connective tissue cells of corpus albicans (white arrowheads) and in connective tissue cells of stroma (arrow) (magnification ×330). **Fig. 8**. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). The border between corpus albicans (CA) and stroma. TUNEL-positive nuclei can be seen in connective tissue cells of corpus albicans (white arrowheads) and in connective tissue cells of stroma (arrows) (magnification ×330).

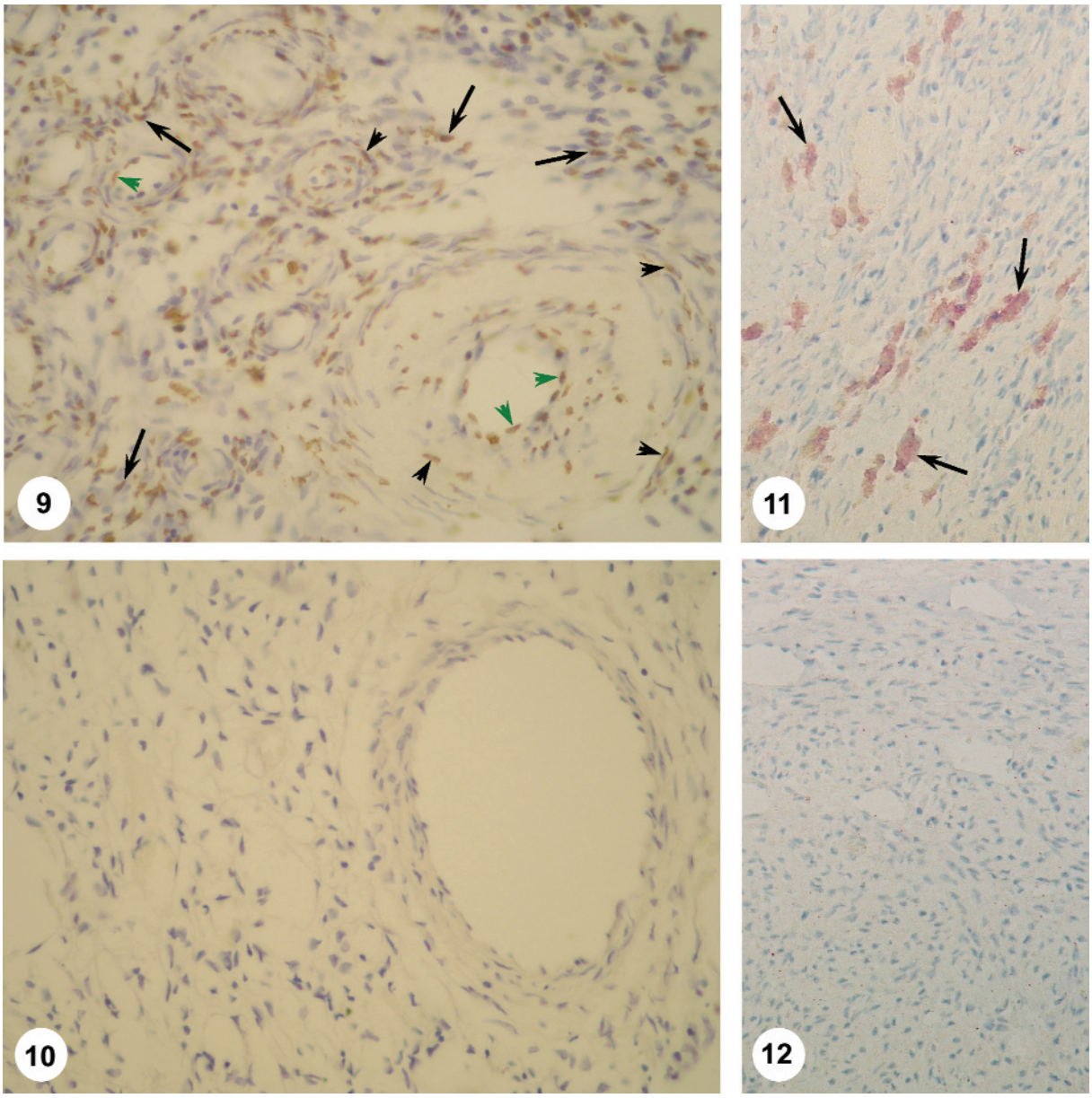

Fig. 9. Ovary of group B postmenopausal women (examined from 5 to 10 years after menopause). Stroma of the ovary and the hyalinizated artery. TUNEL-stained nuclei appear in connective tissue cells (arrows) and in vascular endothelial cells (green arrowheads). Only single TUNEL-positive nuclei can be seen in vascular myocytes of the hyalinizated artery (black arrowheads) (magnification ×330). **Fig. 10.** Ovary of group C postmenopausal women (examined more than 10 years after menopause). Stroma and blood vessel of the ovary. No TUNEL-positive nuclei (magnification ×330). **Fig. 11**. Ovary of group A postmenopausal women (examined no more than 5 years after menopause). The highest level of caspase-3 expression was observed in cytoplasm of connective tissue cells (arrows) (magnification ×160). **Fig. 12.** Ovary of group C postmenopausal women (examined more than 10 years after menopause). No caspase-3-positive cells (magnification ×160).

tia in particular woman depend on the number of ovulations at reproductive age and phagocytic activity, which varies individually and decreases as the time from the last menstruation passes. The size of ovaries in postmenopausal women depends on the number of nonresorbed corpora albicantia.

The results presented in this study show that the structure of female ovaries after menopause depends on the time that passed from the last menstruation. In the ovaries of the patients who had menopause less than 5 years earlier, numerous corpora albicantia and well-developed fibrous connective tissue can be found as well as abundant blood and lymphatic vessels, and nerves. Group B women have fewer, partially fragmentated corpora albicantia in their ovaries. Blood vessels in these ovaries are hyalinizated, which means

that their diameters are reduced, and walls are thicker comparing to the ovarian vessels in group A. In the ovaries of the oldest women from group C a blurring border line between cortex and medulla was observed as well as single corpora albicantia located mainly in the medullary part, few blood and lymphatic vessels and nerves. Our results are consistent with those reported by Focchi *et al.* [14] who, additionally, determined the percentage of corpora albicantia in the whole structure of an ovary.

However, histochemical (the TUNEL method) and immunohistochemical (caspase-3 reaction) analyses that we carried out prove that the process of apoptosis in ovaries of women examined up to 5 years after menopause is most intensive in corpora albicantia, arterial walls and in the area of blood vessels. The longer the time from the menopause, the less active the process is. In group B women, whose mean age was 54 years, the mean serum estradiol concentration was 21.6 pg/mL, and the mean FSH concentration - 55.8 mIU/mL. Less numerous TUNEL-positive cells were found among vascular endothelial cells, connective tissue cells, vascular myocytes and corpora albicantia. Also single caspase-3 positive cells were observed among connective tissue cells, vascular myocytes and corpora albicantia. In group C women, whose mean age was 59 years, the mean estradiol concentration was 13.8 pg/mL, and the mean FSH concentration - 87.6 mIU/mL. Neither TUNEL-positive cells nor caspase-3 positive cells were found. The presented data show the coincidence of the decreasing ovarian apoptosis and increasing serum FSH concentration. Perhaps in women who had the last menstruation over 10 years earlier, the apoptosis does not appear any more or, depending on the patient's age, it is barely active. The written sources lack information on this tissue. It is probable that the decline of apoptosis is also related to the progressing decline of the ovarian steroidogenesis which does not only refer to estrogen, but also androgen production. There are reports on the decreasing activity of key steroidogenic enzymes such as 17-β-steroid and 3-β-steroid dehydrogenase, aromatase P-450 as well as data being the evidence for the decreasing activity of both LH and FSH receptors and steroid hormone receptors [4,5,31]. Hopefully, the research on ovarian apoptosis and steroidogenesis will be carried on, and the results will have clinical implications.

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