

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

The influence of the expression of steroid receptors on angiogenesis, proliferation and apoptosis in myomas of pre- and postmenopausal women

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SUMMARY

Introduction/Objective The aim of this study was to determine the effects of the estrogen and progesterone receptor status on angiogenesis, proliferation, and apoptosis of myoma cells in premenopausal (PreM) and postmenopausal (PostM) women.

Methods This was a cross section; clinical-experimental, retrospective, non-interventional study in the field of the study of fundamental pathogenesis mechanisms of disease using pathohistological materials from the existing archive. The research included 76 patients diagnosed with uterine leiomyomas, operatively treated in the Clinic for Gynecology and Obstetrics, Clinical Centre Kragujevac, Serbia. According to the menstrual status, we formed two experimental subgroups. The first group was PreM women ($n = 35$; 46.2 ± 5.02 years old), and the second group was PostM women ($n = 41$; 60.25 ± 5.41 years old). Hematoxylin-eosin staining for myoma and myometrium was conducted, as well as immunohistochemistry for ER α , ER β , PR α , vascular endothelial growth factor, endoglin, Ki67, and caspase-3.

Results Progesterone receptor was overexpressed in myoma and myometrium of PreM compared to myoma and myometrium of PostM women. Expression of caspase-3 was a statistically significant increase in PostM women compared to PreM group. ER α and ER β were not changed among groups neither in myoma nor in myometrium samples.

Conclusion According to our data, PR α had higher influence on apoptosis and cell growth than estrogen receptors. Since PR α was increased in PreM in both myoma and myometrium, probably this expression led further to lower expression of apoptotic marker in PreM women.

Keywords: steroid receptors; apoptosis; angiogenesis; premenopausal; postmenopausal

INTRODUCTION

Uterine fibroids, also known as uterine leiomyomas or fibroids are well-limited, pseudo-encapsulated, benign, monoclonal tumors, composed mainly of smooth muscle cells of the uterus. Uterine leiomyomas, among the most frequent gynecological tumors in the reproductive period of women. Independently or in association with hyperplasia and adenomyosis, they reach an incidence of 77%, often causing clinically complicated bleeding, which is why they are the leading cause for hysterectomy and a major global health problem [1, 2]. It has been known that uterine leiomyomas are a hormone-dependent disease. However, the mechanism of action is still unknown, and there is increasing evidence that steroid hormones, estrogen and progesterone receptors are not the only modulators of myoma growth [2, 3, 4]. This can be explained with a presence of similar level of circulating hormone in women with and without myoma, with the occurrence of hormone-independent extrauterine leiomyoma

and the possible absence of their regression in postmenopausal women [5].

Throughout the last decade, high effort has been invested to clarify the role of gonadal steroids, the expression of local growth factors, and factors associated with apoptosis in myoma cells. Recent studies showed local tissue-specific factors (for example, growth factors), as well as somatic mutations in pro and antiapoptotic genomes, participate in the pathogenesis and progression of these tumors, with or without cross-linking with mechanisms of action of steroid hormones. Among the environmental factors, particular attention is drawn to the presence and effect of estrogen and progesterone receptors on the endometrium and myometrial cells in the uterine wall with myoma [3].

The key pathological processes involved in myoma growth are proliferation and hypertrophy of leiomyocytes, apoptosis, angiogenesis, stromal and secondary changes [6]. The most reliable marker of cell proliferation is Ki-67 or a proliferation-cell nuclear antigen, which denotes not only the cells in the divide, but also



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all those in the synthetic phase of the cell cycle [7]. A high level of Ki-67 antigen, detected during the secretory phase, suggests that progesterone has a synergistic effect in the pathogenesis of myoma [8].

Apoptosis is a process of programmed cell death that eliminates dysfunctional and undesirable cells. It is highly regulated by the complex interaction between the pro- and the anti-apoptotic molecules, is performed in one cell independently of the surrounding, and is induced by the activation of caspases, specific endoproteases that destroy the essential structural components including the genetic material of the cell [9]. Caspase-3 (cas-3), due to its specificity and sensitivity, is a reliable marker of cells that pass the process of programmed cell death. Its activity cannot be detected before apoptosis. It is registered in early stages and detection grows with progression while is reduced only in the final phase of the apoptotic process [10].

Furthermore, angiogenesis is mediated by numerous angiogenic growth factors; the most powerful among them is the vascular endothelial growth factor (VEGF). VEGF affects the degree of microvascular tumor density by stimulating the proliferation of endothelial cells. Variable concentrations of VEGF, depending on the phase of menstrual cycle, are detected in myometrium, stromal and epithelial endometrial elements [11].

During tumor angiogenesis, endothelial cells intensely express endoglin (CD105), while vascular endothelium, stromal and inflammatory cells barely or do not express at all CD105 [12].

Beside the fact that the prevalence of proliferation over apoptosis is a major condition for myoma growth, with this study we tried to indicate that similarities and differences in angiogenesis, proliferative, and apoptotic indexes in myomas and surrounding tissue, are primarily dependent on the expression of steroid hormone receptors. According to previous, the aim of this study was to determine the effects of the ER and PR status on angiogenesis, proliferation, and apoptosis of myoma cells in premenopausal (PreM) and postmenopausal (PostM) women.

METHODS

This was a cross section; clinical-experimental, retrospective, non-interventional study in the field of the study of fundamental pathogenesis mechanisms of disease using pathohistological materials from the existing archive.

The research included 76 patients diagnosed with uterine leiomyomas, operatively treated in the Clinic for Gynecology and Obstetrics, Clinical Centre Kragujevac, Kragujevac, Serbia, in a three-year-long period (2007–2010). According to the menstrual status, two experimental subgroups were formed. The first group was PreM women ($n = 35$; 46.2 ± 5.02 years old), and the second group was PostM women ($n = 41$; 60.25 ± 5.41 years old).

Clinical data were collected by insight into disease history and operational protocols of examined patients. We collected information related to gynecological status (menstrual cycle, menarche, menopause, number of deliveries, etc.) and

data obtained by macroscopic analysis of the operative preparations (number, position and size of the myoma, changes in the ovaries, morphometric characteristics of the uterus).

The experimental part of the study was carried out on the operative tissue material obtained by hysterectomy.

The study was conducted at Department of Pathology, Clinical Centre of Kragujevac, Serbia. The study was done in accord with standards of the institutional Committee on Ethics of the Clinical Center of Kragujevac, Kragujevac, Serbia.

Hematoxylin-eosin staining

Tissue materials were fixed in formalin, embedded in paraffin, and 5- μ m sections were stained with hematoxylin-eosin (H&E), and further examined by immunohistochemistry [13]. All pictures are taken in original resolution with $\times 200$ magnification. A representative sample of the myoma without regressive changes is separated for immunohistochemical analysis.

Immunohistochemistry

Paraffin-embedded tissue sections were fixed in 10% neutral buffered formalin and embedded in paraffin using standard pathological protocols. Immunohistochemistry was performed on a single representative block from each case or two (when the surrounding myometrium is not visible along with the myoma on the first block). Antigenic retrieval was processed by submerging the sample in 10 mM citrate buffer (pH 6) or commercial buffer 10 mM EDTA Buffer for Heat-Induced Epitope Retrieval (pH 8), AP-9004-125 (Thermo Scientific, Waltham, MA, USA) and microwaving for 20 minutes at 96°C. Primary monoclonal antibodies were directed against ER α Ab11 (mouse: 1:500, MS-354-R7, Thermo Scientific), ER β antibody (mouse/human: 1:200 dilution, MA1-23217, Thermo Scientific), VEGF (rabbit: 1:100 dilution, RB-9031-RQ, Thermo Scientific), Ki-67 (rabbit: 1:100 dilution, RB-9106-R7, Thermo Scientific), PR α Ab-8 (mouse: 1:25 dilution, MS-298-R7, Thermo Scientific), CD105 (rabbit: 1:25 dilution, RB-9291-R7, Thermo Scientific), and cas-3 Ab-3 (human: 1:100 dilution, MS-1123-R7, Thermo Scientific). Tissue sections were incubated with appropriate primary antibody and commercial biotinylated secondary anti-immunoglobulin, at room temperature, according to the manufacturer's instructions (UltraVision LP Large Volume Detection System: HRP Polymer (Ready-To-Use), TL-125-HL, Thermo Scientific). An evaluation of the immunohistochemical analysis was carried out by a semi-quantitative assessment of the expression of the examined markers, by scaling to the scales specific to each marker. All pictures are taken in original resolution with $\times 200$ magnification.

Expression of estrogens, progesterone, vascular endothelial growth factor, and Ki-67

The expression of ER and PR will be quantified based on the Allred score, i.e. by adding parameters that indicate percentage representation (from 0 to 5) and intensity of cell

expression (from 1 to 3) [14]. The sum of these parameters will represent the values of the total score (from 0 to 8), where the values ≥ 3 was considered positive. The expression of VEGF, Ki-67 and cas-3 was determined based on the percentage of immunoreactive cells. Based on this expression, groups with low (0–15%), moderate (16–30%) and high proliferative or apoptotic index (31–100%) were formed.

Expression of endoglin

Immunohistochemical analysis of the expression of endoglin (CD105), an assessment of the degree of angiogenesis will be performed. The right index of intensity of angiogenesis is the density of intra and peritumoral microcirculation or microvessel density. The analysis will be carried out quantitatively by counting blood vessels in zones with their highest density (hot spot areas). We used the recommendations given by Weidner et al. [15] on the magnitude of the field of vision and the counting method. The focal points of the highest density of blood vessels were determined on a small microscopic magnification ($\times 40$). Determination of the focus of the largest microvascular density were performed by two researchers independently, with no clinical and pathohistological data available. After that, the counting of individual blood vessels was performed at a mean microscopic magnification ($\times 200$), which implies an area of 0.739 mm². The mean value of the results obtained by counting in three visible fields was the result. When counting blood vessels in each “hot zone,” the expression of individual endothelial cells, and not just the lumen of a blood vessel with visible red blood cells, was calculated. After obtaining the data on the number of blood vessels for each case separately, the mean value of the three read fields were calculated. Then the median in relation to which all myomas were classified into two groups, those with low degree and those with a high degree of angiogenesis, accordingly whether the number of blood vessels is less than or equal to or greater than the value of the calculated median.

All immunohistochemical staining were carried out with quality control and specificity of colouring, using positive and negative controls according to the UK National Quality Assessment for Immunocytochemistry. Microscopic tumor analysis and evaluation of marker expression were performed on a microscope of the Carl Zeiss, Axioscop 40 type. Preparations with representative fields were painted using three microscopic enlargements ($\times 40$ and $\times 200$) using a Canon PC 1089 camera (Canon Inc., Tokyo, Japan).

Sampling

Regarding the method of selecting a study sample from the entire population, all samples of the material archive will be potentially considered for inclusion. The criteria for the involvement of subjects in the study were a pathohistologically verified uterine leiomyomas disease and PreM or PostM status. Excluding criteria for selecting subjects were associated malignant diseases of the ovary and cervix, incomplete clinical data on menstrual status, use of oral contraceptives and other forms of hormonal therapy.

Statistical analysis

Statistical processing of results will be performed using a commercial software package SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). In the analysis of the obtained results, descriptive statistics was first used to describe the general characteristics of the sample: absolute numbers and proportions (frequencies, percentages), median and variability measures (standard deviation), maximum and minimum. The regularity of the distribution was evaluated by Kolmogorov–Smirnov test. For the comparison of the mean values of the two variable populations, the independent t-test, Kruskal–Wallis and Mann–Whitney tests were used, and for comparison of the mean variables of populations analysis of variance was used. The dependence of two descriptive variables were carried out using the χ^2 test and the Fisher test, the dependence of two numerical variables using Pearson's and Spearman's correlation coefficient, while the influence of more variables on the binary variable were investigated using a multivariate binary logistic regression.

RESULTS

Hematoxylin staining

The standard light microscopic analysis is defined histological type of myoma, the mitotic index expressed through the number of mitotic figures on 10 fields of great enlargement, the presence of regressive changes (necrosis, hyaline and myxomatous degeneration, foci of hemorrhage, etc.), the condition of the endometrium and surrounding myometrium (Figure 1A, B).

Expression of estrogen receptors alpha and beta in myoma and myometrium

Neither ER α nor ER β showed statistically significant expression in myoma of PreM compared to PostM women (Figures 2A, B and Figures 2E, F). Similarly, those parameters were not different among examined groups in myometrium samples (Figures 4A, B and Figures 4E, F).

Expression of progesterone receptor in myoma and myometrium

PR was over expressed in myoma of PreM compared to myoma of PostM women (Figure 2C and 2G). Also, statistically significant increased values of PR α were detected in myometrium of PreM compared to PostM women (Figure 4C and 4G).

Expression of the vascular endothelial growth factor in myoma and myometrium

In myoma tissue, VEGF was not significantly different among groups (Figure 1D and 1H). Similarly, the same result was obtained in myometrium of PreM women compared to PostM women (Figure 4D and 4H).

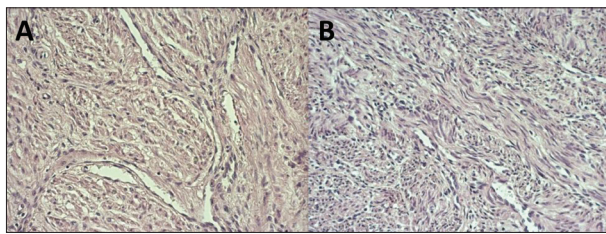


Figure 1. A: representative image of a human myoma (H&E, magnification: $\times 200$); B: representative image of a human myometrium (H&E, magnification: $\times 200$)

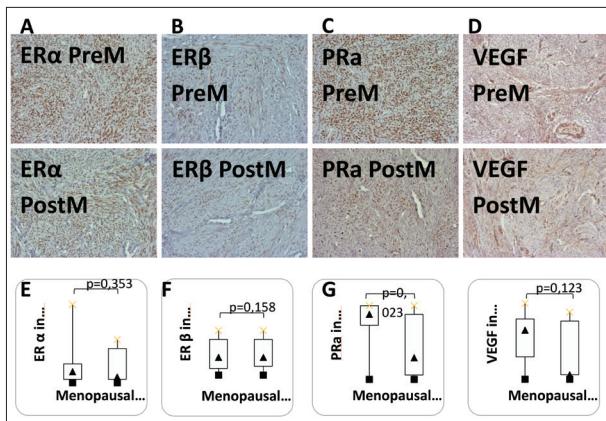


Figure 2. A–D: immunohistochemical expression of ER α , ER β , PR α , and vascular endothelial growth factor (magnification: $\times 200$) in myoma tissue of premenopausal and postmenopausal women, representative tissue sections; E–H: percentage of expression of ER α , ER β , PR α , and vascular endothelial growth factor and statistical difference in percentage of myoma cells between premenopausal and postmenopausal women (\blacktriangle – median; \blacksquare – minimum; \times – maximum)

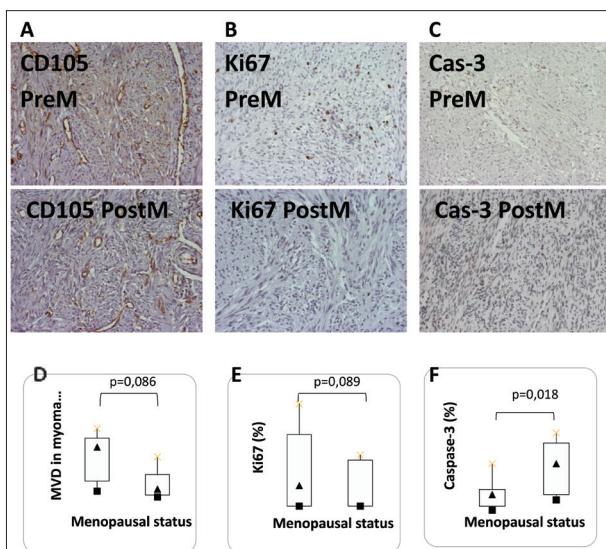


Figure 3. A–C: immunohistochemical expression of endoglin, Ki67, and Cas-3 (magnification: $\times 200$) in myoma tissue of premenopausal and postmenopausal women, representative tissue sections; D–F: percentage of expression of endoglin, Ki67 and Cas-3, and statistical difference in percentage of myoma cells between premenopausal and postmenopausal women (\blacktriangle – median; \blacksquare – minimum; \times – maximum)

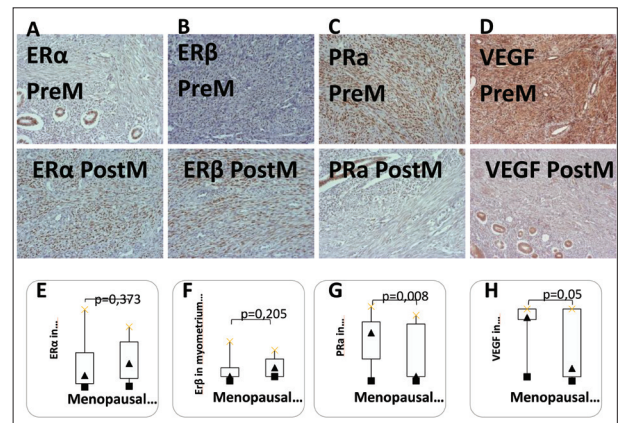


Figure 4. A–D: immunohistochemical expression of ER α , ER β , PR α , and vascular endothelial growth factor (magnification: $\times 200$) in myometrium tissue of premenopausal and postmenopausal women, representative tissue sections; E–H: percentage of expression of ER α , ER β , PR α , and vascular endothelial growth factor and statistical difference in percentage of myometrium cells between premenopausal and postmenopausal women (\blacktriangle – median; \blacksquare – minimum; \times – maximum)

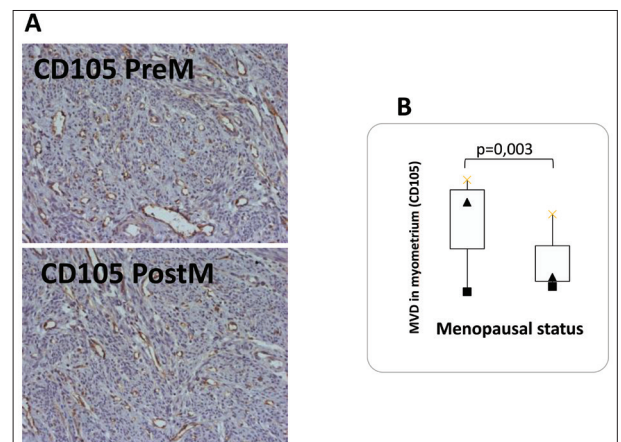


Figure 5. A: immunohistochemical expression of endoglin (magnification: $\times 200$) in myometrium tissue of premenopausal and postmenopausal women, representative tissue sections; B: percentage of expression of endoglin and statistical difference in percentage of myometrium cells between premenopausal and postmenopausal women (\blacktriangle – median; \blacksquare – minimum; \times – maximum)

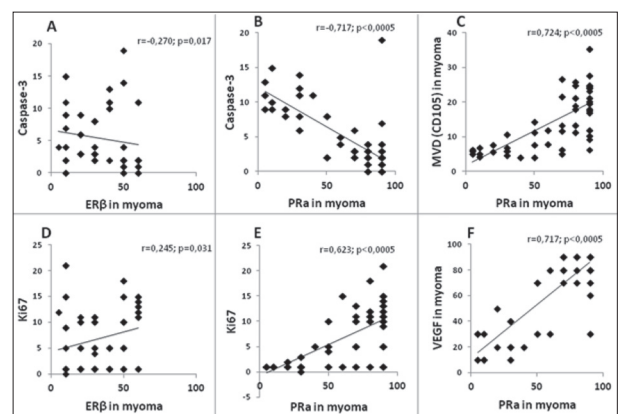


Figure 6. Correlation between all examined parameters in myoma tissue

Expression of endoglin in myoma and myometrium

In myoma of PreM and PostM women CD105 did not show any difference in its expression (Figure 3A and 3D). However, in myometrium CD105 was statistically significant increase in PreM women compared to PostM women (Figure 5A and 5B).

Expression of Ki67 in myoma

Ki67 was not significantly different among PreM and PostM women (Figure 3B and 3E).

Expression of caspase-3 in myoma

Expression of cas-3 was statistically significant increase in PostM women compared to PreM group (Figure 3C and 3F).

Correlation between all examined parameters in myoma tissue

Expression of cas-3 in myoma tissue of all examined groups was weak, but statistically significant negative correlation with expression of ER β in the same tissue (Figure 6A). Moreover, expression of cas-3 was in strong and significant negative correlation with PRa in myoma tissue of both PreM and PostM women (Figure 6B).

In strong correlation with expression of PRa was expression of CD105, VEGF and Ki67 as it is showed in Figures (Figures 6C, E, and F). Moreover, expression of Ki67 was in weak but statistically significant correlation with expression of ER β (Figure 6D) while cas-3 was in negative correlation with ER β (Figure 6A). Correlations between other examined parameters in tissue of myoma did not show to be significant.

DISCUSSION

Uterine leiomyoma is the most common benign tumor; despite its frequent manifestation the etiology and pathophysiology of this abnormality remain unknown. Extensive knowledge has accumulated on the role of hormones in the growth of leiomyomas because the occurrence of uterine leiomyomas during the fertile period and the regression after menopause indicate that gonadal steroids are central for development of these tumors [7, 16]. In the last decade, special attention was given to the role of estrogens and progesterone in the pathophysiology of leiomyomas. Uterine leiomyomas have been considered estrogen-dependent tumors, and his role was supported by the finding that continuous gonadotropin-releasing hormone agonist treatment, significantly decreases ovarian estrogen production, is as well associated with reduction in tumor size [17]. In order to achieve their effects, estrogens act through the activation of estrogen receptors (ER α and ER β). Both of these receptors exhibit DNA- and ligand-binding domain sequence conservation and they are encoded by two distinct genes, they also have different transcriptional activation domains,

as well as different tissue distribution [17]. In our study, we showed that expressions of those receptors were not different in myoma of PreM compared to PostM women (Figure 1E, F). Nevertheless, expression of ER α and ER β was not significantly different neither in myometrium of those women (Figure 3E, F). Similarly, Sakaguchi et al. [18] showed that coordinated expression of ER α and ER β might be necessary for normal estrogen action in myometrium [19]. In addition, it has been shown ER α is phosphorylated at a higher rate on serine in leiomyoma compared with surrounding myometrium, for that reason it is possible that phosphorylated ER α regulated by p44/42 MAPK, will have a role in development of uterine leiomyoma [20, 21].

In recent years, the role of progesterone in uterine leiomyoma pathophysiology has become more established. As in the case of ERs, nuclear PR work as ligand-activated transcription factors and there is two predominant isoforms of PR in humans: PRa and PRb [17]. In our study, PRa showed to be significantly unregulated in myoma of PreM compared to myoma of PostM women, which showed to be same in myometrium tissue (Figure 1G, 3G). Those finding correlate with the fact that progesterone is cyclically elevated during the reproductive years, are significantly elevated during pregnancy, and are suppressed after menopause, however it is still very difficult to distinguishing the relative importance of estrogen versus progesterone [22, 23].

Since it is already a historical fact that leiomyomas are dependent on angiogenesis for their growth and survival, to date it is also found that estrogens and progestins regulate the expression of several potent angiogenic factors, including VEGF and fibroblast growth factor (FGF) [24]. We found that in myoma VEGF was not significantly changed regardless of the menopausal status (Figure 2H); in contrast, VEGF was changed between groups in myometrium (Figure 4H). We found that VEGF is significantly increased in PreM compared to the PostM women. Similarly, Hague et al. [25], according to menopausal status found that VEGF was significantly increased in PreM compared with PostM endometrium. With the PreM tissue, exhibiting a significantly higher level of expression was found in the epithelium but not in the stroma or the blood vessels [26].

Furthermore, one of the most commonly assessed angiogenesis markers is microvessel density, which is determined on the bases of specific endothelial antigen expression (CD34, CD105) [27]. In the tumor we did not notice any change between groups in expression of CD105 regarding menopausal status (Figure 3D), however CD105 was statistically significantly decreased in myometrium of PostM compared to PreM women (Figure 5B).

Moreover, regarding cell death, we evaluated two proteins known as markers involved in growth control of leiomyoma. There was no difference between two examined groups in expression of Ki67 (Figure 3E); however, cas-3 was significantly increased in PostM compared to PreM women (Figure 3F). We noticed that expression of Ki67 did not follow the trend of cas-3 expression, regarding that Plewka et al. [7] showed that the apoptosis was not accompanied by proliferation. There were no immunolocalization of Ki-67 detected in leiomyomas manifesting apoptosis [28].

Additionally, as we can see from Figure 6, PRa is in positive correlation with VEGF, CD105 and Ki67 (Figure 6C, E and F) in myoma cells, and in negative correlation with cas-3 (Figure 6B). On the other hand, ER β was in negative correlation with cas-3 and in positive correlation with Ki67 (Figure 6A, D).

CONCLUSION

Although ER β have effect on cell proliferation and apoptosis, according to all the data, PRa seems to be more rel-

evant. According to our data, PRa had higher influence on apoptosis and cell growth than ER. Since PRa was increased in PreM in both myoma and myometrium, this expression probably led further to lower expression of the apoptotic marker, increased cell proliferation, and angiogenesis in PreM women.

Further studies need to be conducted in order to better understand the mechanisms associated with progesterone-driven growth, according to our data, in order to develop therapies that are more efficient.

Conflict of interest: None declared.

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Утицај експресије стероидних рецептора на ангиогенезу, пролиферацију и апоптозу у миомима пременопаузних и постменопаузних жена

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САЖЕТАК

Увод/Циљ Циљ ове студије је био да се утврди утицај експресије стероидних рецептора на маркере ангиогенезе, пролиферације и апоптозе ћелија миома код жена у пременопаузи и постменопаузи.

Метод Ово је била студија пресека, клиничко-експериментална, ретроспективна, неинтервенцијска студија у пољу истраживања фундаменталних механизма патогенезе болести, коришћењем патохистолошких материјала из постојеће архиве. Истраживањем је обухваћено 76 болесника са дијагностикованим лејомиома утеруса, оперативни лечених на Клиници за гинекологију и акушерство Клиничког центра Крагујевац, Србија. Према менструалном статусу, формиране су две експерименталне подгрупе. Прва група биле су жене у пременопаузи ($n = 35$; $46,2 \pm 5,02$ година), а друга група биле су жене у постменопаузи ($n = 41$; $60,25 \pm 5,41$ година). Коришћено је бојење *H&E* за миом и миометријум, као

и имунохистохемија за *ER α* , *ER β* , *PR α* , васкуларни ендотелни фактор раста, ендоглин, *Ki67* и *caspase 3*.

Резултати Прогестеронски рецептор је био више изражен у миому и миометријуму у пременопаузи у поређењу са миомом и миометријумом жена у постменопаузи. Експресија *caspase-3* је статистички значајно повећана у групи жена које су у постменопаузи у поређењу са групом жена које су у пременопаузи. *ER α* и *ER β* нису били различити између група ни у узорцима миома ни миометријума.

Закључак Према нашим подацима, *PR α* је имао већи утицај на апоптозу и раст ћелија него рецептори за естрогене. Пошто је *PR α* повећан код жена у пременопаузи у миому и миометријуму, вероватно је ова експресија довела до смањења експресије апоптотског маркера код жена које су у пременопаузи.

Кључне речи: рецептори за стероиде; апоптоза; ангиогенеза; пременопауза; постменопауза