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Effects of polyamine synthesis enzymes on angiogenesis and apoptosis during endometriosis

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ABSTRACT

Objectives: Since we assumed that endometriosis is a benign cell division disorder, our study was conducted to investigate the effects of the relationships between polyamine synthesis and angiogenesis in the formation of endometriosis.

Material and methods: Thirty-five patients with endometriosis and 35 healthy female women were included in the study. The patient and the control groups were compared regarding the blood levels of agmatine, argininecarboxylase (ADC), ornithinecarboxylase (ODC), agmatinase, arginase, ornithine, and the vascular endothelial growth factor (VEGF).

Results: There is a statistically significant difference between the patient and the control groups regarding the agmatinase, arginase and VEGF levels (higher in the patient group) ($p < 0.05$). There is no statistically significant difference between the patient and the control groups regarding the ODC, ornithine and the ADC levels ($p > 0.05$). There is a statistically significant difference between the patient and the control groups regarding the agmatine levels (higher in the control group) ($p < 0.05$).

Conclusions: The increase in the serum levels of polyamine synthesis enzymes may contribute to the formation of endometriosis. It is anticipated that the study of the relationship between enzymes and molecules in the polyamine synthesis pathway and angiogenesis in patients with endometriosis will contribute to the literature.

Key words: angiogenesis; apoptosis; endometriosis; polyamine

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INTRODUCTION

Endometriosis is a chronic, benign, often painful disease seen in 8–18% of young women. The disease causes severe physical and mental problems. Endometriosis is a disease that develops due to the formation of a tissue similar to the uterine mucosa in the outer part of the uterus in the sub-abdominal region, and its spread to various organs (such as the uterus, peritoneum, oviducts, ovaries, bladder, and intestines). It has been reported that the number of polyamines, which is also indicated to be associated with the physiopathology of endometriosis, increases in body fluids. In this study, the levels of agmatine, which is essential in the production of major polyamines, the key enzymes in the synthesis of polyamines [arginase, argininecarboxylase (ADC), ornithinecarboxylase (ODC), agmatinase], ornithine, which is an intermediate product, and the vascular endothelial growth factor (VEGF) levels were investigated in cases who has endometriosis.

Polyamines are organic cations found naturally in microorganism, animals, and plants. Putrescine, the precursor of major polyamines, is synthesized from arginine in two different ways through a total of four enzymes (arginase, ADC, ODC and agmatinase). The ability of polyamines to work is related to the electrical charge they carry. Although first detected in semen, polyamines are found in varying amounts in many cell types. They are mostly observed in cells with the highest amount of rapid turn-over [1, 2].Polyamines are essential for normal cell and tissue functions such as development, growth and tissue repair. They have roles in cell proliferation, cell growth, production of proteins and nucleic acids, repair of extracellular matrix, cell adhesion and signal transduction processes [1, 3, 4]. Polyamines are metabolized by aminoxidases and acetyltransferases [2, 5]. They most likely exert this effect through the nuclear phosphoprotein p53. It was observed that the decrease

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in the amount of polyamine increased p53 gene expression and suppressed cell growth [2, 6–10]. Polyamines stimulate NF-KB binding to the specific response elements on DNA [11]. Polyamines reduce macrophage activation, via NO metabolism. [12].

In this study, the relationships between polyamine synthesis and angiogenesis in the formation of endometriosis were investigated.

MATERIAL AND METHODS

The patients and control groups

Between 01.06.2016–01.06. 2017, thirty-five patients, with endometriosis were included in the study. There was no age restriction for inclusion to the study. Our control group consisted of 35 healthy female hospital personnel without any systemic disease (diabetes or hypertension). In this study, patients who underwent surgical operation for benign reasons in gynecology clinic and diagnosed with endometriosis pathological examination were selected as the study group. As the control group, patients who underwent surgery for benign reasons, who had no chronic disease and no endometriosis disease were selected. Local ethics committee approved this study (Ethics Committee no: 2015-11/40; dates: 24.11.2015), and consent of the participants was obtained for the study.

Collection of samples

Ten mL of whole blood were obtained from the participants, centrifuged at 4000 rpm for 15 minutes, and the serum obtained was aliquoted and stored at -80 °C until analysis. The measurements were repeated 3 times and the mean values of the measurements were used.

Determination of arginase activity

We added 9.9 mL of 2.5 mM MnCl2 to 0.1 mL of hemolyzate and left at 55°C for 10 min preincubation. 0.4 mL of 50 mM arginine solution and 0.4 mL of 100 mM carbonate buffer (pH 9.7) were added to the test and zero-time blank tubes. We placed 1 mL of distilled water in the blank tube and 1 mL of urea standard of 0.1 µmol/mL in the standard tube was placed in the blank tube also. In the zero-time tube, 3 mL of the acid mixture (0.12 M FeCl3 and 20% (v/v) H2SO4 mixture in 56.7% H3PO4) was added followed by addition of 0.2 mL of enzyme source and the mixture was vortexed. We placed 3 mL of acid mixture in standard and blind tubes. To reach the same temperature, the tubes and the enzyme source were allowed to stand in the 37°C metabolic water bath for three minutes. Then 0.2 mL of enzyme source was added in the test tubes, vortexed and left in the metabolic water bath for 15 min at 37°C. At the end of this time, the reaction was stopped by adding 3 mL of acid mixture to the test tubes.We added 2 mL of color separator (0.0036 M thiosemicarbazide + 0.0617 M diacetylmonoxime) to all of the tubes and vortexed. The tips of the tubes were closed and kept in a boiling water bath for 10 minutes and the absorbances were read against the blood at 520 nm.

In the calculation; a net absorbance was obtained by subtracting the zero time blank absorbance from the absorbance of each test tube. Thus, the endogenous urine absorbance at the source of the enzyme was excluded from the calculation. A common factor was found from standard absorbance, standard urea amount and dilution coefficients. Factor calculation was done as follows:

$$\text{Factor} = (0.1\ \mu\text{mol urea /mL}) \times 10 \times 5 \times 4$$

The net sample absorbance was multiplied by the factor in the calculation of the enzyme activity. Enzyme activities were found in µmol urea/mL/hour (absorbance of 0.1 µmol urea/mL).

Ornithine assay

Initially, the Serum sample was mixed with 1/1 water and then with 1/1 TCA 10%. The supernatant was removed after centrifugation for five minutes at 3000 rpm. Then 1 mL of supernatant, 1 mL of distilled water and 1 mL of 0.18 µmol/mL ornithine solution were added to the test tube, blind tube and standard tube respectively. Next, 2.5 mL of glacial acetic acid and 0.25 mL of ninhydrin were added to the tubes. The prepared tubes were vortexed and mixed in a boiling water bath for 30 minutes. After the water bath, the tubes were allowed to cool immediately and the absorbances were measured at 515 nm with a spectrophotometer.

The ornithine level was calculated as follows:

$$\text{Ornithine (µmol/L)} = \frac{\text{Test Absorbance} \times 2 \times \text{Standard Concentration}}{\text{Standard Ornithine Absorbance Value}}$$

Determination of agmatine levels in serum by HPLC method

Sample preparation and derivatization

Each of the 2 mL serum samples obtained from the patients and the controls were separated in two different tubes of 1 mL. The first tube was treated with 100µl of deionized water. The same amount of agmatine standard (2 µM concentration) was placed in the second tube. Before the plasma sample in each tube was centrifuged at 1000 × g for 15 minutes at 4˚C, deproteinization was performed with 700 µL of 1M perchloric acid + 0.1 M hydrochloric acid solution and kept in ice for one hour. 1.5 mL supernatant samples were neutralized with 5M NaOH and mixed with 750 µ volume of derivatization reagent. Preparation of o-phthaaldehyde (OPA) and 2-mercaptoethanol (ME) derivatization reagent: 50mg of OPA was dissolved in 1 mL of methanol and ME solution (53 µL ME was mixed into 9 mL volume of 3% KOH + %3H2BO4 solution) was added.

Extraction, concentration and measurement of serum agmatine

Due to the materials in the serum that may interact with OPA, the obtained samples were firstly extracted with the C18 cartridge. and then injected into the HPLC system. (Chromoband, Machery e Nagel, Duren, Germany). First, this cartridge was washed with water and methanol. 2.25 mL derivatized samples were applied to the cartridge. After this, the cartridge is filled with 3 mL of water, methanol and acetonitrile [1/1/1 (v/v/v)], pH13 adjusted with NaOH) and 100 mL of water, methanol and acetonitrile [1/3/5 (v/v/v)], pH: 3.5, adjusted with acetic acid). Agmatine was eluted with a second 100mL of water, acetonitrile and methanol [1/3/5 (v/v/v)], pH3.5, adjusted with acetic acid). We quickly injected 20 µL of the eluate into HPLC. Agmatine levels were determined using the HPLC system. The HPLC system had a quaternary pump and a fluorescence detector. The excitation wavelength of the fluorescent detector was set to 350 nm and the emission wavelength to 450 nm. Chromatography column C18, Nucleosil 250 mm _4 mm i.d. (Hichrom, Berkshire, UK). The column temperature was set to 400C. The mobile phase was formed from a mixture of 10mM KH_2PO_4 (46%), acetonitrile (34%), methanol (20%) and 4.5 mM octyl sulfate sodium salt (pH = 7). The flow rate was set to be 1 mL/min. Agmatine amounts were measured by comparing plasma sample chromatograms and standard chromatograms [13].

VEGF enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay was used to analyze serum VEGF (Cat. No: E0050Hu). The concentration range of the VEGF standard solution was 3–900 ng/L. Added 40 µL sample 10 µL Human VEGFA antibody, and then 50 µL streptavidin-HRP to wells. Plates were incubated at 37 °C for 60 min in the dark. Following incubation, the plates were run through the buffer solution five times and combined with 50 µl of substrate solution A and 50 µL of substrate solution B. Incubation of the plates was continued for 10 minutes in the dark at 37°C. The reaction was terminated with 50 µL of stop solution, after 10 µL the absorbance value was measured at 450 nm.

Statistical analysis

In this study, having $\alpha = 0.05$; $\beta = 0.20$ and $(1-\beta) = 0.80$, the number of individuals constituting the patient and the control group was determined to be 35 and the strength of the test was found to be $p = 0.80555$. The data obtained in the study were loaded on the SPSS (Ver: 22.0) program and evaluated from the data; when the parametric test assumptions were fulfilled (Kolmogorov-Smirnov), the significance test of the difference between the two means was used, and the Mann Whitney U test was used when the parametric test assumptions were not fulfilled. Regression and the correlation analyses were applied and the error level was taken as 0.05.

RESULTS

The descriptive statistics of endometriosis patients (Tab. 1). Dysmenorrhea and dyspareunia symptoms were more common in the patient group than in the control group. Infertility was detected more frequently in the study group than in the control group. In addition, chronic pelvic pain was seen more frequently in study group, the difference was statistically significant (Tab. 1).

Since we hypothesize that endometriosis is a benign cell division disorder, agmatinase, ODC, ADC, agmatine,

Table 1. The descriptive statistics of endometriosis patient

		Endometriosis	Control	p value
Ages	[years] mean ± SD	38.12 ± 9.12	35.82 ± 8.25	
		Endometriosis (n, %)	**Control (n, %)**	**p value**
Family history of endometriosis	No family history	29 (82.9)	32(8.5)	
	First degree relative	6 (17.1)	3(91.5)	
Infertility	Yes	11 (31.5)	2 (5.7)	
	No	24 (68.5)	33 (94.3)	
Dysmenorrhea symptoms	Yes	27 (77.2)	8 (22.9)	
	No	8 (22.8)	27 (77.1)	
Dyspareunia symptoms	Yes	22 (62.9)	5 (14.3)	
	No	13 (37.1)	30 (85.7)	
Cronic pelvic pain	Yes	20 (57.2)	12 (34.3)	
	No	15 (42.8)	23 (65.7)	

n — number, ($p < 0.05$), compared to control group

Table 2. The results of the Mann Whitney test comparing the levels of agmatinase, ornithinecarboxylase, argininecarboxylase, agmatine, vascular endothelial growth factor, ornithine, arginase in the patient and the control groups

	n	Mean	Standard deviation	$\overline{X}_{sira}$	Σ_{sira}	U	z	p
Patient, agmatinase	36	356.92	352.74	46.42	1671.00	291.000	–4.021	0.000
Control agmatinase	36	110.39	73.06	26.58	957.00			
Patient ODC	36	1876.57	2094.92	40.90	1472.50	629.000	–0.214	0.831
Control ODC	36	826.82	527.85	32.10	1155.50			
Patient ADC	36	1006.56	1051.89	41.29	1486.50	475.500	–1.943	0.052
Control ADC	36	519.56	321.15	31.71	1141.50			
Patient agmatine	36	6.58	4.16	29.64	1067.00	401.00	–2.782	0.005
Control agmatine	36	10.85	6.72	43.36	1561.00			
Patient VEGF	36	181.07	174.05	41.89	1508.00	454.000	–2.815	0.029
Control VEGF	36	105.41	111.56	31.11	1120.00			
Patient ornithine	36	0.09	0.01	39.26	1413.50	548.500	–1.160	0.246
Control ornithine	36	0.08	0.01	33.74	1214.50			
Patient arginase	36	13.59	6.86	45.79	1648.50	313.500	–3.768	0.000
Control arginase	36	8.02	5.10	27.21	979.50			

The cut-off value of $p < 0.05$ was used to interpret the analysis results at the 95% confidence level. $p < 0.001$ is used to show that results are less than 0.1% common; ODC — ornithinecarboxylase; ADC — argininecarboxylase; VEGF — vascular endothelial growth factor

VEGF, ornithine, arginase agmatinase levels were examined in the patients with endometriosis and the obtained values were compared with the control group (Tab. 2). There is a statistically significant difference between the patient and the control groups regarding the agmatinase levels (higher in the patient group) ($p < 0.01$). When the ODC levels were compared, no statistically significant difference was found between the study groups. ($p > 0.05$). There is no statistically significant difference between study groups regarding the ADC levels ($p > 0.05$). There is a statistically significant difference between the patient and the control groups regarding the agmatine levels (higher in the control group) ($p < 0.05$). There is a statistically significant difference between the patient and the control groups regarding the VEGF levels (higher in the patient group) ($p < 0.01$). There is no statistically significant difference between study groups regarding the ornithine levels ($p > 0.05$). There is a statistically significant difference between the study groups regarding the arginase levels (higher in the patient group) ($p < 0.01$).

The patient and the control groups were compared regarding the levels of agmatine, ADC, ODC, agmatinase, arginase, ornithine, and the VEGF. Although the patient group's ODC and ADC levels are increased by approximately 200% compared to the control group, there was no statistically significant difference between the two groups in terms of ornithine levels ($p > 0.05$). Agmatinase, arginase, and VEGF levels were higher in the patient group than those in the control group, and the difference was statistically significant ($p < 0.05$). Agmatine levels were lower in the patient group compared to the control group, and the difference was statistically significant ($p < 0.05$).

As shown in Table 1, it is observed that arginase activity, which is the first enzyme of the polyamine synthesis pathway, is increased in the patient group compared to the control group. This increase is statistically significant ($p < 0.05$).

DISCUSSION

Endometriosis is a common gynecological disease of benign, hormonal origin. Pelvic pain, dysmenorrhea, dyspareunia, and infertility are common clinical findings of endometriosis [14]. Histologically, it is accepted that benign endometriotic lesions occur as a result of genetic errors that can also lead to malignant transformations. Studies have detected loss of heterozygosity and the presence of mutations in tumor suppressor genes. This disease shows high genetic instability that plays a role in the cellular phenotypic changes involved in cancer progression [15].

In the studies conducted in different cancer types including lung [16], colorectal [17], prostate [18], pancreatic [19], skin [20] and stomach [21] cancers, increased arginase activity has been detected and it has been directly associated with cancer. Assuming that endometriosis is a benign cell division disorder disease, our findings support the studies showing increasing arginase activity. To the best of our knowledge, arginase activity has not been studied in patients with endometriosis to date, this makes our results meaningful.

Increased arginase activity will increase ornithine levels. However, no increase in ornithine levels was observed in our study. The reason for this finding is the increase of ODC levels by 200% compared to the control group, although it

was not statistically significant. Increased ODC levels convert ornithine, which is formed twice as fast, to putrescine. The studies reporting that ornithine levels do not change in many cancers such as breast cancer [22] and colorectal cancer is in line with our results. On the other hand, the fact that ODC levels are increased in prostate tissue of patients with prostate cancer [23], in breast cancer [24] and in esophageal cancer [25], supports our data.

ADC, which is the first enzyme in the alternative second pathway of the synthesis pathway, was observed to have doubled in the sera of the patients with endometriosis when compared to the control group, but it was not statistically significant (Tab. 2). Although this increase in ADC seems to be in the direction of agmatine formation, in patients with endometriosis, agmatine levels measured by HPLC showed a decrease of approximately 40% when compared to the control group, and it was statistically quite significant ($p < 0.05$). The reason why the increase in ADC levels could not turn in favor of agmatine is the 300% increase in agmatinase enzyme levels that use agmatine as a substrate. Since this increase in agmatinase levels will cause the formation of putrescine, it is significant ($p < 0.001$). Decreased amount of agmatine cannot control ODC and decrease its proliferation rate [26, 27]. The increase in the amount of enzymes in the polyamine synthesis pathways (ornithine and agmatine pathway) caused an increase in putrescine levels. On the other hand, an increase of 200% in VEGF levels, which is an indicator of angiogenesis, shows that vascularization with proliferation is continued [28]. This statistically significant increase indicates that the apoptosis process has started. According to the study of Vodolazkaia et al. [29]; similar to our findings, women with endometriosis had a significantly higher plasma VEGF concentration compared to controls. VEGF is necessary for the growth of eutopic and ectopic endometriotic tissues [30].

CONCLUSIONS

In conclusion, the increase in the serum levels of polyamine synthesis enzymes may be contributing to the formation of endometriosis. The fact that the decreased agmatine levels do not have an inhibitory effect on ODC, the regulatory enzyme in polyamine synthesis, shifts the synthesis pathway to the direction of the synthesis of major polyamines. Thus, the increase in proliferation causes the continuation of angiogenesis. This is confirmed by the demonstration of the increase in VEGF levels. Revealing the relationship between the enzymes and the molecules in the polyamine synthesis pathway and angiogenesis in patients with endometriosis for the first time will close the gap in this area to a great extent.

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Conflict of interest

All authors declare no conflict of interest.

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