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ASEPTIC INFLAMMATION AS THE ESSENTIAL LINK IN THE PATHOGENESIS OF ENDOMETRIOID DISEASE

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Ключові слова: ендометріоз, індукцйбельна NO-синтаза, аргіназа 1, макрофаги фенотипу M1, макрофаги фенотипу M2

Abstract. Aseptic inflammation as the essential link in the pathogenesis of endometrioid disease. Orlova Yu.A., Hromova A.M., Ketova O.M., Liakhovska T.Yu., Martynenko V.B., Krutikova E.I. The paper was aimed at determination of the quantitative activity of iNOS and Arg1, as well as M1 and M2 phenotype macrophages in women with endometrioid disease to establish their role in the pathogenesis of endometriosis. A prospective study was performed in gynecological units of the medical facilities of Poltava city. 140 women of reproductive age who made up the main group (110 women with endometrioid disease) and the control group (30 women without endometrioid disease) voluntarily participated in the study. All women underwent planned surgical treatment for existing gynecological pathology. Before surgical treatment, women were examined in accordance with the current Orders of the Ministry of Health of Ukraine. The spectrophotometric method was used to determine the enzymatic markers of macrophages (in the endometrium and peritoneal fluid) polarized into M1 (iNOS) and M2 (Arg1) phenotypes. The type of macrophages was determined individually in each patient according to the ratios: in iNOS>Arg1, the M1 macrophage type prevailed; in Arg1>iNOS, the M2 macrophage type prevailed. When examining endometrial samplings in women from the main group, the iNOS indicator was by 1.4 times higher compared to women from the control group. The obtained results at the stage of entry into the abdominal cavity showed that mostly women from the main group suffered from the pelvic adhesion, especially stage 3 and stage 4. Among the obtained results, the increased quantitative activity in the peritoneal fluid of both iNOS and Arg1 in women of the main group was significant compared to the control group. When comparing the stages of endometrioid disease to the rates of quantitative activity of macrophage enzyme markers (in peritoneal fluid), it was found that the increase in the stage of the disease (from stage 3 to stage 4) caused an increase in the quantitative activity of Arg1 by 1.9 times and a decrease in the quantitative activity of iNOS by 2.9 times. Therefore, the planning of surgical intervention for women with endometrioid disease should consider a significant percentage of the pelvic adhesive disease, especially at the severe stages. Initiation of the chronic aseptic inflammatory process in endometrioid disease is caused by an increased quantitative activity of iNOS in the endometrium. In the pathogenesis of endometrioid disease, the presence of M2 phenotype macrophages in the peritoneal fluid is important, while the switching of macrophage phenotypes from a pro-inflammatory subpopulation to an anti-inflammatory one is crucial.

Реферат. Асептичне запалення як ключова ланка в патогенезі ендометріюїдної хвороби. Орлова Ю.А., Громова А.М., Кетова О.М., Ляховська Т.Ю., Мартиненко В.Б., Крутікова Е.І. Метою цієї роботи було визначення кількісної активності iNOS і Arg1 та макрофагів фенотипів M1, M2 у жінок з ендометріюїдною хворобою для встановлення їхньої ролі в патогенезі ендометріозу. Проспективне дослідження виконано в гінекологічних відділеннях лікувальних установ міста Полтави. Добровільну участь у дослідженні взяли 140 жінок репродуктивного віку, які склали основну групу (110 жінок з ендометріюїдною хворобою) та контрольну групу (30 жінок без ендометріюїдної хвороби). Усім жінкам було проведено заплановане хірургічне лікування щодо наявної гінекологічної патології. Жінки перед оперативним лікуванням були обстежені згідно з чинними наказами МОЗ України. Для визначення ензимних маркерів макрофагів (в ендометрії та перитонеальній рідині), поляризованих у M1 (iNOS) та M2 (Arg1) фенотипи, використовувався спектрофотометричний метод. Тип макрофагів визначений індивідуально в кожній пацієнтки за співвідношеннями: при iNOS>Arg1 – переважав

тип макрофагів M1, при Arg1>iNOS – превалював тип макрофагів M2. При дослідженні зразків ендометрія в жінок з основної групи показник iNOS був в 1,4 раза більший, ніж у жінок контрольної групи. Отримані результати на етапі входження в черевну порожнину показали, що жінки з основної групи в значно більшій кількості страждали на злуковий процес органів малого таза, особливо 3 та 4 стадії. Серед отриманих результатів вагомою була й збільшена кількісна активність в перитонеальній рідині як iNOS, так і Arg1 у жінок основної групи порівняно з контрольною. При порівнянні стадій ендометріоїдної хвороби з показниками кількісних активностей ензимних маркерів макрофагів (у перитонеальній рідині) було виявлено, що з підвищенням стадії хвороби (з 3 до 4 стадії) виникає зростання кількісної активності Arg1 в 1,9 раза та зменшення кількісної активності iNOS у 2,9 раза. Отже, планування оперативного втручання в жінок з ендометріоїдною хворобою має враховувати значний відсоток злукового процесу органів малого таза, особливо тяжких стадій. Ініціація хронічного асептичного запального процесу при ендометріоїдній хворобі зумовлена підвищеною кількісною активністю iNOS в ендометрії. У патогенезі ендометріоїдної хвороби важливою є не тільки наявність макрофагів фенотипу M2 в перитонеальній рідині, а більш важливим є факт переключення фенотипів макрофагів з прозапальної субпопуляції на протизапальну.

Currently, endometrioid disease (ED), as one of the most common gynecological pathologies worldwide (10% of women are affected according to the WHO), is of great medical and social concern [1].

The targeted management of women with the above disease is complicated not only by the chronic severe course of ED but also by the lack of a complete picture of the pathogenesis of the disease [2, 3, 4, 5, 6].

The scientific community in the 21st century is actively discussing the theories of the development of ED, and it has been stated that ED is a disease with a chronic inflammatory course and the direct involvement of macrophages of various subpopulations [7]. The local aseptic inflammatory process that occurs in the abdominal cavity of women during menstruation, which enters there retrogradely (normally accompanies up to 90% of healthy women), occurs due to the migration of macrophages during the specified physiological process [8].

Therefore, nowadays, many studies are aimed at establishing the dominant phenotype of macrophages in this pathology. Currently, the leading statement is that macrophages of the M2 subpopulation as anti-inflammatory and immunoregulatory macrophages prevail over the M1 subpopulation of macrophages, which in turn have a pro-inflammatory function. On the other hand, the role of M1 polarized macrophages as an important link in the pathogenesis of ED cannot be ignored [9, 10].

Enzyme markers of polarized M1 macrophages are inducible NO synthase (iNOS), whereas arginase 1 (Arg1) is the enzyme marker of polarized M2 macrophages [11].

iNOS is an essential enzyme in the fertility potential of women [12] and arginase contributes to the maintenance of homeostasis during pregnancy by controlling soluble vascular endothelial growth factor receptor 1 (sFlt1) [13].

Therefore, in order to clarify the pathogenesis and further opportunities for diagnosis and treatment, it is important to determine both the enzyme markers of

macrophages and the direct participation of macrophages themselves.

Purpose – to quantify iNOS and Arg1 activity and M1 and M2 phenotype macrophages in women with ED to establish their role in the pathogenesis of endometriosis.

MATERIALS AND METHODS OF RESEARCH

The studies have been carried out in the gynecological units of Poltava city hospitals: Communal Enterprise (CE) “5th City Clinical Hospital” of the Poltava City Council (PCC), CE “Poltava City Clinical Maternity Hospital” of the PCC, CE “Poltava Central District Clinical Hospital” of the PCC and the Department of Pathophysiology of Poltava State Medical University in the period from 2018 to 2022.

All studies were approved by the commission on biomedical ethics of the Ukrainian Medical Stomatological Academy (minutes No. 174 as of May 28, 2019) and were conducted in accordance with the informed consents obtained from the participants of the study and in accordance with the principles of bioethics set forth in the Helsinki Declaration “Ethical Principles of Medical Research Involving Human Subjects” and “General Declaration on Bioethics and Human Rights (UNESCO)”.

The women included in the study, a total of 140 people, have been assigned into two groups: the main group (MG) included women with genital endometriosis (n=110), the diagnosis and the stage of the disease were made based on the “Unified clinical protocol of the primary, secondary (specialized) and tertiary (highly specialized) levels of medical care “Tactics of management of patients with genital endometriosis”) approved by the Order of the Ministry of Health of Ukraine No. 319 as of April 6 2016 [14] and confirmed by the results of pathomorphological examination [15]. The control group (CG) included women without genital endometriosis (n=30).

The inclusion criteria for the subjects to participate in the study were age (reproductive), benign

ovarian and/or fallopian tube neoplasms, infertility, detected and confirmed during ultrasound and laparoscopic (laparotomy) imaging.

Exclusion criteria were a woman's refusal to participate in the study, uterine and ectopic pregnancy, malignant neoplasms of various localization, abnormalities in the development of organs of the reproductive system.

Women from the described groups were examined and underwent surgery for benign pathology of the ovaries and/or fallopian tubes.

To determine the activities of marker enzymes of macrophages of different phenotypes, after preliminary bacterioscopic and culture testing of discharges from the vagina and cervical canal (in the absence of signs of a local inflammatory process), endometrium was sampled from the women of the comparison groups (MG: n=24; CG: n=27) and peritoneal fluid (PF) during surgical treatment (MG: n=24; CG: n=28).

The endometrium was sampled in the first phase of the menstrual cycle using a Pipelle catheter [16]. PF collection (during surgery) in the amount of 5 ml to 15 ml was performed with a sterile catheter and syringe.

Spectrophotometry method (Ulab 101 Spectrophotometer, Germaine Laboratories, Inc.) was used to determine the activity of marker enzymes of macrophages (iNOS, Arg1) in the endometrium and/or PF in each subject separately. In the individual assessment of the ratio of marker enzymes of macrophages, the pro-inflammatory phenotype of macrophages (M1) was determined if the activity of marker enzymes in each individual investigated substrate was equal to $iNOS > Arg1$. Macrophage polarization according to the anti-inflammatory type (M2) was determined in $Arg1 > iNOS$, respectively.

The study of iNOS activity was carried out by calculating the difference between the total activity of NO-synthases and the activity of constitutive isoforms, the latter, in turn, was measured by the difference in nitrite concentration before and after incubation of tissue homogenate in the incubation medium based on a Tris-buffered saline (pH=7.4), containing 0.3 ml of 320 mM solution of L-arginine (which is a donor of NO-synthase [17]), 0.1 ml of 1 mM solution of NADP₂ and 0.1 ml of 1% amino-guanidine hydrochloride solution [18].

To determine the concentration of nitrites, diazo compounds formed during the reaction with sulfanilic acid followed by the reaction with α -naphthylamine (Griess-Ilosvay's reagent) were measured. This led to the formation of a red derivative (the azo dyes). The concentration of nitrites corresponded to the intensity of staining [19].

The activity of Arg1 was determined by the difference in concentrations of L-ornithine before and after incubation in a phosphate buffer saline containing L-arginine. In the reaction with the Chinard reagent in Khramov's modification, the concentration of L-ornithine was determined due to the formed stained product [20].

Statistical processing of the resulting data was carried out using the MedStat software (serial No. MS00019). We used the methods of descriptive statistics. Checking for the normality of the data distribution was carried out using the Shapiro-Wilk and Kolmogorov-Smirnov tests. In the case of quantitative data of the normal distribution, data presentation was used as means (M) and their standard deviations (SD), in the cases when the data followed non-normal distribution they were presented as medians (Me) and 25% and 75% percentiles (Q1; Q3). Qualitative data of research results were presented as frequencies and their percentage ratios.

The differences between the obtained rates were considered statistically significant at $p < 0.05$ and were determined by parametric and non-parametric methods depending on the results of testing the data for normality (Mann-Whitney U test, Student's t-test, Pearson's chi-squared test (if necessary, with Yates's correction), Fisher's exact test (FET) (calculated if one or more values in the 2*2 conjugation table were less than 5). If necessary, confidence intervals were determined [21].

RESULTS AND DISCUSSION

Prior to the scheduled surgical intervention and the obtained informed consents, endometrial samplings (n=24; n=27) were made in women of MG and CG respectively, without local inflammatory process verified both clinically and laboratory.

When calculating the quantitative activity of marker enzymes of macrophages M1 (iNOS) and M2 (Arg1) in the endometrium of women of the studied groups, a probable difference was established only among the rates of the quantitative activity of iNOS, which was higher in MG compared to CG (1.18 (0.716; 2.445) against 0.844 (0.51; 1.38), Mann-Whitney U test=217; $p=0.04$).

The comparison of the stages of ED and the quantitative activity of marker enzymes in women of MG showed no significant difference, which was established during surgery and confirmed by the pathomorphological study.

The women of both MG and CG groups were admitted to the gynecological units at Poltava hospitals for scheduled surgical treatment. 101 (91.8%) women underwent laparoscopy, and 9 (8.2%) women underwent laparotomy (lower median laparotomy). All women of CG (100%) underwent laparoscopy.

Assessment of the abdominal organs during surgery was carried out according to general principles. Special attention was paid to the presence/absence of the pelvic adhesive disease (PAD) and its stage, endometrioid heterotopias (Ehs) (if present), its size, localization, distribution and number were evaluated.

During the surgical intervention, the visual assessment of pelvic adhesion (the final determination and measuring of PAD stage was performed on the basis

of the American Society for Reproductive Medicine score classification [22]) revealed significant prevalence of PAD in women of MG (n=87 (79 %)) by 1.7 times compared to women of CG (n=14 (46,7%)) ($\chi^2=10,77$; p=0,001).

A comparative assessment of PAD stage among women of both groups showed stage 3 and stage 4 PAD in women of MG compared to controls (Table 1).

Table 1

Pelvic adhesion disease in women of comparison groups, abs.v. (%)

PAD stage	CG (n=30=100%)	MG (n=110=100%)	Differences between the groups, χ^2 (p)* / FET (p)*
1	8 (26.7%)	12 (10.9%)	3.58 (0.06)
2	6 (20%)	16 (14.5%)	0.2 (0.65)
3	-	21 (19.1%)	0.007 (0.04)
4	-	38 (34.5%)	0.00003 (p<0.001)

Note. * – the differences were considered significant at p≤0.05 between the indicators of women in the main group and the control group.

Thus, the resulting data on the predominance of PAD in women of MG by 1.7 times can be explained by the fact that the pelvic adhesion occurs due to EHS in the PF, which, depending on the phase of the menstrual cycle, can bleed, thus creating the conditions for the formation of an inflammatory process, which is present in ED [23, 24].

During surgery, a visual assessment of the ED women of the main group showed that stage 3 and stage 4 of ED prevailed.

Importantly, EHS were not visualized in the abdominal cavity in 11 (10%) women of the MG; however, ovarian tumor-like masses were noted and ED was histologically verified. The severity of ED in the women was determined after the results of histological study.

In the main group, stage 1, stage 2, stage 3 and stage 4 ED was detected in 6 (5.4%), 2 (1.8%), 53 (48.2%) and 49 (44.6%) women, respectively.

During the surgical intervention, PF samplings were made to count the marker enzymes of macrophages (iNOS and Arg1) in the MG (n=24) and CG (n=28).

It has been established that the quantitative activity of iNOS in PF prevailed by 1.7 times in women of MG, the same parameter in women of CG was (0.826±0.683 μmol/min/1 g of protein (95% CI 0.538-1.115) vs. 0.477±0.235 μmol/min /1 g of protein (95% CI 0.386 – 0.569) respectively; p=0.02). Arg1 in the PF of women of MG was also significantly increased compared to the values of women from CG (0.511 (0.434; 0.817) μmol/min/1 g of pro-

tein versus 0.293 (0.243; 0.672), respectively; Mann-Whitney U test=226, p=0.04).

In our previous study, calculation of the marker enzymes of macrophages of M1 and M2 phenotypes in PF established that in the pathogenesis of ED, the leading role was assigned to M2 phenotype macrophages. It has been confirmed that in PF the polarization of macrophages according to the M2 phenotype was by 2 times higher in MG women compared to controls ($\chi^2=4,59$; p=0,03). However, it was notable, that the number of MG women in whom M1 type of macrophage polarization and M2 type of macrophage polarization were determined using their marker enzymes in PF was almost the same (41.7% and 58.3%, respectively) [10]. This fact led us to the idea of correlation the stages of ED with the rates of marker enzymes of macrophages of M1 and M2 phenotypes in PF. Table 2 shows the results of the correlation of the rates of women of the MG.

Noteworthy, one woman (4.2%) of the MG out of the rest subjects (n=24=100%) had stage 1 ED, therefore, the rates of quantitative activity of marker enzymes were not taken into account (statistically small number of samples).

Table clearly showed significant decrease in the quantitative activity of the marker enzyme of macrophages of the M1 phenotype in PF of women of the MG with deterioration of the stage of ED, namely, iNOS in the PF was by 2.9 times lower in stage 4 ED compared to the stage 3 ED.

Table 2

The ratio of the quantitative activities of the marker enzymes of macrophages M1 (iNOS) and M2 (Arg1) in the peritoneal fluid of women with endometrioid disease to its stage, (Me (Q1; Q3))

The rates of quantitative activity of marker enzymes of macrophages	Women with stage 3 endometrioid disease (n=17)	Women with stage 4 endometrioid disease (n=6)	Reliability, Mann-Whitney U test (p)
iNOS μmol/min/1 g of protein	0.859 (0.340; 1.546)	0.297 (0.208; 0.347)	U=21 (0.02)
Arg1 μmol/min/1 g of protein	0.453 (0.346; 0.571)	0.844 (0.766; 0.979)	U=24 (0.03)

Note. * – the differences were considered significant at $p \leq 0.05$ between the indicators of women of the main group with different stages of endometrioid disease.

With the marker enzyme of M2 macrophages, namely Arg1, the opposite pattern occurs, that is, a significant increase in the quantitative activity of this enzyme by 1.9 times is noted in PF with progression from stage 3 to stage 4 ED.

We hypothesize that significant increase in the quantitative activity of the macrophage marker enzyme iNOS in the endometrium of women with ED by 1.4 times, as compared to women of CG, is the manifestation of the existing aseptic inflammation of endometrial cells in endometriosis. The findings of our study do not contradict the data from the available scientific literature. It is believed that this enzyme increases in people with a wide variety of non-infectious diseases with underlying inflammation, such as chronic diseases of the heart, skin, lungs, kidneys, etc. [25]. Another very important aspect is the fact that ED due to the similarity of some pathogenetic processes (including the ability to proliferate and avoid apoptosis) can be considered, according to the up-to-date data, as one of the types of oncology [26], and it is currently known that in female oncological diseases of the reproductive system, an increase in iNOS is also noted [27].

We grounded not only on the theory of the underlying ED inflammation, but also the implantation theory, which is based on the effect of retrograde blood flow on the pathogenesis of ED [1]. However, taking into account that according to the latest data, up to 90% of women have a physiological reverse flow of menstruation to abdominal cavity [8], we hypothesize that aseptically endometrial inflamed cells migrate to the abdominal cavity with retrograde blood flow, where a local aseptic inflammatory process develops.

Noteworthy, we detected a 1.7-fold increase in the quantitative activity of iNOS even in PF. Notwith-

standing the confirmed fact of the influence of M2 macrophages on the pathogenesis of ED [10], we have found that inflammation in endometriosis begins with the switching of polarization from the pro-inflammatory phenotype of M1 macrophages to the anti-inflammatory phenotype of M2 macrophages in more severe stages of ED.

CONCLUSIONS

1. Planning of surgical intervention for women with endometrioid disease should consider a significant percentage of the pelvic adhesion, especially at the severe stages.
2. Initiation of the chronic aseptic inflammatory process in endometrioid disease is caused by an increased amount of iNOS in the endometrium.
3. In the pathogenesis of endometrioid disease, the presence of M2 phenotype macrophages in the peritoneal fluid is important, while the switching of macrophage phenotypes from a pro-inflammatory subpopulation to an anti-inflammatory one is crucial.

Contributors:

Orlova Yu.A. – conceptualization, writing – original draft, writing – review and editing, formal analysis, investigation);

Hromova A.M. – project administration, supervision, validation;

Ketova O.M. – data curation, visualization;

Liakhovska T.Yu. – methodology;

Martynenko V.B. – investigation, resources;

Krutikova E.I. – writing – review and editing, funding acquisition.

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