

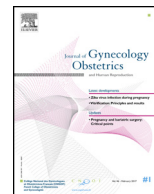


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Original Article

Phagocyte function of peripheral neutrophil granulocytes and monocytes in endometriosis before and after surgery

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ABSTRACT

Introduction: Endometriosis is a chronic systemic disease, which influence negatively the quality of life of affected women and responsible for infertility and chronic pelvic pain. Pathophysiology of the disease is still enigmatic, but insufficient immune surveillance may play a role in it. Peripheral natural immune cell function is rarely examined. The aim of the study was to examine phagocyte function of peripheral neutrophil granulocytes and monocytes, whether this phagocytic activity is affected by the presence or removal of endometriotic lesions in women with endometriosis.

Material and methods: Twenty-six preoperative, 13 postoperative samples from women with endometriosis, 23 samples from healthy women, 14 pre- and postoperative samples from the surgical control group were enrolled. Cells were isolated from peripheral blood samples, marked and evaluated for the phagocytosis index with immunofluorescent microscope after phagocytizing the zymosane molecules.

Results: Phagocyte function of monocytes and neutrophil granulocytes decreased significantly women with endometriosis before surgery compared to healthy controls. However, 7 days after surgery the postoperative values showed significant improvement compared to the preoperative results of women with endometriosis. This increment reached the values of the healthy women. In the surgical control group no difference was detected between the pre- and postoperative outcomes.

Discussion: Decreased phagocyte function of the examined cells, which can be the result of the circulating immunosuppressive factors, may play a role in the deficient clearance of ectopic endometrial tissue. Based on the postoperative results, these immunosuppressive factors may be reduced or eliminated 7 days after surgery in women with endometriosis.

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Introduction

Endometriosis is a chronic benign disease which causes subfertility, chronic pelvic pain and dysmenorrhea among women in fertile ages. It is characterized by endometrial glands and stroma outside the uterine cavity, mainly on the pelvic peritoneum, ovaries and rectovaginal septum [1].

The pathophysiology of the disease is still unknown, but retrograde menstruation remains the primary mechanism put forth to account for it. It was described by Sampson [2] at first and

recent studies have supported the theory by documenting that retrograde menstruation and peritoneal presence of endometrial cells occur frequently in cycling women [3]. Although most women have retrograde menstruation, not all women with retrograde menstruation have endometriosis [4]. It raises the possibility that endometriosis is a complex and probably systemic disease and altered immunity should have play essential role in pathophysiology. After retrograde menstruation the implant should attach to the peritoneal surface, invasion, proliferation and neovascularization should occur to survive in the abnormal localization [5].

The function of the immune system is to detect and destroy foreign or abnormal cells. Some data are available which support the theory, that impaired cleansing of the endometrial cells from the peritoneal cavity plays a crucial role in the implantation. Next to the impaired immunological function, chronic inflammation can be also part of the disease. Evidence of these changes are the proliferation and activation of macrophages, secretion of

Abbreviations: DAPI, 4', 6-diamidino-2-phenylindole; EDTA, Ethylenediamine tetraacetic acid; FITC, fluorescein-isothiocyanate; FITC-OZ, opsonized and labeled with fluorescein-isothiocyanate Zymosan A particles; PI, phagocytosis index.

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proinflammatory, growth and angiogenic factors, and increase natural killer (NK) and T lymphocytes, their dysfunction and reduced cytotoxic activity (in peritoneal fluid and peripheral blood of women with endometriosis) [6,7].

Most of the publications are deal with the functional changes of macrophages and NK cells. Not only these cells, but also lymphoid cells, neutrophil granulocytes and monocytes have been thought to play a role in maintaining homeostasis within the peritoneal cavity with endometriosis. As well as the existence of local inflammation, adhesion formation and altered peritoneal fluid cytokine composition [8]. Other changes can occur with the different immune cells in the peritoneal cavity and in the peripheral blood.

Neutrophil granulocytes and monocytes are immune cells which are part of the innate immune system. These cells may contribute the pathology of endometriosis. Takamura et al. [9] shown, that depletion of neutrophil granulocytes in mice with early stage of endometriosis cause a significant reduction in number and total weight of endometriotic lesions. One of the main function of neutrophils and monocytes is the phagocytosis, which is a complex process involving recognition of an antigen and internalization of the foreign molecule or cell into a phagosome and destroy it. Macrophages which are originated from monocytes have important role in intra-ovarian events during different stages of the cycle too [10].

We speculated that neutrophil granulocytes and monocytes are taking part in the cleansing of peritoneal cavity from ectopic endometrial cells, and because endometriosis is a systemic disease, the phagocyte function of these immune cells are changed in the peripheral circulation also.

Our aim was to measure the phagocyte function of neutrophil granulocytes and monocytes in patients with endometriosis before and after surgery and compare it to the same results of healthy women, for the better understanding of pathophysiology of endometriosis. Because there is absolutely no available data regarding the effects of laparoscopic interventions to the phagocyte function in benign gynecological disorders a surgical control group was also selected in order to exclude the immunoenhancement effect of the surgery. It consisted of women who needed surgery because of myomas where altered phagocyte function couldn't be detected.

Materials and methods

Study population

After obtaining informed consent with the permission of the Ethics Committee of the University of Debrecen, peripheral blood was collected in vacutainer test tubes containing EDTA (Becton-Dickinson, Cedex, France) from 26 women with endometriosis, 23 healthy women as a control and 14 women with myomas as a surgical control group.

Endometriosis was evaluated and completely removed during laparoscopic surgery, and confirmed by histologically. Endometriosis was staged according to the revised criteria of the Revised American Society for Reproductive Medicine classification of endometriosis [11]. All of the women ($n=26$) had stage I-II endometriosis.

In surgical control group ($n=14$) patients with myomas underwent laparoscopic myomectomy and had the same preoperative preparations as patients with laparoscopically removed endometriosis.

Preoperative peripheral venous samples were collected in the morning before surgery and from the same person postoperative peripheral venous samples were also taken 7 days after surgery in both group. Blood collection was performed once in healthy women.

All patients and healthy women ($n=23$) were in the follicular phase of the menstrual cycle at the time of blood sampling to rule out the potential effect of hormonal status. All patients had normal menstrual cycle, normal blood count and no history of any chronic systemic disease. None of the women had undergone any hormonal or immunomodulatory therapy for at least six months before the blood sampling.

Preparation of FITC labeled and opsonized zymosan particles

Zymosan particles were labeled with fluorescein isothiocyanate (FITC) as described previously [12]. Briefly, the particles (1×10^8 /mL) were incubated in carbonate buffer, pH 9.6, containing FITC at a final concentration of 0.01 mg/mL for 60 min at 37 °C. Then they were washed three times and opsonized in Hanks' solution containing 50% human AB serum at 37 °C for 30 min. The labeled and opsonized particles (FITC-OZ) were washed three times and stored at -20 °C in Hanks' solution (3×10^7 /mL) until the phagocytosis assay.

Separation and examination of phagocyte function of granulocytes and monocytes

Mononuclear cells and granulocytes were separated as described previously [13,14]. Blood samples were mixed with an equal volume of Hanks' solution, pH 7.4 and layered on the top of a discontinuous Ficoll gradient (1.077 and 1.119 g/mL) and centrifuged at 400 g at 20 °C for 30 min. Mononuclear and polymorphonuclear cells were collected from the top and interface of the Ficoll layers, respectively. Then the cells were washed twice with Hanks' solution. Their viability was determined by trypan blue exclusion test and found to be 96–98%. The purity of granulocyte suspensions varied between 95% and 98%, as judged by morphology.

In the phagocytosis assay, phagocytosis of FITC-OZ was determined as described previously [15,16]. Briefly, 1×10^5 granulocytes and mononuclear cells in 300 μ L aliquots of Hanks' solution containing 5% heat inactivated human AB serum were placed into the wells of chamber slides to allow the cells to adhere for 30 min at room temperature. Subsequently, the non-adherent cells were removed by washing, the adherent cells and FITC-OZ (3×10^6 /well) were incubated in 300 μ L aliquots of Hanks' solution at 37 °C in 5% CO₂/95% humidified air for 60 min. Following incubation, the fluorescence of non-ingested zymosan particles was quenched by trypan blue solution [17,18]. Monocytes were identified by indirect immunofluorescent method. The cells were labeled with anti-CD14 monoclonal antibody and then with immunoglobulin G-conjugated Dylight 594 fluorescent dye. The

Table 1

Clinical characteristics of women with endometriosis, healthy women and surgical control group.

Clinical data	Endometriosis (preop. $n=23$) (postop. $n=13$)	Healthy control($n=23$)	Surgical control (preop. $n=14$) (postop. $n=14$)	p-value
Age (years)	335 $\pm$ 6,5	3013 $\pm$ 358	3608 $\pm$ 913	NS
BMI ^a (kg/m ²)	2195 $\pm$ 281	2116 $\pm$ 107	2234 $\pm$ 338	NS
Parity ^b	1 (0–3)	1 (0–3)	2 (0–13)	NS
Gravidity ^b	0 (0–1)	0 (0–2)	1 (0–2)	NS

Mean values $\pm$ SD are presented. NS: not significant. ^a BMI: body mass index. ^b Values are expressed as median (range).

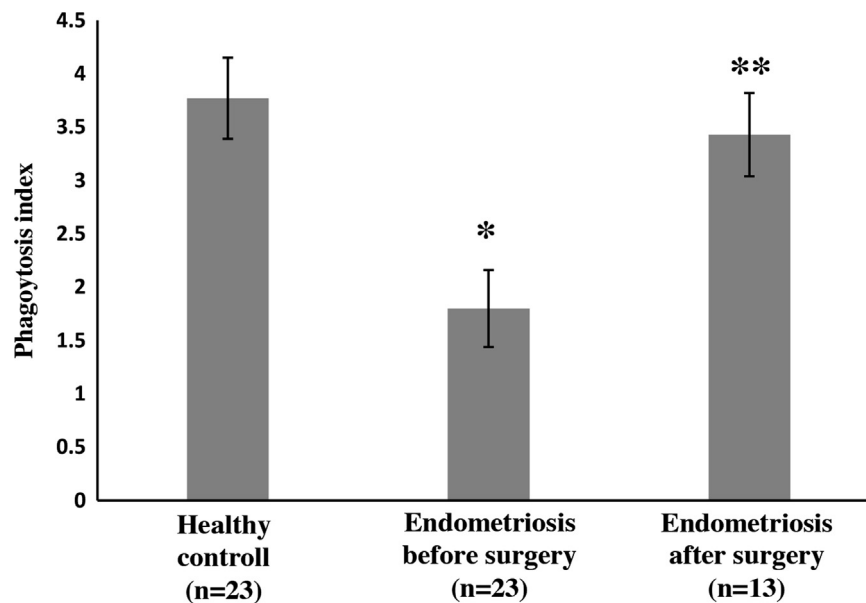


Fig. 1. A: Phagocytosis index of neutrophil granulocytes separated from healthy women and patients with endometriosis before and after surgery. Mean values $\pm$ SD are shown. * $p < 0.0001$ preoperative values of patients with endometriosis versus healthy controls; ** $p < 0.0001$ preoperative values of patients with endometriosis versus postoperative values of patients with endometriosis.

B: Phagocytosis index of neutrophil granulocytes separated from healthy women and surgical control patients before and after surgery. Mean values $\pm$ SD are shown.

nuclei of the granulocytes and monocytes were stained with DAPI and the slide was then removed from the chamber for microscopic evaluation. The number of FITC-OZ/cell was determined with an Axioplan fluorescent microscope (Zeiss Oberkochen, Germany) by examining 2×100 cells in randomly selected microscopic fields. Then the phagocytosis index (PI), the average number of ingested particles/cell, was calculated and presented as mean $\pm$ SD.

Statistical analysis

Shapiro–Wilk test was used to verify normal distribution of the data. For comparison of the average values of continuous variables the Student's *t*-test and analysis of variance were used. Values of $p < 0.05$ were considered as statistically significant.

Results

The clinical characteristics of the study population are presented in Table 1. As shown, there were no significant differences in age, gravidity, parity and body mass index (BMI) at the time of blood sampling women with endometriosis, healthy women and surgical controls.

Phagocyte function of neutrophil granulocytes

Phagocytosis index of neutrophil granulocytes from peripheral blood of women with endometriosis and healthy women is illustrated in Fig. 1A, results of surgical control group and healthy women illustrated in Fig. 1B. As shown, preoperatively isolated granulocytes from women with endometriosis demonstrated a significantly decreased phagocytosis index (PI: 180 ± 036 ; $p < 0.0001$) compared to healthy controls (PI: 377 ± 038). However phagocytosis indexes of granulocytes from women with endometriosis 7 days after surgery showed significant improvement (PI: 343 ± 039 ; $p < 0.0001$) compared to the results of granulocytes separated before surgery (180 ± 036). The postoperative improvement regarding phagocyte function was detected to be so high it reached the level of healthy granulocytes. Phagocytosis indexes of pre- (347 ± 028) and postoperative

(356 ± 039) values of granulocytes derived from the surgical control group did not differ significantly.

Phagocyte function of monocytes

Phagocytosis index of monocytes from peripheral blood of women with endometriosis and healthy women is illustrated in Fig. 2A, results of surgical control group and healthy women illustrated in Fig. 2B. As shown, monocytes from women with endometriosis demonstrated a significantly decreased phagocyte index (PI: 1.73 ± 0.32 ; $p < 0.0001$) compared to healthy controls (PI: 3.68 ± 0.37). Nevertheless, monocytes isolated 7 days after surgery (340 ± 036 ; $p < 0.0001$) illustrated significant increment compared to the preoperative results (PI: 1.73 ± 0.32). Comparing the postoperative phagocytosis index of monocytes from women with endometriosis and the results of healthy women no difference was detected. Phagocytosis indexes of pre- (347 ± 028) and postoperative (356 ± 039) monocytes derived from the surgical control group did not differ significantly.

Discussion

The public health significance of endometriosis is significant. This disease affects 10–15% of women in reproductive age, 50–60% of women with pelvic pain and dysmenorrhea and up to 50% of women with infertility [1,19]. It is a complex and probably multisystem disease, with multiple factors involved in its pathophysiology. In order to the survival of endometrial tissue outside of the uterine cavity altered function of adaptive and natural immune system may involve, besides genetic and environmental factors. Despite the intensive research, clearing the endometrial cells from the peritoneal surface is poorly understand.

Endometriosis is associated with systemic low grade chronic inflammation state, which should be also responsible for the mechanism of pelvic pain, too [20]. Besides, ectopic endometrial deposits generate inflammation with increased number of activated immune cells and causes extensive expression of inflammatory cytokines in peritoneal fluid [21].

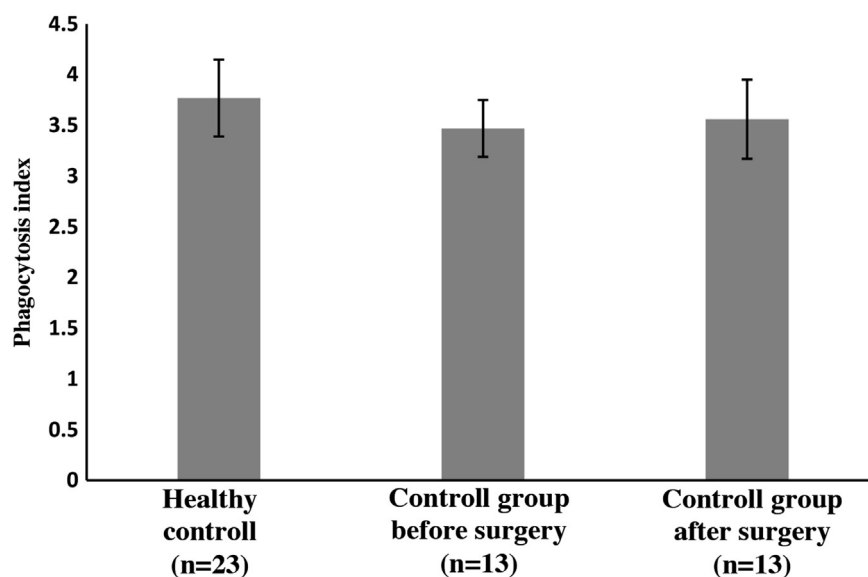


Fig. 2. A: Phagocytosis index of monocytes separated from healthy women and patients with endometriosis before and after surgery. Mean values $\pm$ SD are shown. * $p < 0.0001$ preoperative values of patients with endometriosis versus healthy controls; ** $p < 0.0001$ preoperative values of patients with endometriosis versus postoperative values of patients with endometriosis.

B: Phagocytosis index of monocytes separated from healthy women and surgical control patients before and after surgery. Mean values $\pm$ SD are shown.

Most of the natural immune system related research is with the immune cells in the peritoneal cavity, and some of it are contradictory. There are data about decreased NK cell cytotoxic activity, increased macrophage number and cytokine production in peritoneal fluid of endometriosis patients [22]. Neutrophils and monocytes plays pivotal roles in all inflammatory diseases including inflammatory and non-inflammatory. Neutrophil granulocytes intraperitoneal number is increasing in early stage of endometriosis, and decreasing with advanced stages [23].

Peripheral immune cell function in endometriosis is rarely examined. Macrophages and monocytes have been identified as important cells in the regulation and promotion in endometriosis generation. Monocytes shown an increased cytokine production, macrophage number, concentration and activation status are enhanced [24]. Neutrophil to lymphocyte ratio correlates positively with the severity of endometriosis, but this result is contradictory [25]. Next to the several examined functions of these peripheral cells the phagocyte function has not been examined yet in scientific literature in patients with endometriosis.

Due to our results, peripheral neutrophil granulocytes and monocytes phagocyte function is decreased significantly in women with endometriosis compare to healthy women. This alteration in phagocyte function may leads to the decreased clearance of endometrial tissue from the abdominal cavity. Neutrophils and monocytes also play a role in the defending mechanisms against tumor cells or infections. Decreased phagocyte function of these immune cells also can be an explanation of increased risk of lower genital tract infections, endometrial cancer and, especially in case of endometriomas, ovarian cancer in patients with endometriosis [26–28]. We speculate that the alteration in phagocyte function in endometriosis patients may be caused by circulating factors in peripheral blood. These may be produced in connection with the presence of ectopic endometrial cells since the postoperative values revealed significant improvement and the possible effects of the intervention was ruled out by the results of the surgical controls.

Limitation of our study is the relative low number of patients and the lack of identification of suspected plasma factors. In a multicentre study with higher number of cases and additional

methods for the identification of plasma factors would make the results much more effective.

As a conclusion, peripheral neutrophil granulocytes and monocytes phagocyte function is decreased in patients with endometriosis compare to healthy women, which can be a part of immunological changes observed in endometriosis and also the effect of the ectopic endometrial lesions itself. Level of some anti-inflammatory factors, such as IL-10, are increased in peripheral blood of women with endometriosis so the potential influence of IL-10 to this function cannot be excluded but factors that mediate phagocyte function, their origin and identification of these require further investigation [29]. Our results contribute to the better understanding of the still enigmatic pathophysiology of endometriosis.

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CRediT authorship contribution statement

Luca Lukács: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Anna Rebeka Kovács:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **László Pál:** Formal analysis, Investigation, Writing - review & editing. **Sándor Szűcs:** Conceptualization, Methodology, Writing - review & editing. **Ágnes Kövér:** Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Rudolf Lampé:** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

None.

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