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A Comparative Analysis of Oxidative Stress and Inflammatory Biomarkers in Different Stages of Endometriosis

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Abstract

Background: The fundamental mechanisms behind the causes and development of endometriosis are still poorly understood. Therefore, identifying biomarkers that can help with early detection and targeted treatment is crucial for effective management of this disease. This study aimed to compare total antioxidant capacity (TAC), the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx), and the concentrations of interleukin-6 (IL-6) and phenylalanine (Phe) across different stages of endometriosis.

Methods: The plasma samples were collected from women with endometriosis who had undergone laparoscopic surgery. The stages were confirmed by a gynecologist, with 30 plasma samples from stages I-II (mild) and 30 from stages III-IV (severe). The obtained measurement data were first normalized and tested for normality, followed by analysis using the t-test and Mann-Whitney U test. The p-value below 0.05 was considered statistically significant. The sample size was determined based on Cohen's guideline of 30. Biomarker levels were assessed using ELISA and colorimetric techniques.

Results: TAC levels, GPx, and SOD activities, as well as Phe concentration significantly differed between endometriosis stages I-II and III-IV ($p<0.05$). These measured biomarkers were higher in stage I-II. On the other hand, although IL-6 levels were higher in stages III–IV, the differences between stages were not statistically significant.

Conclusion: The potential of TAC, SOD, GPx, and Phe as biomarkers for the early diagnosis and treatment of endometriosis underscore the roles of inflammation and oxidative stress in the pathogenesis of the disease, providing insights that may aid in developing more targeted diagnostic and therapeutic strategies.

Keywords: Biomarkers, Endometriosis, GPx, IL-6, Phenylalanine, SOD, TAC.
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Introduction

Endometriosis is a prevalent and non-malignant chronic gynecological condition that is estrogen-dependent. It is characterized by the presence of endometrial tissue outside its normal location (1). Pelvic pain and infertility are the primary symptoms, impacting 6%−10% of women in

their reproductive years. Studies have indicated that the occurrence of endometriosis in women with infertility might reach up to 20%−30% (2).

Oxidative stress has been suggested as a potential contributing factor in the pathogenesis of endometriosis. Reactive oxygen species (ROS) can enhance cell proliferation, potentially reducing the effectiveness of treatments in alleviating symptoms and prolonging the time to recurrence (3). Furthermore, endometriosis is an inflammatory condition triggered by immune-related cells releasing cytokines that recruit additional immune cells and stimulate ectopic endometrial cell formation (4), with elevated levels of IL-6 being associated with the disease (5). Additionally, researchers suggest that phenylalanine could be related to inflammatory markers and it may also play a role in the development of endometriosis by altering serum levels, which can inhibit the invasion and migration of tumor cells and reduce cell proliferation (6-8).

Several potential biomarkers have been suggested to be involved in endometriosis, including CA125, IL6, and IL17 (9). However, these biomarkers are not yet utilized in diagnostic tests (10). The purpose of the current study was to investigate the correlation between levels of oxidative stress and inflammation biomarkers in the mild and severe stages of endometriosis (11, 12).

Methods

Subjects: The subjects were female patients aged 20 to 40 years who were diagnosed with endometriosis and underwent laparoscopic surgery at Avicenna Fertility Center affiliated to Avicenna Research Institute, Tehran, Iran between September 2022 to September 2023. All participants provided their consent by signing the enrollment forms for the study. The exclusion criteria were alcohol, drugs, and caffeine consumption, obesity, diabetes, and history of breast cancer. The stage of disease was confirmed by the gynecologist and the conclusive diagnosis was verified through a histopathological report. The classification was based on ESHRE guidelines. Stages I-II were considered mild, and stages III-IV were classified as severe (13). A total of 60 subjects were enrolled in this study with 30 patients in the mild stage, and 30 patients in the severe stage. This study was approved by the Avicenna Ethics Committee (IR.ACECR.Avicenna.REC1399.007).

Plasma samples: Prior to laparoscopic surgery, a blood sample was collected from each patient into an EDTA tube and kept on ice. All samples were immediately ultra-centrifuged for 10 *min* at 1000× *g* and 4°*C*. After collection, the plasma samples were frozen at -80°*C* until analysis of plasma enzyme activity.

TAC levels, SOD, and GPx activities alongside Il-6 and phenylalanine concentrations were assessed using commercially available assay kits according to the manufacturer's instructions. The following kits were used in the current study: TAC assay kit (Navand Salamt, Iran); the superoxide dismutase (SOD) activity assay kit (Navand Salamat, Iran); glutathione peroxidase (GPx) activity assay kit (Navand Salamat, Iran); human interleukin-6 ELISA kit (Navand Salamt, Iran); and PKU ELISA kit (Kimia Pajoohan, Iran).

Plasma TAC levels were measured by colorimetry employing 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), a next-generation, more stable reagent. Antioxidants reduce the ABTS radical in a manner proportional to their concentration and antioxidant capacity. The alteration in color was quantified by the variation in absorbance at 660 *nm*. This procedure was used with an automated analyzer and the assay was calibrated using Trolox.

SOD activity in plasma was measured by inhibiting pyrogallol auto-oxidation reaction. The chromophore produced had a maximum absorbance at 405 *nm*. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

GPx activity was measured based on the glutathione peroxidase–catalyzed reduction of cumene hydroperoxide, during which reduced glutathione (GSH) was oxidized to glutathione disulfide (GSSG). Subsequently, GSSG was converted back to GSH by glutathione reductase, consuming NADPH in the process. In this assay, the consumption of NADPH served as an indicator of GPx activity, which was measured spectrophotometrically at 340 *nm*. IL-6 concentration was measured using anti-IL-6 human monoclonal antibodies. Absorbance was measured at 450 *nm* using a microplate reader. IL-6 concentrations in the samples were determined by comparing absorbance values to a standard curve using IL-6 standards.

Phenylalanine concentration was measured using PKU screening kit, which employs engineered recombinant microbial phenylalanine dehydrogenase (PheDH) to catalyze the NAD^+-dependent oxidative deamination of blood phenylalanine. The NADH produced was converted to NAD^+ by the

coupled diaphorase/tetrazolium reaction system, preventing product inhibition and driving the dehydrogenase reaction toward completion. Absorbance was measured at 490 *nm* using a microplate reader.

Statistical analysis: All data were analyzed using Prism software version 9 (GraphPad Software, USA). Data were analyzed for significance using normality tests, followed by Student's t-test or the Mann–Whitney U test, as appropriate. Receiver operating characteristic (ROC) curves for the biomarkers were generated using Prism software. The sample size was calculated based on the effect size of 1, a power of 0.8, and an alpha level of 0.05, resulting in a required sample size of 30.

Results

Demographics: From November 2021 to June 2023, a total of 80 blood samples were prospectively collected from female patients who underwent laparoscopic surgery for suspected endometriosis at Avicenna Fertility Center. Among these patients, 70 were diagnosed with endometriosis. Of the 70 diagnosed patients, 60 met the eligibility criteria for further analysis, with 30 classified as having mild stages of endometriosis and the remaining 30 classified as having severe stages. After evaluating their medical reports, 10 were excluded from the study group due to prior hormonal treatment and abnormal body mass index (BMI). Data is shown in table 1.

Oxidative stress and inflammation biomarkers: Each group consisted of 30 samples, and colorimetric techniques were used to measure TAC levels as well as SOD and GPx activities. TAC, SOD, and GPx levels differed significantly ($p< 0.05$) between mild and severe stages (Table 2).

ELISA was used to quantify IL-6 and phenylalanine as inflammatory biomarkers, as shown in table 2. IL-6 levels were higher in severe stages, but the difference was not statistically significant (Table 2). In contrast, phenylalanine levels were significantly decreased in severe stages ($p<0.05$). The area under the ROC curve for the biomarkers is shown in table 2.

Discussion

Noninvasive diagnostic techniques can reduce surgical risks, improve accessibility to diagnostic testing, and optimize treatment efficacy by facilitating earlier and more precise detection of endometriosis (14-16). Efforts to identify potential biomarkers for endometriosis have leveraged insights from genomics, transcriptomics, proteomics, and metabolomics. Thus far, none of the identified biomarker candidates have demonstrated the required level of specificity and sensitivity necessary to match the diagnostic accuracy of lap-

Table 1. Summary of patient enrollment and classification by endometriosis stage

	Mild stages (n=30)	Severe stages (n=30)	p-value
Age	36	36.6	0.9
BMI	23.74	25.44	0.8
Cycle length	28.7	29.125	0.56
Dysmenorrhea severity (from 1 to 10)	7.75	7.77	0.77
CA-125 levels	63.705	40.953	0.3

Table 2. Measured total antioxidant capacity, proteins, and metabolite across different stages

Name	Stage I-II			Stage III-IV			p-value	Area under the ROC curve
	Mean±SD	Lower 95%CI	Upper 95%CI	Mean±SD	Lower 95%CI	Upper 95%CI		
TAC	0.5452±0.19 (*mM*)	0.433	0.6574	0.3476±0.18 (*mM*)	0.254	0.441	0.0059	0.7857
SOD1	0.9111±0.053 (*U/ml*)	0.8906	0.9316	0.8746±0.037 (*U/ml*)	0.9316	0.8884	0.0053	0.7107
GPx	6.486±3.279 (*mU/ml*)	4.738	8.233	4.039±1.167	3.392	4.685	0.0122	0.7604
IL-6	45.38±24.44 (*Pg/ml*)	34.25	56.50	46.53±27.92 (*Pg/ml*)	34.15	58.91	0.9568	0.5054
Phe	44.63±27.42 (*mg/dL*)	30.87	59.06	21.51±18.51 (*mg/dL*)	10.82	32.20	0.0105	0.7164

TAC: Total Antioxidant Capacity, SOD1: Superoxide Dismutase, GPx: Glutathione Peroxidase, IL-6: Interleukin-6, Phe: Phenylalanine Levels Measured at Different Stages of Endometriosis.
Data were analyzed using normality tests, followed by Student's t-tests or Mann–Whitney U tests, as appropriate

aroscopy (17). In recent years, the significance of identifying noninvasive blood biomarkers and the role of oxidative stress in endometriosis has gained increasing attention (18). The purpose of the current study was to find early-stage diagnostic biomarkers to avoid the risk of surgery. Additionally, to the best of our knowledge, this is the first study that targeted phenylalanine in endometriosis as a potential biomarker.

Endometriosis is a condition characterized by persistent pelvic pain, infertility, inflammation, and altered reactive oxygen species levels. Disease progression can lead to oxidative stress and tissue damage. Previous studies have suggested that reduced total antioxidant capacity and its components may contribute to mild endometriosis, promoting chronic inflammation and tissue remodeling (19). Our study confirmed these findings.

SOD has a crucial role in protecting cells against oxidative damage caused by ROS. Nonetheless, the hydrogen peroxide produced remains reactive and in the presence of free ferrous iron can generate hydroxyl radicals that worsen disease progression (20). The involvement of SODs, particularly Cu and Zn SOD, has been implicated in several illnesses, including cancer, Parkinson's disease, and several neurological disorders. SOD activity appears essential to mitigate oxidative stress. Studies have demonstrated that SOD rises at mild stages of endometriosis compared to severe stages (21). Consistent with previous studies, our results showed a statistically significant up-regulation of SOD in stages I-II in comparison to stages III-IV ($p<0.002$).

GPx, a selenoenzyme, regulates cellular ROS, protecting against numerous illnesses. It eliminates hydrogen peroxide and prevents ROS-induced ROS generation in both mitochondria and cytoplasm. GPx catalyzes the reduction of H_2O_2 and low-molecular-weight hydroperoxides, functioning as a peroxidase (22, 23). Some studies have shown that GPx levels do not change in endometriosis patients (21). In contrast, our findings showed that GPx activity was upregulated in stages I-II.

ROS production is increased in mild stages of endometriosis, while ROS scavengers are down-regulated. This implies that there may be heightened oxidative stress and damage in endometriosis patients, particularly in the early stages of the condition. These findings may indicate a potential role of oxidative stress in the pathogenesis of endometriosis and highlight the importance of antioxidant defenses in mitigating ROS-induced damage (24).

The cytokine network plays a critical role in endometriosis, as elevated levels of inflammatory cytokines in the peritoneal fluid suggest tumor-like growth and disease progression in affected patients. The viability of IL-6 as a biomarker for endometriosis is still a subject of discussion. Researchers have reported that there are no statistically significant variations in IL-6 levels between patients with endometriosis and control groups in peritoneal fluid, follicular fluid, or serum (25, 26). In contrast, other researchers noted higher levels of IL-6 in infertile patients with endometriosis compared to those without endometriosis. Studies have also indicated that women with endometriosis-related infertility have higher levels of IL-6 in their serum (27). In the present study, IL-6 was measured in stages I-II compared to stages III-IV. It was shown that IL-6 level was not statistically significant in stages III-IV compared to stages I-II ($p>0.05$). However, a higher concentration was observed in stages III-IV compared to stages I-II. Therefore, the elevated concentration of IL6 aligns with previous studies indicating increased inflammation in patients' clinical presentation.

Phenylalanine is an essential amino acid, indicating that it cannot be endogenously synthesized by humans or other animals. Therefore, phenylalanine or phenylalanine-containing proteins must be obtained through dietary sources. Research indicates that phenylalanine may contribute to endometriosis by modifying serum levels and exerting effects such as preventing tumor cell invasion and migration, as well as suppressing cell proliferation (28). A study has shown that phenylalanine levels in the serum for the mild-stage diagnosis of endometriosis are significantly changed (29, 30). Our results showed that phenylalanine is significantly down-regulated in the severe stage compared to the mild stage ($p<0.05$). It enhances inflammation and immune responses, as phenylalanine promotes the innate immune response and stimulates the release of pro-inflammatory cytokines (31, 32).

Conclusion

In this study, it was shown that ROS production is increased in the mild stage of endometriosis compared to the severe stage. Additionally, ROS scavengers are down-regulated. This result indicates ROS-induced damage across different stages

of endometriosis, suggesting the activation of inflammatory pathways in affected patients. Our research reveals the possible use of these biomarkers in understanding the underlying causes of endometriosis and discovering potential markers for diagnosis or prognosis. Our results demonstrate significant alterations in TAC levels, as well as SOD, and GPx activity in conjunction with phenylalanine concentrations among endometriosis patients, with variability observed across different stages of the disease. Furthermore, a larger sample size is required to confirm the potential biomarkers proposed in this study.

Conflict of Interest

The authors declare no conflict of interest.

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