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Increased oxidative stress markers may be a promising indicator of risk for primary ovarian insufficiency: a cross-sectional case control study

Marcadores de estresse oxidativo aumentados podem ser um indicador de risco promissor para insuficiência ovariana primária: um estudo transversal caso-controle

Original Article

Keywords
Oxidative stress
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Risk factors

Palavras-chave
Estresse oxidativo
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Abstract

PURPOSE: The aim of this study was to evaluate serum levels of inducible nitric oxide synthase (INOS), myeloperoxidase (MPO), total antioxidant status (TAS), and total oxidative status (TOS) in women with primary ovarian insufficiency (POI) and to compare them with healthy fertile women. We also examined the possible risk factors associated with POI. **METHODS:** This cross-sectional case control study was conducted in Zekai Tahir Burak Women's Health Education and Research Hospital. The study population consisted of 44 women with POI (study group) and 36 healthy fertile women (control group). In all patients, serum levels of INOS, MPO, TAS, and TOS were determined. INOS and MPO levels were measured by enzyme-linked immunosorbent assay whereas colorimetric method was used for evaluating TAS and TOS levels. Age, body mass index (BMI), obstetric history, smoking status, family history, comorbidities, sonographic findings, complete blood count values, C-reactive protein and baseline hormone levels were also analyzed. Student's t-test or Mann-Whitney U test was used to compare continuous variables between the groups; categorical data were evaluated by using Pearson χ^2 or Fisher exact test, when appropriate. Binary logistic regression method was used to identify risk factors for POI. **RESULTS:** We found significantly elevated levels of INOS (234.1±749.5 *versus* 133.8±143.0; p=0.005), MPO (3,438.7±1,228.6 *versus* 2,481.9±1,230.1; p=0.001), and TOS (4.3±1.4 *versus* 3.6±1.4; p=0.02) in the sera of the study group when compared to the BMI-age matched control group. However, difference in serum levels of TAS were not significant between the 2 groups (1.7±0.2 *versus* 1.6±0.2; p=0.15). Logistic regression method demonstrated that BMI <25 kg/m^2, nulliparity, family history of POI, smoking, and elevated serum levels of INOS, MPO, and TOS were independent risk factors for POI. **CONCLUSION:** We found an increase in INOS, MPO, and TOS in women with POI. These serum markers may be promising in early diagnosis of POI. Further large-scale studies are required to determine whether oxidative stress markers have a role in diagnosing POI.

Resumo

OBJETIVO: Avaliar os níveis séricos da sintetase nítrica induzível (INOS), da mieloperoxidase (MPO), do estado antioxidante total (EAT) e do estado oxidante total (EOT) em mulheres portadoras de insuficiência ovariana primária (IOP) e compará-las às mulheres férteis. Também examinamos os possíveis fatores de risco associados à IOP. **MÉTODOS:** Trata-se de estudo transversal caso-controle desenvolvido no *Zekai Tahir Burak Women's Health Education and Research Hospital*. A população de estudo abrangeu 44 mulheres portadoras de IOP (grupo de estudo) e 36 mulheres férteis hígidas (grupo controle). Em todas as pacientes, foram determinados os níveis séricos de INOS, MPO, EAT e EOT. Os níveis de INOS e MPO foram determinados com o uso do teste ELISA e os níveis de EAT e EOT foram determinados mediante método colorimétrico. Analisou-se também a idade, o índice de massa corporal (IMC), os antecedentes obstétricos, tabagismo, histórico familiar, comorbidades, achados sonográficos, valores completos do hemograma, proteína C-reativa e níveis hormonais basais. O teste *t* de Student ou o teste U de Mann-Whitney foi utilizado para comparar as variáveis contínuas entre os grupos; os dados categóricos foram avaliados pelo teste do χ^2 de Pearson ou o teste exato de Fisher, conforme o caso. O método de regressão logística binária foi utilizado para identificar os fatores de risco para IOP. **RESULTADOS:** Encontramos níveis significativamente elevados de INOS (234,1±749,5 *versus*

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133,8±143,0; p=0,005), MPO (3.438,7±1.228,6 *versus* 2.481,9±1.230,1; p=0,001) e EOT (4,3±1,4 *versus* 3,6±1,4; p=0,02) nos soros do grupo estudo em relação ao grupo controle pareado por IMC e idade. Entretanto, as diferenças entre os níveis séricos de EAT nos dois grupos não foram significantes (1,7±0,2 *versus* 1,6±0,2; p=0,15). O método de regressão logística demonstrou que IMC <25 kg/m^2, nuliparidade, histórico familiar de IOP, tabagismo e níveis séricos elevados de INOS, MPO e EOT foram fatores de risco independentes para IOP. **CONCLUSÃO:** Foram encontrados níveis aumentados de INOS, MPO e EOT em mulheres portadoras de IOP. Estes marcadores séricos podem ser promissores para o diagnóstico precoce de IOP. Novos estudos em larga escala são necessários para determinar se os marcadores de estresse oxidativo desempenham um papel no diagnóstico da IOP.

Introduction

Premature ovarian failure is defined as the cessation of the ovarian function under the age of 40 years and is characterized by the development of hypergonadotrophic hypogonadism. A follicle stimulating hormone (FSH) level greater than 40 mIU/mL is indicative of ovarian failure. Recently, it has been suggested that the term premature ovarian failure should be replaced by the term primary ovarian insufficiency (POI) since its course can be long, variable and reversible[1]. The incidence of spontaneous POI was found to be 1/100 under the age of 40 years[2]. Although the exact etiology is unknown in 90% of the cases with POI, genetic factors play a key role, with family history reported in 10–15% of the cases[3]. Women with POI are confronted with a variety of health problems such as cardiovascular diseases which were caused by decreased estrogen during their lifetime[4]. One of the most important problems is infertility in the majority of these women. Although different assisted reproductive methods were defined for the treatment, oocyte donation still remains the most successful method[5].

Oxidative stress had been proposed to be a determinant for apoptosis in a reproductive cell system[6]. Increased reactive oxygen species (ROS) levels inhibit follicle growth in antral follicles and antioxidants such as N-acetyl sistein restores ROS levels and protect ovaries from damaging mediated by free oxygen radicals[7]. The production of ROS, although important for physiologic processes, may also induce pathologic conditions. The cyclical production of ROS may result in cumulative overlong periods and contributes to increased risk of ovarian diseases such as primary ovarian insufficiency[8].

In this study, we aimed to evaluate serum levels of some oxidative stress markers including inducible nitric oxide synthase (INOS), myeloperoxidase (MPO), total anti-oxidant status (TAS), and total oxidative status (TOS) in women with POI and to compare them with healthy fertile women. We also examined the possible risk factors associated with POI.

Methods

This study was designed as a cross-sectional case control and performed between October 2013 and March 2014 in Zekai Tahir Burak Women's Health Education and Research Hospital, Ankara, which is a tertiary referral hospital in the middle region of Turkey. The study group was comprised of 44 women who were referred to the infertility clinic and diagnosed with POI. The control group included 36 healthy fertile women who were referred to the family planning clinic for contraception. The institutional review board approved the study, and the universal principles of the Helsinki Declaration were applied. All women in the study gave written Informed Consent to participate.

The POI group selection criteria were as follows: female aged between 20 and 40 years who had no menses or experienced irregular menses within the last 4 months, FSH levels >40 mIU/mL recorded during at least 2 readings 1 month apart, no history of pelvic surgery, no exposure to chemotherapy or radiotherapy that could lead to ovarian dysfunction or failure, a normal female 46,XX karyotype in women ≤30 years of age, and no estrogen, progestogen, or other steroid hormonal therapy for at least 4 months. The inclusion criteria for the control group were age and body mass index (BMI) matched women with regular menstrual cycles who presented to our hospital requesting contraception on the third day of their menstrual cycle, no use of hormonal contraceptive methods, having had at least two children and with no history of infertility. The patients with acute and chronic infections, rheumatic and other inflammatory diseases, malignancies, metabolic disorders, and cardiovascular diseases were excluded from the study. We also excluded women who reported use of gonadotropin-releasing hormone agonists/antagonists, anti-androgens, oral anti-diabetics, and multivitamin containing pills.

Gynecological and general histories were obtained, and demographic and obstetrical characteristics were recorded for each woman. The main parameters recorded were: age, BMI, gravidity, parity, smoking status, family history of POI, comorbidities, sonographic findings, complete blood count values, baseline hormone levels, and serum levels of some oxidative stress markers including INOS, MPO, TAS, and TOS. A complete physical and pelvic examination was performed in all women. Uterine and ovarian structures were evaluated using transvaginal ultrasound (ALOKA Prosound 4 Ultrasound device with a 5–6.5-MHz endovaginal probe). Blood samples were taken after an overnight fasting from

the antecubital vein of each participant. Hematologic parameters were immediately investigated using the Coulter LH-780 hematology blood analyzer (Beckman Coulter Inc., Brea, CA). Serum samples were evaluated for some hormonal and blood parameters; then they were separated by centrifugation at 3,000 rpm for 10 minutes. C-reactive protein (CRP) levels were measured with a nephelometric method using an IMAGE 800 analyzer (Beckman Coulter Inc.) and serum baseline hormone levels were measured by electrochemiluminescence immunoassay (Roche Diagnostics GmbHx, Mannheim, Germany) on the same day after blood samples were taken. The rest of the serum samples was filled into polypropylene tubes and immediately frozen in aliquots at -80°C until analysis.

Analysis of oxidative stress markers

Serum MPO levels were analyzed by enzyme linked immunosorbent assays (ELISA) method using MPO ELISA kit (Insight Genomics Co., VA, USA) — measurement range: 312 to 20,000 pg/mL). Serum INOS levels were also analyzed by ELISA method using INOS/NOS2 ELISA kit (Hangzhou Eastbiopharm Co., Ltd., Zhejiang, PRC) — measurement range: 0.2 to 60 ng/mL). Colorimetric method was used for determining serum TAS and TOS levels. TAS measurements were performed by application of TAS assay kit (Rel Assay Analysis Diagnostics Co., Gaziantep, TR) on Beckman Coulter AU680 auto-analyzer (measurement range: 1.2 to 1.5 mmol/L). TOS measurements were also performed by application of TOS assay kit (Rel Assay Analysis Diagnostics Co., Gaziantep, TR) on Beckman Coulter AU680 auto-analyzer (measurement range: 4 to 6 µmol/L).

Statistical analysis

Statistical Package for the Social Sciences version 21 (SPSS Inc., Chicago, IL, USA) and Medcalc 9 (Acacialaan 22, B-8400 Ostend, Belgium) software were used for statistical analysis. Compliance with the normal distribution of data was analyzed considering the Kolmogorov-Smirnov test and the Shapiro-Wilk test. Parametric methods were used to analyze the variables with normal distribution whereas in the analysis of non-normally distributed variables non-parametric methods were used. In order to compare the two independent groups, independent-samples of Student's t-test and Mann-Whitney U (exact) test were used. Differences between categorical data were evaluated using Pearson χ^2 and Fisher exact test. Receiver operating characteristic (ROC) analysis of the area under the curve (AUC) was used to determine the discriminative values of INOS, MPO, and TOS. Binary logistic regression method was used to identify risk factors for POI. Mean±standard deviation and median±interquartile range for quantitative data, as well as numbers and percentages for qualitative data were computed. Data were examined in the 95% confidence level, and statistical significance was set at $p<0.05$.

Results

A total of 80 women (44 POI cases and 36 BMI-age matched healthy controls) were included in the study. There was a significant difference between the groups in terms of obstetric characteristics. Women with POI had significantly lower values for gravidity and parity. Nulliparity was also more common among the POI group ($p<0.001$). Sonographic findings including endometrial thickness and antral follicle count (AFC) were significantly lower in the POI group than in the control group ($p=0.001$). No significant difference in hematologic parameters was found between the groups. There was also no significant difference in mean serum CRP levels between the study group and the control group. We found significantly elevated levels of INOS (234.1±749.5 *versus* 133.8±143.0; $p=0.005$), MPO (3,438.7±1,228.6 *versus* 2,481.9±1,230.1; $p=0.001$), and TOS (4.3±1.4 *versus* 3.6±1.4; $p=0.02$) in the sera of the study group compared to the control group. But serum levels of TAS were statistically insignificant between the 2 groups (1.7±0.2 *versus* 1.6±0.2; $p=0.15$). The comparison of demographics, clinical and laboratory features of the groups are presented in Table 1. The smoking habit was more common among the patients in the study group (40.9 *versus* 11.1%, $p=0.005$). A history of POI in the mother or sister was found in 18 (40.9%) cases in the POI group and 2 (5.6%) cases in the control group ($p=0.001$). The only comorbidity detected in the history was thyroid disorder (3 *versus* 2), but none of these patients was receiving any medical treatment. The most frequent causes of previous surgery were cesarean, appendectomy and hernia repair, respectively. There were no significant differences between the groups in terms of history of surgery and comorbidity.

ROC analyses demonstrated that increased INOS, MPO, and TOS were the statistically significant discriminative factors for POI cases. According to the highest Youden's index, cut-off values were found to be 178 at 65.9% sensitivity and 69.4% specificity for INOS. For MPO, the cut-off value was calculated as 2,768.2 at 77.3% sensitivity and 66.7% specificity. The cut-off value for TOS was determined to be 3.6 with 55.6% sensitivity and 77.3% specificity. The AUCs and their standard errors were 0.680±0.059 ($p=0.002$), 0.708±0.057 ($p<0.001$), and 0.643±0.063 ($p=0.022$), respectively.

In binary logistic regression analysis, normal body weight (BMI <25 kg/m^2), nulliparity, family history of

POI, smoking, and higher serum levels of INOS, MPO, and TOS than the calculated threshold values were found to be independent risk factors for POI (Table 2).

Table 1. Comparison of demographics, clinical characteristics and laboratory parameters between the groups

Variables	POI group (n=44)	Control group (n=36)	p-value*
	Median±IQ range	Median±IQ range	
Age (years)	32.5 (±4.4)#	31.0 (±5.8)#	0.2
≤35/<35 – n (%)	16 (36.4)/28 (63.6)	11 (30.6)/25 (69.4)	0.6
BMI (kg/m²)	24.2±4.1	26.6±5.9	0.06
≤25/<25 – n (%)	16 (36.4)/28 (63.6)	24 (66.7)/12 (33.3)	0.01
Gravidity	0.0±2.0	2.0±1.0	0.002
Parity	0.0±2.0	2.0±0.5	<0.001
Nulliparity			
No/yes – n (%)	17 (38.6)/27 (61.4)	36 (100)/0	<0.001
Smoker			
No/yes – n (%)	26 (59.1)/18 (40.9)	32 (88.9)/4 (11.1)	0.005
Family history			
No/yes – n (%)	26 (59.1)/18 (40.9)	34 (94.4)/2 (5.6)	0.001
FSH (mIU/mL)	55.0±22.5	6.1±2.0	<0.001
LH (mIU/mL)	29.7 (±14.1)#	3.6 (±1.5)#	<0.001
Prolactin (ng/mL)	8.0±4.2	12.1±10.0	0.003
TSH (mIU/mL)	1.5±1.4	2.2±1.5	0.04
E_2 (pg/mL)	29.0±45.0	37.4±14.4	0.06
Leukocyte count (x 10^3/µL)	6,350±2,100	6,800±2,000	0.8
CRP (mg/L)	4.0±2.4	2.3±2.8	0.1
Ultrasound parameters			
Endometrium (mm)	4.0±3.0	10.0±3.5	<0.001
Right ovary (AFC)	2.0±3.0	6.0±2.0	<0.001
Left ovary (AFC)	2.0±3.0	6.0±2.0	<0.001
Oxidative stress markers			
INOS (ng/mL)	234.1±749.5	133.8±143.0	0.005
MPO (pg/mL)	3,438.7 (±1,228.6)#	2,481.9 (±1,230.1)#	0.001
TOS (µmol H_2O_2 Equiv./L)	4.3±1.4	3.6±1.4	0.02
TAS (mmol H_2O_2 Equiv./L)	1.7±0.2	1.6±0.2	0.15

#mean (±standard deviation); *p<0.05 is considered as statistically significant.

IQ: interquartile; POI: primary ovarian insufficiency; BMI: body mass index; FSH: follicle stimulating hormone; LH: luteinizing hormone; TSH: thyroid stimulating hormone; E_2: estradiol; CRP: C-reactive protein; AFC: antral follicle count; INOS: inducible nitric oxide synthase; MPO: myeloperoxidase; TOS: total oxidative status; TAS: total antioxidant status.

Table 2. Logistic regression method of risk factors for primary ovarian insufficiency

Group (study)	p-value	*Odds Ratio* (95%CI)
TOS (3.6)	0.002	4.6 (1.71–12.35)
INOS (178)	0.002	4.3 (1.7–11.29)
MPO (2,768.2)	<0.001	6.8 (2.5–18.27)
BMI (<25)	0.008	3.5 (1.3–8.83)
Parity (no)	<0.001	12.7 (3.8–42.33)
Smoker (yes)	0.005	0.1 (0.05–0.60)
Family history (yes)	0.001	11.7 (2.5–55.31)

Logistic regression (Method = Enter).

95%CI: confidence interval of 95%; TOS: total oxidative status; INOS: inducible nitric oxide synthase; MPO: myeloperoxidase; BMI: body mass index.

Discussion

The exact pathophysiologic mechanism underlying POI still remains unclear. It is an indisputable fact that further investigations are required for early detection and treatment of POI. In this study, we aimed to evaluate serum levels of some oxidative stress markers including INOS, MPO, TAS, and TOS in women with POI and to compare them with healthy fertile women. The main finding of this study is that serum levels of INOS, MPO, and TOS, but not TAS, are significant discriminative markers for POI. We also found that a BMI less than 25, nulliparity, family history of POI, smoking, and increased serum levels of INOS, MPO, and TOS are independent risk factors for POI.

Apoptosis or programmed cell death is an essential phenomenon to human physiology, occurring at all periods of life. The rate of apoptosis should be balanced by the proliferation rate of the cell division. This mechanism has a greater importance for ovarian physiology; from the settlement of primordial cells in the genital cresta up to the cessation of ovarian activity at menopause, ovarian germ cells and follicle somatic cells are eliminated by activation of apoptotic processes[9]. A cell may initiate intracellular apoptotic signaling in response to a stress. Both intracellular and extracellular factors may cause this stress. Most of the morphological and functional changes seen in apoptotic cells can be accounted for by a family of enzymes which become activated in a reaction cascade[10].

Reactive oxygen and nitrogen species are a family of antimicrobial molecules derived from nitric oxide and superoxide anions generated during normal metabolic reactions[11]. However, if these molecules and their metabolites required for many systems in the body, particularly the immune system, are not sufficiently neutralized, they may pose a serious threat to cellular viability. Proteins in the body undergo a variety of post-translational modifications. Among these modifications, oxidative modifications are substantially involved in aging and certain diseases[12]. The transduction pathways that link toxic oxidant accumulation to apoptosis include peroxidative perturbations in membrane phospholipid dynamics, cytosolic calcium accretion, microskeletal disruption, DNA (deoxyribonucleic acid) damage, and endonuclease activation. MPO is a biomarker of oxidative stress causing halogenations in activated immune cells such as neutrophils[13]. INOS is derived from nitric oxide and can be a marker indicating activation of macrophages. Although it was primarily identified in macrophages, the expression of the enzyme can be virtually stimulated in any cell or tissue, provided that the appropriate agents get deployed for inducing its synthesis[14].

The function of oxidative stress in pathogenesis of POI has not been investigated extensively. It has been reported that female reproductive system maintains a delicate balance

of pro- and anti-oxidants to minimize oxidative stress[15]. In a recent study, it was reported that administration of coenzyme Q having antioxidant properties in POI patients with high ROS levels improves the embryo quality[16]. We know that the development of oocyte occurs under the hypoxic condition in the ovarian cortex, however exposure to supra-physiological concentrations of ROS are detrimental to developing oogonia. The mammalian oocyte and embryo are very sensitive to oxidative stress; physiological levels sub-serve several important functions whereas high levels impair oocyte maturation[17]. In another study, it was suggested that increased production of ROS contributes to oophoritis associated with POI[8]. Kumar et al.[18] found an increase in the number of nucleotide alterations in mitochondrial DNA and supra-physiological ROS levels in POF cases compared to age-matched controls. They concluded that high levels of ROS lead to mitochondrial DNA damage results in mitochondrial dysfunction and increase apoptosis. This situation could lead to less adenosine triphosphate production due to impaired oxidative phosphorylation and thus to deteriorated oogenesis, low oocyte number and POI.

Increased endogenous hormone levels in obese women may lead to faster follicle consumption due to persistent stimulation of follicular growth. However, previous epidemiological studies on the relationship between increased BMI and natural menopausal age have disclosed no conclusive results[19]. A recent study reported that low BMI is associated with earlier onset of natural menopause[20]. However, the same authors could not find such a risk for POI cases. In the present study, we found no significant difference between the two groups in terms of mean BMI. On the other hand, when we classified groups according to their BMI as overweight and normal weight, having a lower BMI, it was found as an independent risk factor for POI.

Although the association between smoking and POI has not been established yet definitely, studies indicate that smoking is toxic for ovarian germ cells and cigarette smoke contains polycyclic hydrocarbons that exert a negative impact on follicle development. Hayatbakhsh et al.[21] reported that smoking during reproductive age was associated with earlier menopause. In addition, smoking was associated with decreased Antimüllerian hormone (AMH)[22], AFC and increased serum FSH[23] levels in fertile women. Consistent with the literature, our study showed that smoking frequency was higher in the POI group than the control group, and that smoking was an independent risk factor for POI.

Several studies have shown evidence of a strong relationship between the menopausal age of mothers and their daughters and genetic factors in the determination of menopause. In a case series including 71 idiopathic POI cases, familial transmission was found in 31% of the patients[24]. A genetic etiology is suggested by the occurrence of families with two or more affected females. Although they assumed the mode of inheritance is autosomal recessive in some POI cases, Davis et al.[25] have proposed the X chromosome defects as the main reason for POI. The present study showed that POI history was more common in families of women with POI than the control group, even despite excluding major chromosomal anomalies such as fragile X syndrome. In a previous study evaluating risk factors for POI, it was found that nulliparity is associated with an increased risk for POI[26]. Similarly, Harlow and Signorello[27] showed that multiparous women had a later age at natural menopause in their study. In our study, we also found nulliparity to be an important risk factor for the development of POI.

In conclusion, predicting POI before development or the early detection of POI could be useful for developing appropriate treatment options that would solve infertility problem. To the best of our knowledge, the present study is the first to suggest that the INOS, MPO, and TOS may be significant markers of POI. However, serum levels of TAS were not significantly different from healthy controls in women with POI. This study indicates that the balance between prooxidants and antioxidants has shifted in favor of the prooxidants in women with POI. While the etiologic factors of disease are being evaluated, analysis of serum markers of oxidative stress may also be included in the initial work up of a woman with POI. If these cases are diagnosed at an early stage, appropriate treatment, such as antioxidants, which prevents DNA damage and slows down germ cell apoptosis, can be initiated. Further studies with more participants are required to determine whether these oxidative stress markers have a role in diagnosing POI.

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