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Copper, ceruloplasmin and oxidative stress in patients with advanced-stage endometriosis

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Abstract. – AIM: To compare patients with advanced stage endometriosis with control patients without endometriosis with respect to serum Copper (Cu) and Ceruloplasmin (Cp) levels and oxidative stress markers in order to evaluate the importance of these parameters in the pathogenesis of endometriosis.

PATIENTS AND METHODS: A total of 72 women who underwent laparoscopy or laparotomy for evaluation of infertility, pelvic pain, pelvic mass, tubal ligation or endometriosis were enrolled for this prospective clinical study. Patients were divided into two groups by visual diagnosis at surgery and histological confirmation of endometriosis: control patients (n=41) without endometriosis and study group (n=31) with stage III/IV (advanced stage) endometriosis. Serum Cu, Cp, total antioxidant status (TAS), total oxidant status (TOS), oxidative stress index (OSI), paraoxonase-1 (PON-1), malondialdehyde (MDA), triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) levels were compared between the two groups. Correlations between Cu, Cp and oxidative stress markers were determined.

RESULTS: Serum TOS, OSI, Cu, Cp, TG, TC, LDL were significantly higher, whereas TAS, PON-1 activity and HDL were significantly lower, in women with advanced-stage endometriosis than in control groups. There was no difference in serum MDA activities between the two groups. Positive correlations were found between Cu and TOS, Cu and OSI, Cu and Cp, while a negative correlation was found between Cu and PON-1 in the advanced-stage endometriosis group. Positive correlations were found between Cp and TOS, and Cp and OSI in the advanced-stage endometriosis group.

CONCLUSIONS: Cu and Cp appear to be associated with the etiopathogenesis of and oxidative stress in endometriosis.

Key Words:
Copper, Ceruloplasmin, Oxidative stress, Endometriosis.

Introduction

Endometriosis is a prevalent gynecological disease characterized by extrauterine implantation and growth of endometrial gland and stroma[1]. The prevalence of endometriosis in asymptomatic women ranges between 2 and 22%, while among infertile women it climbs to 35-50 percent[2]. Although little is known about its etiology, the important role of oxidative stress and inflammation in its pathogenesis has been accepted[3-7].

Reactive oxygen species (ROS) are oxygen-containing molecules produced during normal metabolic processes. In the organism, derangements in some conditions lead to abnormalities in the enzymatic and non-enzymatic systems that neutralize the deleterious effects of these endogenous ROS. This disruption of the balance between oxidants and antioxidants, and the resultant generation of reactive oxygen metabolites and subsequent oxidative stress, leads to destructive changes in circulating lipoproteins, proteins, carbohydrates, and nucleotides[7].

Cu is a redox-active essential metal of vital importance. It has both antioxidant and prooxidant effects related to oxidative stress and inflammation[8,9]. In mammalian serum, the predominant Cu containing protein is Cp, a glycosylated multi-Cu ferroxidase synthesized primarily in the liver, which carries 95% of total serum Cu[10]. Cp is an acute phase response protein. Similar to Cu, it is related to inflammation and oxidative stress and has both antioxidative and prooxidative effects[8,9]. Even though endometriosis is a disease that manifests both inflammation and oxidative stress, an adequate number of studies looking at levels of Cu and Cp in endometriosis has not been performed.

Serum paraoxonase-1 (PON-1) is an antioxidant enzyme which exerts its direct and indirect effects through HDL and LDL. Its deficiency leads to an increase in inflammation and oxidative stress with an impaired lipid profile[7,11]. Malondialdehyde (MDA) is an important degradation product of lipid peroxidation that occurs in endometriosis due to oxidative stress. A limited

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number of studies have been done concerning MDA activity in endometriosis, and outcomes differ widely[12,13]. Determination of oxidative stress via multiple, isolated, and different methods, such as through PON-1 and MDA activities, is costly in terms of both time and financial resources. TAS, TOS and OSI is a novel automated and colorimetric measurement method of oxidative stress developed by Erel[14,15]. With this method, assessments of total antioxidant and oxidant states can be accomplished without restricting the evaluation to only certain antioxidant or oxidant parameters. It has not been used in patients with endometriosis.

The objective of our study was to compare patients with advanced stage endometriosis to controls with respect to serum Cu and Cp levels, new oxidative stress measurement methods (TAS, TOS, and OSI), which have not been investigated in the endometriosis in previous studies, and also PON-1 and MDA.

Patients and Methods

This prospective case-controlled study was performed on 95 patients who had undergone laparoscopy or laparotomy in Dicle University, Faculty of Medicine, Clinics of Gynecology and Obstetrics between March 2012 and November 2012 in order to evaluate infertility, pelvic pain, pelvic mass, tubal ligation and endometriosis. The study included premenopausal women between 19-41 years of age with regular menstrual cycles between 21-35 days. Women with malignant gynecological diseases, those who were pregnant, those under hormonal therapy (e.g.: oral contraceptives, GnRH agonists, progestins, estrogens), smokers, alcoholics, patients with coronary artery disease, unstable angina, heart attacks, those with a history of any cardiovascular intervention or operation, autoimmune or endocrine/metabolic diseases, polycystic ovary syndrome, anemia, systemic and/or local inflammatory disease within the last 3 months, history of malignant disease, and those who used drugs with an antioxidant effect within the previous 5 years were excluded from the study. Of the 95 women recruited for the study, 81 (85.3%) were eligible and consented to participation in the research. Endometriosis was diagnosed by laparoscopy/laparatomy with histological confirmation of the disease and severity classified according to the revised American Fertility Society classification for endometriosis. Only nine patients with endometriosis were stage I/II. Because the small sample size of this group and did not meet a sufficient and reliable number for statistical analysis, we excluded these patients from the study. Women with endometriosis stage III/IV (advanced stage) were enrolled as the study group. A total of 72 patients (31 cases with stage III/IV endometriosis and 41 control subjects without endometriosis) were enrolled. The protocol was approved by the Medical Ethics Committee of Dicle University in accordance with the Helsinki Declaration of Human Rights and informed consent was obtained from all subjects involved.

Serum Cu, Cp, TG, TC, HDL and LDL levels, TAS, TOS and OSI values, MDA and PON-1 activities were measured. Demographic data of the patients (age, height, body weight, body mass index (BMI), stage of the disease) were evaluated. BMI was calculated as weight (kg) per height^2 (m).

Serum Sampling

Blood samples were drawn from the patients on days 2-4 of the follicular phase of the menstrual cycle, the time during the menstrual cycle when endometrial activity is at its peak. The phases of the menstrual cycle were determined by the last and next menstrual period as well as basal body temperature, and corroborated with ultrasound findings on the endometrium and ovarian follicles. Blood was collected in the morning hours (08:00-10:00 AM) after an overnight fasting period before the surgery. Each collected blood sample was immediately centrifuged at 4000 rpm at +4°C for 10 min and then transferred into an Eppendorf tube. Samples were transferred on ice and kept at –70°C until analysis.

Biochemical Analysis

Cu was determined by Shimadzu 6401S atomic absorption/emission spectrometer (Shimadzu Co., Kyoto, Japan). The acetylene flow rate and the burner height were adjusted in order to obtain the maximum absorbance signal with a slit of 0.5 nm at a wavelength 324.8 nm for Cu. The radiation sources were hollow cathode lamps (Shimadzu, Tokyo, Japan). The operating conditions were those recommended by the manufacturer (Operation Manual-Atomic Absorption Spectrophotometer AA-6800, Shimadzu, 2000). Serum Cp was assessed by nephelometric assay on the automatic Image analyzer (Beckman Coulter, Inc. Fullerton,

CA, USA). The TAS and TOS of serum were evaluated by using a novel automated and colorimetric measurement method developed by Erel (REL assay diagnostics, Mega Tip, Gaziantep, Turkey)[14,15]. The TOS level to TAS level ratio was regarded as the oxidative stress index (OSI)[16]. Serum PON1 levels were measured spectrophotometrically by modified Eckerson method. Initial rates of hydrolysis of paraoxon (0.0-diethyl-0-p-nitrophenylphosphate; Sigma Chemical Co. London, UK) were determined by measuring liberated- p-nitrophenol at 405 nm at 37°C[17]. Malonyldialdehyde (MDA) concentrations were measured spectrophotometrically with a Shimadzu UV-1201 spectrophotometer (Shimadzu Co., Kyoto, Japan) as described previously[18].

Serum total-cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride levels were measured with an autoanalyzer (Abbott aeroset autoanalyzer, Toshiba, Kanajawa, Japan). The intra and interassay coefficients of variation (CV) for TAS, TOS and PON-1 activity were < 3%, for Cu and MDA activity were < 7%, for Cp were < 5% and for TG, TC, HDL and LDL were < 2%.

Statistical Analysis

Data were stored in an Excel 2003 (Microsoft, Redmond, WA, USA) worksheet and loaded into SPSS 18.0 statistical software (SPSS Inc., Chicago, IL, USA) for analysis. Comparisons of continuous variables between two groups were done using Student's *t* test. Pearson's correlation analyses and scatter graphs were performed to determine and graph the magnitude of correlations. Results were presented as mean±SD (standard deviation). A *p* value of less than 0.05 was accepted as statistically significant.

Table I. Surgical approach and preoperative indications of the patients included in the study.

Parameters	Distribution among total subjects (n=72)
Surgical approaches	
Laparoscopy	54 (75%)
Laparotomy	18 (25%)
Preoperative indications	
Infertility	35 (48.6%)
Pelvic mass	19 (26,4%)
Pelvic pain	15 (20.8%)
Tubal ligation	3 (4.2%)

Results

There was no significant difference in mean age between women with endometriosis (31.3±4.6 years) and controls (30.4±7.8 years) (p = 0.581). Moreover, no significant difference was seen in body mass index (BMI) between women with endometriosis (22.2±1.8 kg/m^2) and controls (22.1±1.7 kg/m^2) (p = 0.645). All of the participants underwent laparoscopy (75%) or laparotomy (25%). Preoperative indications and surgical approach of the patients were listed in Table I.

Table II summarizes serum TAS, TOS, OSI, PON-1 and MDA activities, Cu and Cp levels and lipid profiles in women with advanced-stage endometriosis and controls.

Serum TOS (p < 0.001), OSI (p < 0.001), Cu (p < 0.001), Cp (p < 0.001), TG (p = 0.014), TC (p = 0.009), and LDL (p = 0.003) levels were significantly higher, whereas TAS (p < 0.001), PON-1 (p = 0.020) activity and HDL (p = 0.011) were significantly lower, in women with advanced-stage endometriosis compared to control

Table II. Comparison of mean level of parameters in women with advanced-stage endometriosis and controls.

Parameters	Advanced-stage endometriosis (n = 31) (mean ± SD)	Controls (n = 41) (mean ± SD)	*p* value
TAS (mmol Trolox Equivalent/L)	1.01 ± 0.10	1.15 ± 0.17	< 0.001
TOS (μmol H_2O_2 Equiv/L)	25.40 ± 9.35	15.98 ± 6.97	< 0.001
OSI (H_2O_2/Trolox)	25.07 ± 9.21	14.05 ± 6.69	< 0.001
PON-1 (u/l)	73.38 ± 44.34	98.47 ± 44.46	0.020
MDA (mmol/L)	220.87 ± 41.84	205.49 ± 43.57	0.136
Cu (μg/ml)	1088.00 ± 273.58	811.20 ± 265.77	< 0.001
Cp (mg/dl)	38.41 ± 9.58	26.50 ± 8.63	< 0.001
TG (mg/dl)	121.65 ± 43.11	97.83 ± 33.47	0.014
TC (mg/dl)	183.13 ± 23.98	164.80 ± 31.59	0.009
HDL (mg/dl)	49.58 ± 9.06	56.20 ± 11.72	0.011
LDL (mg/dl)	108.52 ± 23.12	89.09 ± 28.33	0.003

SD = standard deviation.

women. There was no difference in serum MDA activities between endometriosis and control groups ($p = 0.136$).

Positive correlations were found between Cu and TOS (r = 0.343, $p = 0.003$), Cu and OSI (r = 0.358, $p = 0.002$), Cu and Cp (r = 0.783, $p < 0.001$), and a negative correlation was found between Cu and PON-1 (r = –0.314, $p = 0.007$) in the advanced-stage endometriosis group (Figures 1 and 2).

Positive correlations were found between Cp and TOS (r = 0.296, $p = 0.012$), Cp and OSI (r = 0.323, $p = 0.006$) in the advanced-stage endometriosis group (Figure 3).

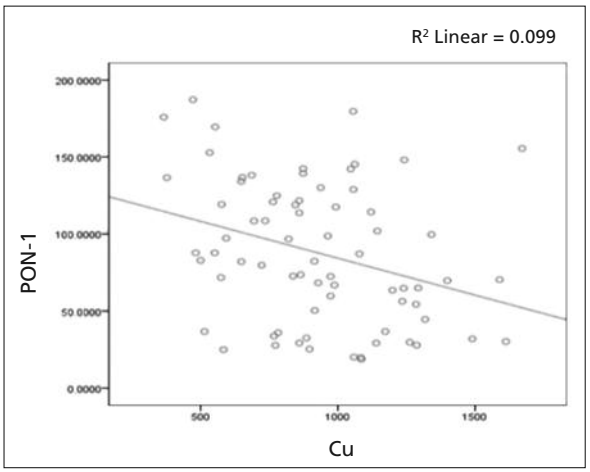

Figure 2. Correlation of level PON-1 and Cu in patients with end-stage endometriosis ($p = 0.007$).

Discussion

In our study, Cu and Cp levels in patients with advanced stage endometriosis were significantly higher compared to the control group. In addition, there was a strong relationship between Cu and TOS, OSI, and PON-1 activity and between Cp and TOS and OSI. Cu is a redox-active metal. Oxidative stress increases in both its deficiency and toxicity[9]. Cp, which requires Cu for its ferroxidase function, is synthesized in the liver[10]. Cp can have antioxidant as well as prooxidant effects *in vitro*[19-22]. The Cu-protein Cp exhibits potent oxidant activity against LDL *in vitro*. Cp is a plasma glycoprotein containing seven Cu atoms per molecule and contains 95% of the total plasma Cu. It was reported that the prooxidant activity of Cp and Cu could become result via the binding of Cu to a specific protein binding site on Cp[22]. Cp is an acute phase response protein whose synthesis and secretion can be markedly increased during inflammation, infection and in diseases such as diabetes mellitus, cancer and cardiovascular disease, as well as during pregnancy[8,9]. The function of Cp prooxidant activity in inflammation is not known, but it may be involved in host defense. A bactericidal activity indicates that Cp may participate in the inflammatory response to foreign agents; inflammatory cytokines induce production of Cp by monocytic cells[22]. A similar and common etiopathogenic

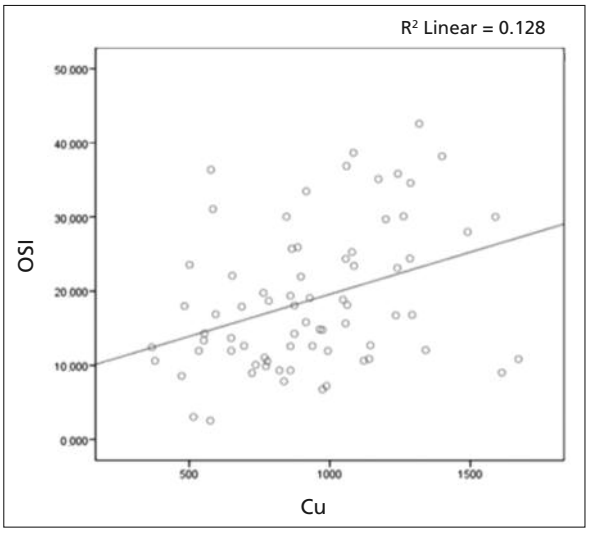

Figure 1. Correlation of level OSI and Cu in patients with advanced-stage endometriosis ($p = 0.002$).

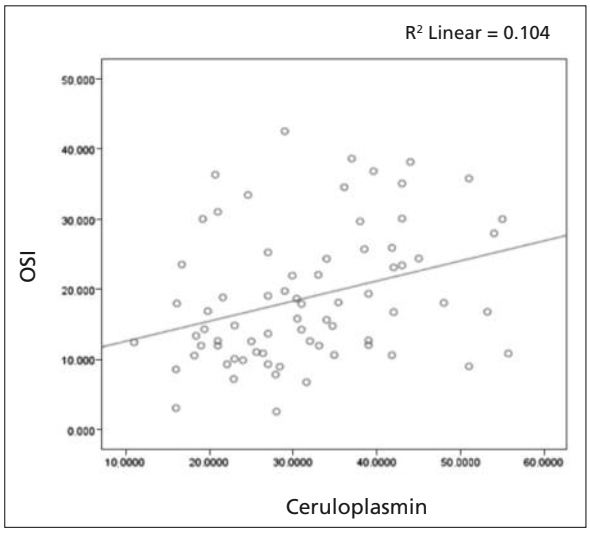

Figure 3. Correlation of level OSI and Ceruloplasmin in patients with advanced-stage endometriosis ($p = 0.006$).

mechanism involving oxidative stress and inflammatory markers is shared by endometriosis and atherosclerosis. Oxidative stress plays a role in the development and progression of atherosclerosis[23]. Detection of macrophages, cytokines, lipoproteins (LDL and HDL), T-cells, and oxidized LDL in both peritoneal fluids of the patients with endometriosis and atherosclerotic lesions points to a common etiopathogenic mechanism shared by both entities[24]. In addition, higher serum Cu and Cp levels were detected in patients with atherosclerosis[25]. In our work, the significantly higher serum Cu and Cp levels detected in patients with endometriosis reinforce this similarity. The association between Cu, Cp and oxidative stress markers might suggest their roles in the etiopathogenesis of oxidative stress in endometriosis. Endometriosis is a disease which manifests signs and symptoms of inflammatory and oxidative stress similarly to atherosclerosis. However, any investigation has not been conducted about significance of serum Cu and Cp levels in endometriosis so far. These findings suggest that, because of the strong relation between increased Cu and Cp levels and oxidative stress in endometriosis, Cu, Cp and components of oxidative stress may be used as markers for therapeutic purposes in the near future. The levels of Cu and Cp may be expected to decrease simultaneously with oxidative stress after treatment of endometriosis aimed at preventing oxidative damage. They also may be useful for monitoring endometriosis during pharmacological therapy.

TAS, TOS and OSI have not been studied as oxidative stress markers in endometriosis before. In our study, significantly lower TAS, but higher TOS and OSI, values were detected. Endometriosis is associated with a widespread inflammatory response, and oxidative stress has been thought to play an important role in the pathophysiology of this condition[1,5,26]. The localized macrophage response and neutrophilic activation against endometriotic processes lead to an increase in ROS[1,26,27]. Lower levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase, which have crucial roles in the degradation of free oxygen radicals and ROS, were detected in the peritoneal fluids of the patients with endometriosis[1]. While some reports did not find any association between endometriosis and oxidative stress[28,29], Wo et al and Wang et al analyzed these markers only in the peritoneal fluid of limited number of patients (n=34 and 39, respectively). Oxidative stress has been evaluated using markers such as TAS, TOS and OSI in many disease states, i.e. chronic inflammatory diseases, diabetes mellitus and coronary artery disease, and lower TAS and higher TOS and OSI levels were observed[30-32]. We have similar findings concerning TAS, TOS and OSI, suggesting they might be a valid indicator of deranged oxidative/antioxidative equilibrium in endometriosis.

In our study we also observed lower serum PON-1 activity and HDL, but higher TG, TC and LDL levels in the endometriosis group compared to the control group. Serum PON-1 is an antioxidant enzyme associated with HDL that prevents development of oxidative changes of LDL. Besides blocking oxidization of HDL, PON-1 substantiates the antiatherogenic role of HDL in the reverse cholesterol transport[11,33]. It also facilitates faster clearance of LDL[34]. Therefore, with PON-1 deficiency, the impact of oxidative stress, levels of TG, cholesterol and LDL, and predisposition to atherosclerotic diseases increase, while levels of HDL decrease[7,35]. Also, in this work, our findings underline the association, in advanced stage endometriosis, between increased inflammation, oxidative stress and lower levels of PON-1, a component of the antioxidant system that has a role in the prevention of inflammation[7].

In our study we analyzed the activity of MDA in serum as another marker of oxidative stress; a difference between the advanced-stage endometriosis group and the control group could not be found. Several reports have indicated increased levels of MDA in peritoneal fluid without any change in serum levels in endometriosis[12]. In one study, no significant difference in levels of MDA in peritoneal fluid or serum was found between infertile patients with peritoneal endometriosis and the control group[13]. In another report, MDA activities in the peritoneal fluid of patients with endometriosis and the control group were not found to be significantly different[36]. MDA, a lipid peroxidation product, is expected to rise in oxidative stress and inflammation. Conversely, that serum MDA levels remain unmodified despite increased MDA concentrations in the peritoneal fluid might be due to the presence of a relatively local inflammatory event or of unknown mechanisms in endometriosis. These complex mechanisms should be investigated further in larger scale investigations. Our study does not support the use of MDA as a peripheral oxidative stress marker in endometriosis.

Conclusions

This research is the first study looking Cu and Cp levels in endometriosis, with TAS, TOS and OSI used in the determination of oxidative stress. Cu and Cp appear to be associated with the etiopathogenesis of and oxidative stress in endometriosis. These novel markers may be useful for therapeutic purposes and follow up during pharmacological therapy in the near future.

In endometriosis, oxidative stress indicators such as TAS, TOS, OSI can be used as relevant markers similarly to PON-1. PON-1 only provides information about antioxidant state, while TAS, TOS and OSI provide information about the total antioxidative and total oxidative load. However, investigations that are more comprehensive are needed to confirm our findings.

Conflict of Interest

Authors declare no conflict of interest or financial disclosure for this manuscript.

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