Research Communication

Impaired Antioxidant Enzyme Functions with Increased Lipid Peroxidation in Epithelial Ovarian Cancer

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Abstract

We aimed to identify the possible role of oxidant–antioxidant status in epithelial ovarian cancer (EOC) by measuring (a) antioxidant enzyme (AOE) activities [total superoxide dismutase (SOD_{total}), manganese-SOD (Mn-SOD), copper,zinc-SOD (Cu,Zn-SOD), catalase (CAT) and glutathione peroxidase (GPx1)], (b) Mn-SOD protein expression, (c) lipid peroxidation markers [malondialdehyde (MDA), 8-epi-prostaglandin-F2α (8-epi-PGF2α)] and by evaluating the possible correlations between tumor biomarkers, reproductive hormone levels and all measured parameters, comprehensively. The data obtained from the patients with EOC (M, $n = 26$) evaluated according to the histopathological/clinical characteristics of tumors and compared with data of healthy controls [C_{tissue} (C1) and $C_{blood/urine}$ (C2), $n = 30$, respectively). Significantly, low activities of tumor SOD_{total} (52%), Mn-SOD (42%), Cu,Zn-SOD (55%); high activities of tumor and erythrocyte CAT (66%, 33% respectively) and tumor GPx1 (60%); high levels of tumor Mn-SOD protein expression; tumor MDA (193%) and urinary 8-epi-PGF2α (179%) were observed in serous EOC tumors (M1, $n = 18$) compared with controls ($P < 0.05$). However, higher levels of tumor MDA, Mn-SOD protein expression and urinary 8-epi-PGF2α were observed along with lower tumor CAT activity in poorly differentiated or undifferentiated (grade 3, G 3) *versus* well or moderately well differentiated (grade 1-2, G 1-2) serous EOC tumors. Obtained data indicate the presence of a severe redox imbalance in EOC and draw attention to the criticial role of AOEs in the pathogenesis of the disease. © 2017 IUBMB Life, 69(10):802–813, 2017

Keywords: *epithelial ovarian cancer; oxidative stress; lipid peroxidation; superoxide dismutase; catalase; glutathione peroxidase*

Abbreviations: *EOC, epithelial ovarian cancer; SOD_{total}, total superoxide dismutase; Mn-SOD, manganese superoxide dismutase; Cu, Zn-SOD, copper,zinc superoxide dismutase; CAT, catalase; GPx1, glutathione peroxidase; LPO, lipid peroxidation; MDA, malondialdehyde; 8-epi-PGF2α, 8-epi-prostaglandin-F2α; AOEs, antioxidant enzymes; OS, oxidative stress; ROS, reactive oxygen species; FSH, follicle stimulating hormone; LH, luteinizing hormone; FIGO, internatiol federation of gynecology and obstetrics; G1–2, grade 1 and 2; G 3, grade 3; S I-II, stage I and II; S III, stage III; BMI, body mass index*

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Introduction

Ovarian carcinoma is a dangerous disease with respect to its histopathology, clinical characteristics and fatal outcomes. Early ovarian cancer usually has no obvious symptoms; so the disease is usually diagnosed at advanced stages despite to more promising developments in the area of medicine. Although it accounts for 5% of cancer deaths among women and is less prevalent than uterine carcinomas (corpus uteri, cervix uteri), it is the leading cause of death from gynecological cancers (1–3). It is the fifth most frequent cause of death from cancer in women in European Union countries (EU-28), whereas ranks seventh among most common female cancers and second among gynecological cancers in Turkey. From 2003 to 2012, ovarian cancer incidence and mortality rates decreased by 0.9% and 2%, respectively. However, ovarian cancer incidence is 3.6% (age-standardized rate is 6.1) and mortality is 4.3% (age-standardized rate is 3.8) in all over the world, whereas the incidence is 3.9% (age-standardized rate is 6.3) and mortality is 4.8% (age-standardized rate is 4.2) in Turkey according to the GLOBOCAN 2012 data (1,4–6). As most ovarian cancers occur after menopause due to cessation of normal physiological ovarian functions, the incidence and mortality rates increase with age. Epithelial ovarian cancer (EOC) comprises 90% of malignant and 60% of all ovarian tumors and has been associated with oxidative stress (OS) due to epithelial inflammation of the ovaries arising from incessant ovulation, which might be an etiological factor that makes ovaries more vulnerable to the deleterious effects of ROS repeatedly (7–9).

OS is an imbalance between toxic reactive species [reactive oxygen species (ROS) and reactive nitrogen species] and antioxidant defense system. ROS are products of normal cellular metabolism and play a dual role with their both harmful and beneficial effects to the body (10–12). Increased generation of ROS and a decreased removing capacity of antioxidant system are the features of a malignant tumor cell (13). Although normal levels of ROS are associated with normal cellular signal transduction, normal growth and development, increased levels of ROS cause cellular damages such as genomic instability, uncontrolled growth and perturbed differentiation, which are the characteristics of malignant phenotype. In addition, high ROS levels might activate signaling pathways that contribute to tumor cell proliferation, chemoresistance, invasion and metastasis (14–16). However, ROS may have a regulatory role in various physiologic functions of female reproductive system as oocyte maturation, folliculogenesis, ovarian steroidogenesis and luteolysis. They influence both reproductive and menopausal periods of women in some conditions and diseases such as pregnancy, normal parturition, age-related infertility, premature labor, preeclampsia, menopause, endometriosis, uterine leiomyoma and some gynecological cancers (17,18). Thus, the balance between ROS and antioxidant mechanisms in healthy female reproductive system is critical.

Antioxidant enzymes (AOEs) protect aerobic cells against the biological damage of free radicals and regulate the intracellular signaling pathways to provide cancer prevention by maintaining normal cell cycle progression, inducing apoptosis, activating phase-II detoxification enzymes and inhibiting proliferation, tumor invasion, angiogenesis and inflammation (11). Superoxide dismutases (SODs) along with catalase (CAT) and glutathione peroxidase (GPx) play a critical role against lipid peroxidation (LPO). When the studies about AOEs were evaluated, marked alterations were found in the enzyme activities with elevated LPO levels in most of the cancer cells due to disturbed redox status. Initially, AOEs may be supplied through the antioxidant system in response to acute OS, but over a period of time, prolonged OS elevates ROS production and induces consumption and/or decreases activity of AOEs, which in turn further increase the risk of developing cancer due to aggravation of ROS. In this regard, manganese SOD (Mn-SOD), a conspicuous member of AOEs, was found to play a critical role in a wide range of OS-induced pathological conditions. CAT and GPx1 are also important AOEs which modulate intracellular ROS and have complex effects on cancer development and progression due to dual role of hydroperoxides in cancer (19–23).

Despite the availability of general knowledge and various data on the fundamental importance of OS in the development and progression of cancer, there is no study assessing the possible relationships and correlations between oxidant–antioxidant parameters, tumor markers, reproductive system hormones, comprehensively in EOC both according to the histopathological and clinical characteristics of the disease. To understand the critical role of AOEs in the pathogenesis of EOC, we aimed to obtain the oxidant-antioxidant status alterations in the tumor tissue, blood and urine samples of patients with particularly serous along with other types of EOC and to evaluate the data according to the tumor grading and staging of the disease. In addition, we planned to reveal all the possible correlations between AOEs, LPO markers, tumor biomarkers and reproductive system hormones.

Materials and Methods

Chemicals and Reagents

Prolactin, free testosterone, progesterone, estradiol, follicle stimulating hormone (FSH), luteinizing hormone (LH) and total testosterone kits were purchased from Abbott (Abbott Laboratories, Abbott Park, IL) and Siemens (Los Angeles, CA). Tumor biomarker kits were purchased from Roche (Roche Diagnostics, Mannheim, Germany). Mouse SOD-2 (4F10) primary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and rabbit β-actin primary antibody was purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse IgG and goat anti-rabbit IgG seconder antibodies used for Mn-SOD and β-actin analysis were both purchased from Thermo Fisher Scientific (Waltham, MA) and reagents were of the highest analytical grade for high pressure liquid chromatography (HPLC)

TABLE 1 ***Histopathological and clinical characteristics of primary epithelial malignant ovarian tumors (n = 26), classified according to the FIGO***

Histopathological and clinical characteristics		
Variables		n *(%)*
Primary epithelial ovarian tumors (M)		*26 (100)*
Histology	Serous adenocarcinoma	18 (69)
	Endometrioid adenocarcinoma	4 (15)
	Mixed adenocarcinoma	3 (12)
	Clear cell adenocarcinoma	1 (4)
Histologic grade (G)	1–2	14 (54)
	3	12 (46)
Clinical stage (S)	IIIc	17 (65)
	IIa	5 (19)
	IIb	1 (4)
	Ia	3 (12)
Primary epithelial serous ovarian tumors (M1)		18 (100)
Histologic grade (G)	1–2	9 (50)
	3	9(50)
Clinical stage (S)	IIIc	12 (67)
	IIa	4 (22)
	IIb	1 (5.5)
	Ia	1 (5.5)

and were as follows: 1,1,3,3-tetraethoxypropane, trichloroacetic acid, phosphoric acid, 2-thiobarbituric acid, butylated hydroxytoluene and potassium dihydrogen phosphate and methanol were all obtained from Sigma Aldrich (St. Louis, MO). Commercially available competetive enzyme-linked immunoassay (ELISA) kit BIOXYTECH 8-Epi-Prostaglandin-$F_{2\alpha}$ (8-epi-PGF2α) was obtained from OxisResearch (Portland, OR). All reagents and chemicals used to measure AOEs activities were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich (St. Louis, MO).

Study Groups

The cases consisted of malignant epithelial ovarian tumors (M, $n = 26$) obtained from patients who underwent surgical resection at the Hacettepe University Adult Hospital, Department of Obstetrics and Gynecology (Ankara, TURKEY): serous adenocarcinomas (M1, $n = 18$) and miscellaneous adenocarcinomas (M2, $n = 8$). The tumor SOD_{total}, Mn-SOD, Cu,Zn-SOD, CAT and GPx1 activities along with malondialdehyde (MDA) levels were compared with data of a $control_{tissue}$ group (C1) comprised of 30 normal healthy ovarian tissue specimens which were obtained by abdominal or vaginal hysterectomy and oophorectomy due to various conditions such as uterine leiomyoma, intractable menorrhagia and pelvic relaxation. The levels of tumor biomarkers, reproductive system hormones, plasma MDA and urinary 8-epi-PGF2α were compared with results of $control_{blood/urine}$ group (C2) comprised of blood and urine samples of 30 healthy individuals without any diease including gynecological cancers. Blood and urine parameters of M1 and M2 were compared with data of C2 to exclude the possible oxidant-antioxidant status alterations due to existing pathologies such as uterine leimyoma in C1. The patients diagnosed for the first time were involved in the study and those receiving radiation therapy for metastases were excluded. Tumor samples were classified separately according to the Internatiol Federation of Gynecology and Obstetrics (FIGO) staging and grading (Table 1). Written informed consent was obtained from all the patients before participation and a comprehensive questionnaire has been carried out to collect data about age, height, body weight, body mass index (BMI), education level, smoking history and reproductive system information of participants, The study was approved by the Hacettepe University Clinical Research Ethics Committee.

Preparation of Tissue Homogenates, Blood and Urine Samples

All ovarian tissues, blood and urine samples were immediately transferred to the laboratory with a cold chain. Fifteen milliliter venous blood samples were obtained in the morning after an overnight fasting. Heparinized blood samples were centrifugated at 3,000*g* for 15 min to obtain plasma and after separating the plasma, the packed cells (red blood cells) were washed thrice with cold physiological saline. Ten milliliter of urine samples were also obtained before the surgery for the measurement of 8-epi-PGF2α levels. All the samples were kept at −80 °C until analyses. Tumor biomarkers and reproductive hormone levels were assessed by the Hacettepe University Adult Hospital Central Laboratory and the oxidant-antioxidant status parameters were determined by our toxicology laboratory.

Ovarian tissue homogenates were prepared in a volume of ice-cold Tris–diethylenetriaminepentaacetic acid (DTPA)–phenylmethanesulfonyl fluoride (PMSF) buffer (10 mM Tris, 1 mM DTPA and 1 mM PMSF, adjusted to pH:7.4) to obtain a 10% (w/v) whole tissue homogenate. After centrifugation of the homogenates at 4,000*g*, 4 °C for 10 min, the supernatant was divided into two fractions: MDA levels were measured in Supernatant I by HPLC technique. Supernatant II was recentrifugated at 13,000*g*, 4 °C for 20 min and the SOD_{total}, Mn-SOD, Cu,Zn-SOD, CAT and GPx1 activities were determined in the latter supernatant.

Measurement of Tumor Biomarkers and Hormone Levels

Serum CA125 and CA15–3 levels were determined using electrochemiluminescence immunoassay. Serum prolactin, total testosterone, free testosterone, progesterone, estradiol, FSH and LH levels were measured using chemiluminescent microparticle immunoassay.

Determination of AOE Activities and Manganese SOD Expression

The activities of SOD_{total}, Mn-SOD and Cu,Zn-SOD were assessed by monitoring the auto-oxidation of pyrogallol at 420 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of pyrogallol auto-oxidation by 50% (24). The specific activities of all SODs were expressed as units of enzyme per milligram protein and hemoglobin (U mg^{-1} protein and U mg^{-1} Hb, respectively). Erythrocyte Cu,Zn-SOD specific activity is equal to erythrocyte SOD_{total} activity. CAT activity was determined spectrophotometrically by following the enzymatic decomposition of hydrogen peroxide (H_2O_2) directly at 240 nm (25). One unit of CAT activity was defined as the amount of enzyme required to decompose 1 μM H_2O_2 per minute and expressed as U mg^{-1} protein and U mg^{-1} Hb. GPx1 activity was measured in a coupled reaction with glutathione reductase as described earlier (26). One unit of GPx1 activity is defined as the amount of enzyme that oxidized 1 μM NADPH to NADP per minute and one unit of enzyme was expressed as U mg^{-1} protein and U g^{-1} Hb. Total soluble protein concentrations in ovarian tissue homogenates were determined according to a standart method (27).

The Mn-SOD expression was determined using a Western blot analysis in ovarian tissue samples. The malignant ovarian tumor samples and healthy ovarian tissues were homogenized and tissue extracts were loaded into the gel. Total protein was electrophoresed in a 12% sodium dodecyl sulfate polyacrylamide running gel and a 4% stacking gel. Actin was used for loading controls. After electrophoresis, proteins were then electrotransferred onto poly(vinylidene fluoride) membranes. Mouse SOD-2 (4F10) primary antibody, goat anti-mouse IgG, rabbit β-actin primary antibody and goat anti-rabbit IgG were used for Mn-SOD protein detection by Western blotting. Primary antibodies were diluted 1:1,000 and secondary antibodies were diluted 1:100,000 to identify the Mn-SOD and β-actin. Relative signal intensities of immunoreactive bands were determined by a public domain Java image processing program called Image J.

Determination of LPO

The MDA levels were determined quantitatively by HPLC (28) equipped with an autosampler (Hewlett Packard Agilent, 1100 series, Vienna, Austria) using a fluorescence detector (excitation wavelength of 515 nm and emission wavelength of 550 nm). The analytical column was a reverse phase silica-based C_{18} column (ACE, Scotland) with length of 25 cm × 4.6 mm i.d., 5 μm particle size. The mobile phase was %65 50 mM KH_2PO_4-KOH, pH 7.0, and %35 MeOH. The flow rate was 0.6 mL min^{-1}, and the injection volume was 100 μL. The average retention time of the MDA-$(TBA)_2$ complex was 4.7 min. The levels were calculated directly by calibration curves of peak area prepared for MDA standards and results were given as μM.

The 8-epi-PGF2α levels of urine samples were determined using a competitive ELISA. Assays were performed as per manufacturers' instructions and a standard curve was obtained by fitting the standard absorbances at 450 nm to the concentration of 8-epi-PGF2α by the 4-parameter logistic curve fit method. Samples were run in duplicate to assure consistency, and intra-sample variability was less than 10%. Results were given as nanogram per milliliter.

Statistical Analyses

Experimental data were analyzed with Kruskal–Wallis test followed by Mann–Whitney *U* test using a using a Statistical Package for Social Sciences Programme (SPSS programme v23.0, SPSS Inc., Chicago, IL). Non parametric Chi-square test was used to determine relationships between categorical variables. The correlations between all the parameters were assessed using Spearman correlation test. The level of significance was defined as ($P < 0.05$) and values are given as mean ± standard error of mean (SEM).

Results

Demographic and Reproductive System Data

Demographic and reproductive system data of patients with EOC and controls are summarized in Table 2. In our study, as the diet profile of cases and controls were enquired before the study and no statistically significant differences were found with respect to their dietary habits, patients with M were found to be overweighted and very close to obese category with their mean BMI (28.66 ± 1.11 kg/m^2). Early age at menarche, late age at menopause, heavy premenstrual syndromes and long and incessant ovulation are classified as menstrual risk factors, whereas nulliparity, decreased parity, abortus, delayed childbearing, infertility or infertility treatment and short-term breastfeeding are admitted as fundamental reproductive risk factors for EOC. The individuals in M, C1 and C2 were matched in terms of their mean age, BMI, age at menopause, fertility period, gravida and parity and only early age at menarche was noted as a risk factor in M compared with controls ($P < 0.05$). On the other hand, total breastfeeding period of M have been found to be significantly higher (60–95%) than that of controls ($P < 0.05$) (Table 2).

We also planned to identify whether any association exists between education level and EOC risk and a significant difference was observed between M and C2 with respect to their education levels (χ^2 =11,956; $P < 0.05$). Approximately 30% of the patients with EOC were illiterate, whereas this ratio decreased to approximately 8% in healthy individuals. In addition, although there are conflicting reports about cigarette smoking as a risk factor for developing EOC, a significant association was found between M and C2 groups with respect to

TABLE 2 *Demographic and reproductive system data of patients with malignant epithelial ovarian tumors and controls*

Data/study groups	*Primary epithelial ovarian tumors (M) (n = 26)*	*Primary epithelial serous ovarian tumors (M1) (n = 18)*	*Miscellaneous epithelial ovarian tumors (M2) (n = 8)*	*$Control_{tissue}$ (C1) (n = 30)*	*$Control_{blood/urine}$ (C2) (n = 30)*
Mean age (year)	55.6 ± 1.75[a]	57.3 ± 2.35[a]	52.8 ± 2.12[a]	49.9 ± 0.91[a]	43.2 ± 1.09[a]
Range	41–75	41–75	44–60	44–61	38–60
BMI (kg/m^2)	28.66 ± 1.11[a]	29.80 ± 1.57[a]	26.36 ± 1.24[a]	31.32 ± 1.30[a]	26.89 ± 0.75[a]
Age at menarche (year)	13.00 ± 0.32[a]	13.00 ± 0.32[a]	12.90 ± 0.63[a]	14.30 ± 0.24[b]	13.7 ± 0.19[b]
Age at menopause (year)	49.20 ± 0.91[a]	49.90 ± 1.18[a]	48.40 ± 1.12[a]	48.50 ± 1.72[a]	47.5 ± 0.97[a]
Fertility period (year)	36.20 ± 1.15[a]	37.10 ± 1.51[a]	35.20 ± 1.62[a]	34.40 ± 1.65[a]	33.7 ± 1.03[a]
Total breastfeeding period (month)	50.40 ± 9.48[a]	59.00 ± 12.70[a,c]	29.70 ± 8.69[b]	31.40 ± 3.92[b]	25.9 ± 3.58[b]
Gravida	4.62 ± 0.57[a]	5.44 ± 0.70[a,c]	2.75 ± 0.71[b]	4.63 ± 0.42[a]	4.85 ± 0.37[a]
Parity	3.04 ± 0.37[a]	3.50 ± 0.47[a,c]	2.00 ± 0.32[b]	2.97 ± 0.26[a]	2.04 ± 0.26[a]

All data are given as mean ± SEM.
[a]*Mean values within a line sharing a common superscript letter were not significantly different (P > 0.05).*
[a,b]*The different superscript letters (a–b) within a line indicate significant differences (P < 0.05).*
[c]*M1* versus *M2 (P < 0.05).*

their smoking habits (χ^2 =13,894; $P<0.05$) and women who smoke more than half a pack of cigarettes per day were found to be more at risk to develop malignant tumors.

Tumor Biomarkers and Reproductive System Hormone Levels

Tumor biomarker and reproductive system hormone levels of patients with M and C2 are summarized in Table 3. The serum CA125 levels of M were found markedly higher (~60%) than that of C2 ($P<0.05$). The elevation was markedly higher (95%) in M1, whereas (30%) in M2 compared with C2. In addition, CA125 levels of patients with S III (2561.8 U mL^{-1}) were nine times higher than that with S I–II (288.9 U mL^{-1}) and the levels of G 3 (2,344.9 U mL^{-1}) were 1.6 times higher than that of G 1–2 tumors (1,417.9 U mL^{-1}). Although there was no any other cancer diagnosis (breast cancer, etc.) in patients with M, the CA15–3 levels were also obtained higher compared with C2 ($P<0.05$), while the elevation of CA15–3 levels were not as high as of CA125 levels (Table 3). The mean CA15–3 levels were also markedly comparable among M1 and M2 ($P<0.05$). Prolactin and free testosterone levels were found to be markedly high (50% and 25%, respectively, $P<0.05$) along with increases in FSH and LH levels (30% and 51%, respectively, $P>0.05$), whereas progesterone and estradiol levels were low (34% and 33%, respectively, $P>0.05$) in M compared with C2. On the other hand, total testosterone levels in M were approximately similar to levels in C2 ($P>0.05$). The mean prolactin and free testosterone levels were also markedly comparable among M1 and M2 ($P<0.05$).

AOE Activities and Manganese SOD Protein Expression

All the alterations of tumor and erythrocyte AOE activities are illustrated in Table 4. Significant decreases in tumor SOD_{total}, Mn-SOD and Cu,Zn-SOD activities were observed in M1 compared with C1 (52%, 42%, 55%, $P<0.05$, respectively), whereas activities of these tumor SODs were found to be high (38%, $P<0.05$; 34%, $P>0.05$; 25%, $P>0.05$, respectively) and erythrocyte Cu,Zn-SOD activity was found to be low (23%, $P<0.05$) in M1 compared with M2. Both tumor and erythrocyte CAT activities were found to be high (66%, 33%, $P<0.05$, respectively) in M1 and (60%, 52%, $P<0.05$, respectively) in M2 compared with control. Tumor GPx1 activity was observed higher (60%), whereas erythrocyte GPx1 activity was found to be lower (27%) in M1 compared with control ($P<0.05$). In addition, tumor GPx1 activity of M1 was comparably higher (78%) than the activity of M2 (Table 4).

The tumor activities of AOEs were also evaluated according to the tumor grading and staging of disease in M1. High SOD_{total}, Mn-SOD and Cu,Zn-SOD activities were observed in G 1–2 compared with G 3 serous tumors ($P>0.05$). The tumor CAT activity of G 1–2 was significantly higher (50%) than G 3, whereas erythrocyte CAT activity was lower (21%) than G 3 ($P<0.05$). No alterations were obtained both in the tumor and

TABLE 3 *Tumor biomarker and reproductive system hormone levels of patients with malignant epithelial ovarian tumors and controls*

Variables/study groups	*Primary epithelial ovarian tumors (M) (n = 26)*	*Primary epithelial serous ovarian tumors (M1) (n = 18)*	*Miscellaneous epithelial ovarian tumors (M2) (n = 8)*	*$Control_{blood}$ (C2) (n = 30)*
CA-125 (U mL^{-1})	1145 ± 319.27[a]	1804 ± 505.45[a,c]	564.07 ± 210.55[a]	18.34 ± 1.84[b]
CA15–3 (U mL^{-1})	118.72 ± 22.29[a]	172.70 ± 29.44[a,c]	38.43 ± 4.92[a]	15.04 ± 1.44[b]
Prolactin (ng mL^{-1})	32.64 ± 8.27[a]	25.67 ± 7.60[a,c]	48.57 ± 21.67[a]	15.96 ± 1.17[b]
Total testosteron (ng dL^{-1})	31.58 ± 3.14[a]	31.24 ± 3.18[a]	32.52 ± 8.72[a]	33.50 ± 3.26[a]
Free testosteron (pg mL^{-1})	2.08 ± 0.23[a]	1.89 ± 0.33[a,c]	2.24 ± 0.19[a]	1.59 ± 0.11[b]
Progesteron (ng mL^{-1})	1.46 ± 0.31[a]	1.29 ± 0.74[a]	1.84 ± 1.25[a]	2.22 ± 0.13[a]
Estradiol (pg mL^{-1})	58.25 ± 17.74[a]	51.24 ± 22.42[a]	75.29 ± 28.50[a]	86.50 ± 18.05[a]
FSH (mIU mL^{-1})	37.27 ± 5.47[a]	40.20 ± 6.42[a]	30.16 ± 9.95[a]	28.67 ± 6.79[a]
LH (mIU mL^{-1})	20.52 ± 3.20[a]	21.70 ± 3.91[a]	17.64 ± 5.42[a]	13.59 ± 2.16[a]

All data are given as mean ± SEM.
[a]Mean values within a line sharing a common superscript letter were not significantly different (P > 0.05).
[a,b]The different superscript letters (a–c) within a line indicate significant differences (P < 0.05).
[c]M1 versus *M2 (P < 0.05).*

erythrocyte GPx1 activities according to the tumor grading and staging of the disease ($P > 0.05$) (Table 6).

After immunoblotting detection, a immunopositive band (25 kDa) was recognized in the ovarian tissue protein lysates, based upon immunoreactivity with specific Mn polyclonal antibody. The band with the molecular weight of 25 kDa, corresponded to the monomeric Mn-SOD form that exists in human as a homotetramer with an individual subunit molecular weight of about 23 kDa. Tumor Mn-SOD protein was significantly more expressed in M1 compared with M2 and C1 (77%, 67%, $P < 0.05$, respectively). Besides this, the elevation was higher (74%, $P < 0.05$) in G 3 *versus* G 1–2 of M1, whereas only a slight induction of tumor Mn-SOD expression (~20%) was observed in patients with S III compared with S I–II serous EOC ($P > 0.05$).

LPO, MDA and 8-Epi-Prostaglandin $F_{2\alpha}$ Levels

Tumor and plasma MDA levels along with urinary 8-epi-PGF2α levels are shown in Table 5. Tumor MDA levels were higher (193%, 127%, $P < 0.05$, respectively) in all histologic subtypes of malignant epithelial ovarian tumors (M1, M2), whereas plasma MDA levels of M1 and M2 were not found to be different compared with C1 ($P > 0.05$). Elevations (179%, $P < 0.05$; 35%, $P > 0.05$, respectively) were also obtained for urinary 8-epi-PGF2α levels with respect to tumor histologic subtype (M1, M2) compared with C2. Tumor MDA and urinary 8-epi-PGF2α levels of M1 were found to be significantly higher in G 3 (127%, 216%, $P < 0.05$, respectively) compared with G 1–2 and only urinary 8-epi-PGF2α levels were found to be higher in S III (233%, $P < 0.05$) compared with S I–II (Table 6).

Correlations

Marked correlations were observed among tissue AOEs and between AOEs, LPO markers, tumor markers and reproductive system hormones of M1: (a) a positive correlation was observed between tissue SOD_{total} activity and tissue Cu,Zn-SOD activity ($r = 0.869$, $P = 0.000$). (b) A negative correlation was obtained between tissue GPx1 activity and serum CA-125 levels ($r = -0.591$, $P = 0.027$), (c) some significant positive correlations were noted between tissue CAT activity and serum total testesterone levels ($r = 0.697$, $P = 0.038$), tissue CAT activity and serum progesterone levels ($r = 0.856$, $P = 0.003$), plasma MDA and serum prolactin levels ($r = 0.528$, $P = 0.021$), plasma MDA and serum estradiol levels ($r = 0.675$, $P = 0.008$). (d) A significant negative correlation was noted between erythrocyte Cu,Zn-SOD activity and free testesterone ($r = -0.588$, $P = 0.019$). In addition, no significant positive correlation was found between urinary 8-epi-PGF2α levels and MDA levels, whereas insignificant but inverse correlations were obtained between urinary 8-epi-PGF2α levels and all measured tissue AOEs activities of M1.

Discussion

Evaluation of Reproductive System Hormone Levels

The pathogenesis of the ovarian cancer has not been fully clarified yet. However, based on various hypotheses (incessant

TABLE 4 *Tissue and erythrocyte AOE activities of patients with malignant epithelial ovarian tumors and controls*

	Epithelial ovarian tumors				*Control tissue and blood samples*	
	Primary epithelial serous ovarian tumors (M1) (n = *18*)		*Miscellaneous epithelial ovarian tumors (M2)* (n = *8*)		*Control$_{tissue}$ (C1)* (n = *30*)	*Control$_{blood}$ (C2)* (n = *30*)
Parameters/study groups	*Ovarian tumor*	*Erythrocyte*	*Ovarian tumor*	*Erythrocyte*	*Ovarian tissue*	*Erythrocyte*
SOD_{total} activity	5.24 ± 0.41[a]	–	3.80 ± 0.25[b]	–	10.55 ± 0.48[c]	–
Mn-SOD activity	2.22 ± 0.21[a]	–	1.54 ± 0.08[a]	–	3.83 ± 0.17[b]	–
Cu,Zn-SOD activity	3.03 ± 0.35[a]	1.33 ± 0.07[d]	2.42 ± 0.25[a]	1.72 ± 0.08[e]	6.72 ± 0.42[b]	1.31 ± 0.09[d]
CAT activity	27.16 ± 4.31[a]	295.91 ± 16.28[d]	26.12 ± 5.02[a]	338.05 ± 35.49[d]	16.36 ± 1.53[b]	221.78± 13.59[e]
GPx1 activity	0.16 ± 0.01[a]	27.11 ± 2.00[d]	0.09 ± 0.01[b]	29.78 ± 2.70[d]	0.10 ± 0.01[b]	37.00 ± 1.75[e]

All data are given as mean ± SEM. Tissue activities were expressed as (U mg^{-1} protein) for SOD_{total}, Mn-SOD, Cu,Zn-SOD, CAT and (U mg^{-1} protein) for GPx1. Erythrocyte activities were expressed as (U mg^{-1} Hb) for Cu,Zn-SOD, CAT and (U g^{-1} Hb) for GPx1.

[a,b,c]Mean values within a line not sharing a common superscript letter (a-c) were significantly different ($P < 0.05$).

[d,e]Mean values within a line not sharing a common superscript letter (d,e) were significantly different ($P < 0.05$).

ovulation, gonadotropin, androgens and progesteron), hormones can play an etiologic role in the development of ovarian cancer by regulating the cell proliferation, differentiation and apoptosis. Androgens may play an important role in the ovarian cancer pathogenesis by stimulating ovarian cancer formation due to excessive androgenic stimulation of androgenic receptors. Excessive stimulation of ovarian tissue due to decreased levels of peripheral estrogen and increased levels of pituitary FSH and/or LH are associated with ovarian tumor development (9,29–31). On the other hand, progesterone may have a preventive role against the ovarian cancer risk (9,32). In our study, androgen, gonadotropin and progesterone hypotheses were supported by elevated free testosterone, FSH, LH levels (31%, 30% and 51%, respectively) and decreased progesterone and estradiol levels (34%, 33%, respectively) of M compared with control. Prolactin plays a causal role in many cancer types via its local synthesis and/or accumulation (33,34). The high levels obtained in some types of cancers (*e.g.*, breast, endometrial and ovarian) diminish the use of prolactin as a biomarker alone for ovarian cancer (35,36). However, elevated levels were accepted as a risk factor for endometrial and ovarian cancers because of prolactins' triggering effect on tumor growth via *Ras* oncogene activation or increasing effect on the malignant transformation of the mutated cells in the tumor supressing genes (33). Elevated serum prolactin levels (104%) of M compared with control supported the hypothesis claiming prolactin as a risk factor for EOC development.

Genotoxic effects and mitotic effects of estrogen were reported as a potent factor for the neoplastic transformation of ovarian surface epithelium cells. In addition, estrogens in peripheral circulation and/or local production area play an important role in the initiation and progression of tumor by supressing apoptosis (9,29–31). In accordance with these knowledge, estradiol levels were found high and progesterone levels preventively effective against to the EOC development were found low (approximately two times for both) in G 3 compared with G 1–2 of M1. However, only prolactin and estradiol levels were high in S III compared with S I–II (~2.5 and 1.6 times, respectively). All these changes observed in the hormonal status with respect to the tumor grading and staging of disease were in support of the hypothesis related to ovarian cancer patogenesis.

Evaluation of Oxidant and Antioxidant Status

Although there are several studies evaluating the role of OS in various cancer types, to our knowledge, this is the first study assessing the AOEs and OS-induced LPO together in ovarian cancer comprehensively according to the histopathological and clinical characteristics of tumors and evaluating the correlations between AOEs activities, Mn-SOD expression, LPO markers, tumor biomarkers and reproductive hormone levels. The major oxidant and antioxidant status alterations observed in our study were summarized in detail: (a) tumor SOD_{total} (52%), Mn-SOD (42%) and Cu,Zn-SOD (55%) activities were

TABLE 5 *Tissue, blood and urine levels of LPO markers of patients with malignant epithelial ovarian tumors and controls*

	Epithelial ovarian tumors				*Control tissue, blood and urine samples*			
Parameters/study groups	*Primary epithelial serous ovarian tumors (M1) (n = 18)*			*Miscellaneous epithelial ovarian tumors (M2) (n = 8)*	*Control$_{tissue}$ (C1) (n = 30)*	*Control$_{blood}$ (C2) (n = 30)*		*Control$_{urine}$ (C2) (n = 30)*
Ovarian MDA levels	0.88 ± 0.17[a]	–	–	0.68 ± 0.04[a]	–	0.30 ± 0.03 [b]	–	–
Plasma MDA levels	–	2.52 ± 0.38[c]	–	–	1.89 ± 0.27[c]	–	2.12 ± 0.23[c]	–
Urinary 8-epi-PGF2α levels	–	–	3.24 ± 0.66[a]	–	–	1.57 ± 0.35[b]	–	1.16 ± 0.15 [b]

All data are given as mean ± SEM. Tumor and plasma levels of MDA were expressed as (μM); urinary 8-epi-PGF2α level was expressed as (ng mL^{-1}).
[a,b]Mean values within a row not sharing a common superscript letter (a–b) were significantly different ($P < 0.05$).
[c]Mean values within a line sharing a common superscript letter (d,e) were not significantly different ($P > 0.05$).

decreased, (b) tumor GPx1 (60%) and CAT (66%) and erythrocyte CAT (33%) activities were increased, (c) tumor MDA levels (%193) and urinary 8-epi-PGF2α levels (%179) were increased, whereas no changes were observed in plasma MDA levels, (d) tumor Mn-SOD protein expression was increased (67%), in M1 compared with controls. However, the same trend was also observed for M2 with the decreases in tumor SOD_{total} (64%), Mn-SOD (60%) and Cu,Zn-SOD (64%) activities and increases in tumor CAT activity (60%). Tumor MDA levels were also higher (127%) than control in M2, whereas no alterations were observed in plasma levels.

OS plays a causative and/or a consequential role in the pathogenesis of various diseases including cancer (11). Epidemiological, *in vivo* and *in vitro* studies have demonstrated OS appears to be a major source of carcinogenesis due to its contribution to tumor initiation, promotion and progression by inducing genomic instability. Perturbed redox status due to high cell metabolic activity because of high ROS generation exhibits extensively in malignant tumor tissues and OS existence in these cells is the primary stimulus for AOE induction (16,37,38).

Most malignant tumor cells likely have high cell metabolic activity and, thereby may have high oxidant generation. As OS is a primary stimulus for the induction of AOE, the AOE induction most commonly remains insufficient in response to enhanced OS and can reduce but not fully compensate the OS. The imbalance reveals a slightly pro-oxidant state and over a time creates a tumor supportive oxidant environment in which tumor suppressor elements are downregulated, and tumor cells show enhanced proliferation and are more aggressive. In this regard, DNA and cell damages occur as a result of enhanced OS and decreased AOE levels in early stages of cancer (19). The most conspicuous AOE is Mn-SOD, and it is diminished or elevated in various cancers. However, the question of when and how Mn-SOD expression is regulated still remains unclear (39). In the literature, Mn-SOD activity is generally lower in cancer cells and a possible association between decreased Mn-SOD activity and malignant phenotype was reported in solid tumors in several studies including our study (39–42). In addition, Mn-SOD overexpression with low Mn-SOD levels in transformed cells was shown to restore cells to a growth pattern (39).

In our study, tumor Mn-SOD activity was found lower (42%), whereas protein expression was found higher (67%, 77%) in M1 compared with C1 and M2. Besides this, higher levels were observed in G 3 (74%) compared with G 1–2 and S III (~20%) compared with S I-II. There might be some post-translational modifications on Mn-SOD protein due to high levels of OS. As majority of Mn-SOD is localized in mitochondria, possible explanation for the high Mn-SOD protein expression levels observed in M1 might be originated from higher content of mitochondria in the epithelial serous malignant cells and Mn-SOD protein expression might be a discriminative factor between serous and other type of malignant epithelial tumors. Early reported studies suggested that Mn-SOD might act as a

TABLE 6 *Significant AOE activities and LPO levels according to the grading and staging in primary epithelial serous ovarian cancer*

Parameters/grades and stages	*Grades*		*Stages*	
	G 1–2 (n = *9*)	*G 3* (n = *9*)	*S I–II* (n = *6*)	*S III* (n = *12*)
Tumor CAT activity	44.31 ± 10.08	21.78 ± 3.20[a]	33.73 ± 5.01	31.82 ± 6.09
Erythrocyte CAT activity	255.71 ± 12.08	323.28 ± 27.28[a]	264.74 ± 24.57	300.37 ± 21.72
Tumor MDA levels	0.51 ± 0.08	1.16 ± 0.14[a]	0.92 ± 0.39	0.86 ± 0.19
Urinary 8-epi-PGF2α levels	1.45 ± 0.24	4.58 ± 0.83[a]	1.17 ± 0.31	3.90 ± 0.82[b]

All data are given as mean ± SEM. Tumor and plasma levels of MDA were expressed as (μM); urinary 8-epi-PGF2α level was expressed as (ng mL^{-1}). Tumor CAT activity was expressed as (U mg^{-1} protein); erythrocyte CAT activity was expressed as (U mg^{-1} Hb).
[a]*G 3* versus *G 1–2* ($P < 0.05$).
[b]*S III* versus *S I-II* ($P < 0.05$).

tumor supressor gene on the basis of the low SOD expression in tumors (43,44). However, several studies with different known cancers including our study with EOC challenged the tumor supressor concept by showing higher tumor Mn-SOD protein expression levels and emphasized the key role of Mn-SOD in malignant tumor progression and metastasis (19,45,46). Moreover, tumor cells acquire defects in mitochondrial function and, therefore, use aerobic glycolysis as an alternative source of ATP production. Lower Mn-SOD levels may be a threat for impairment of mitochondria and this transition in early stages of cancer. On the other hand, as cancer cells progress and become more agressive, higher Mn-SOD levels and aerobic respiration were observed (39). Thus, higher levels of this enzyme may contribute to the repair of mitochondrial function. Our results were in consistent with the knowledge above as high expression levels observed in poorly or undifferentiated tumors (G3) and latter stages (S III) of M1. In addition, Hu et al. (45) showed substantially higher levels of Mn-SOD and Cu,Zn-SOD expression in malignant ovarian lesions. Ishikawa et al. (47,48) observed markedly high serum Mn-SOD levels in epithelial ovarian tumors and by staging of the disease. Our study was also consistent with those studies above. However, we also suggest that increased ROS stress might induce Mn-SOD expression as a cellular response to intrinsic OS.

Some scientific researches suggested high 8-OH-dG levels due to high hydroxyl radical-induced OS in ovarian cancer (49,50). Neurauter et al. (51) found high 8-isoprostane levels in S III–IV compared with S I–II and levels were increased also by tumor grading in serous cystadenocarcinomas. Our study was compatible with these studies in terms of pointing out to the existence of an oxidative metabolism towards a pro-oxidation state in EOC and increased urinary 8-epi-PGF2α levels of M1 in accordance to tumor grading and staging of the disease. In this study, 8-epi-PGF2α levels might be a non-invasive tumor biomarker for patients with suspected serous EOC due to high 8-epi-PGF2α levels measured in M1 compared with C2 and M2 and more marked elevations observed by tumor grading (216%, G 3 *vs*. G 1–2) and staging (233%, S III *vs*. S I–II) of the disease. However, tumor MDA levels were increased markedly in all types of EOC, but the increase was found more marked in M1. Induced LPO with higher tumor MDA and urinary 8-epi-PGF2α levels (127%, 216%, respectively) in G 3 compared with G 1–2 might be associated with high Mn-SOD expression observed in G 3 serous tumors. Increased OS-induced LPO along with elevated Mn-SOD levels might be an effective factor contributing to the tumor progression.

Moreover, elevated antioxidant levels or enzyme activities such as CAT and GPx1 were also reported in response to OS as an adaptive mechanism in some cancer cells (20,52–54). GPx1 is counteracting OS with its ability to prevent oxidative DNA mutations and production of proinflammatory mediators such as prostaglandins and leukotrienes. Thus, GPx1 may prevent carcinogenesis at least in the initiation phase. Loss of GPx1 in early stages of carcinogenesis may contribute to cancer initiation, whereas may promote proliferative responses in latter stages of cancer. Possibly, the major role of GPx1 in normal cells is the prevention of oxidant-mediated cytotoxicity and inflammatory response, while in transformed cells, almost same functions prevent oxidant-mediated initiation of apoptosis and increase cell growth. In contrast, excess GPx1 may prevent oxidative damage such as DNA oxidation and inflammation, but may also block apoptotic cell death, leading to enhanced survival of transformed cells. Thus, GPx1 has a complex effect on the cancer development and progression due to dual role of hydroperoxides and its role on modulating intracellular ROS (21,55,56). In our study, as a discriminative factor between serous and other malignant epithelial ovarian tumors, tumor GPx1 activity increased significantly only in patients with M1, and this result might contribute to the serous tumor cells growth and survival of transformed cells. Further clinical studies with a large number of study population are needed to clarify the pathophysiological role of GPx1 as it may be a discriminative factor between different types of EOC tumors. In addition, high tumor MDA and urinary 8-epi-PGF2α levels

might cause high tumoral GPx1 and CAT antioxidant adaptive responses in order to inhibit tumor cell apoptosis in M1 compared with C1. However, higher SOD and CAT activities can inhibit tumor cell growth and metastasis (20), and our study supported this knowledge in the literature with lower tumor CAT activity (51%) measured in tumor cells which tend to grow quickly and more likely to spread (G3) compared with G 1–2 of M1.

In the literature, the limited available researches about oxidant–antioxidant status in ovarian cancer are not comprehensive and the results are conflicting. In studies using ovarian tumor tissue samples, Sanchez et al. (50) observed low levels of tumor SOD_{total}, Mn-SOD and CAT activities and high levels of GPx1 activity and MDA levels. In contrast, Falfushynska et al. (57) obtained high Mn-SOD activity, but low CAT activity and induced oxy-radical formation compared with normal ovarian tissue. On the other hand, in studies using blood samples of patients with EOC, Senthil et al. (58), Manimaran and Rajneesh (59) and Bandebuche and Melinkeri (60) observed low SOD, CAT activities and high plasma MDA levels, whereas Gorozhanskaia et al. (61) measured high SOD and CAT activities along with high MDA levels in ovarian cancer patients compared with control. As extensively discussed above with the general knowledge in literature, we believe that it is considerably difficult to evaluate the role of oxidant-antioxidant status in EOC tumorigenesis and progression with these limited studies due to lack of knowledge about tumor histologic subtypes, tumor gradings, staging of the disease and reproductive hormonal alterations. Besides this, we also suggest that the measurements at tumor tissue level may be more reliable than the data obtained from blood samples of patients with EOC in terms of clear reflection of oxidant-antioxidant imbalance in tumor microenvironment. Moreover, evaluation of possible correlations may also be useful to clarify the links between tumor biomarkers, hormonal changes, OS and AOEs in EOC. From our point of view, the negative correlation between tumor GPx1 activity and serum CA125 demonstrated the importance of AOEs in EOC. Inverse correlations obtained between urinary 8-epi-PGF2α levels and all measured AOEs activities also emphasized the importance of OS in EOC due to reduced ROS scavenging capacity of AOEs in malignant epithelial tumors of the ovary. Positive correlations between tumor CAT activity and total testosterone or progesterone levels suggested the alterations in AOEs activities might contribute to the several hormonal hypothesis proposed for EOC.

Conclusions

In conclusion, this study pointed out to a severe OS and a stepwise elevation of redox imbalance particularly by tumor grading in serous EOC. Perturbed AOE activities and increased MDA and 8-epi-PGF2α levels might be due to excessive OS caused by epithelial inflammation regarding to incessant ovulation. However, we also demonstrated some discriminative marked differences between serous and other type of epithelial ovarian tumors in terms of investigated parameters.

Our data supported the general knowledge and approaches related to OS and AOE interactions in various cancers. However, advanced stage, higher tumor grade and pre-operative high CA-125 levels were associated with OS-induced LPO in serous EOC. The OS may be a causative or consequential factor for the impairment of AOEs' expressions and/or activities. Further clinical researches are needed with a large study population to confirm the results of this study and to identify potential underlying mechanisms related to redox imbalance which may play an important role in the pathogenesis of EOC. This study also supports the hypothesis related to the role of reproductive system hormones in ovarian cancer. Moreover, we draw attention to the importance of antioxidant defense system, as alterations in tumoral oxidant–antioxidant status along with impaired reproductive hormone levels may have a crucial role in the pathogenesis of EOC. In addition, development and use of specific, sensitive and differential biomarkers is a focus of interest in cancer research. As epithelial ovarian tumors are typically sensitive to redox imbalance, the development of therapies targeting mainly Mn-SOD and CAT may provide an important insight for the prevention and/or treatment of EOC and contribute to the new improvements in cancer treatment researches.

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Conflicts of Interest

The authors indicate no potential conflicts of interest.

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