Original Article

Antioxidant Status in Blood of Gynaecological Patients: Influence of Diagnosis and Reproductive Factors

(antioxidant enzymes / reproductive factors / gynaecological patients)

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Abstract. Cancer of the reproductive tract is an important cause of morbidity and mortality among women worldwide. In this study we evaluated the influence of diagnostic categories, age and reproductive factors on antioxidant enzymes and lipid hydroperoxides in the blood of gynaecological patients diagnosed with endometrial polyp, myoma, hyperplasia simplex, hyperplasia complex and endometrial adenocarcinoma. Multivariate regression analysis was used to assess the association of diagnosis, age, parity, abortions and abnormal uterine bleeding with the examined parameters. Diagnosis provided the best predictive model for superoxide dismutase, catalase and glutathione peroxidase activities, and also for the lipid hydroperoxide level. Abortions fitted the best predictive model for superoxide dismutase activity. A significant correlation was also found between the predictor variables themselves. This study showed that reproductive and other factors may be associated, at least partially, with antioxidant capacity and ability to defend against the oxidative damage in gynaecological patients with various diagnoses.

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Abbreviations: ACE – adenocarcinoma endometrii, AUB – abnormal uterine bleeding, AO – antioxidant, CAT – catalase, CH – hyperplasia complex endometrii, EC – endometrial cancer, EH – endometrial hyperplasia, GPx – glutathione peroxidase, LOOH – lipid hydroperoxide, PE – polypus endometrii, ROS – reactive oxygen species, SH – hyperplasia simplex endometrii, SOD – superoxide dismutase, UM – uterus myomatosus.

Introduction

Cancer of the reproductive tract is an important cause of morbidity and mortality among women worldwide, with endometrial cancer (EC) as the fourth most common cancer among women in developed countries. So far, it has been known that several factors may participate in pathogenesis of various gynaecological diseases. Obesity is considered to be strongly associated with the risk of developing endometrial cancer and it depends on the obesity degree, expressed on the relative 2–10 scale (Olson et al., 1995; Purdie, 2003). It is also believed that the association between high body weight and EC is more pronounced in postmenopausal women (Trentham-Dietz et al., 2006); however, about 5–30 % of women are pre- or perimenopausal at the time of diagnosis (Yamazawa et al., 2000; Soliman et al., 2005).

Diagnoses before the age of 50 has been linked to a number of risk factors including age of menarche, parity, failure to ovulate and tamoxifen use (Straughn and Partridge 2009; Zucchetto et al., 2009). Other factors that may contribute to increased EC risk are age, late menopause, hyperandrogenaemia (Cline, 2004). Factors affecting EC and endometrial hyperplasia (EH) are known to be, at least in part, similar (Ricci et al., 2002). However, women with benign gynaecological conditions such as endometriosis, uterine fibroids (leiomyomas) or endometrial polyps may also experience increased risk of developing hyperplasia and malignancy (Brinton et al., 2005; Silberstein et al., 2006; Rowlands et al., 2011). Studies indicate that completed or uncompleted pregnancy may be protective against EC but not against EH (Parslov et al., 2000; Pike et al., 2004; Xu et al., 2004). Association of history of abortions with the EC risk is still unclear since both positive and inverse relationships were reported (Xu et al., 2004).

Abnormal uterine bleeding (AUB) is one of the most common symptoms of endometrial cancer in postmenopausal women (Epstein and Valentin, 2004), but it also represents a risk factor for the occurrence of endometrial hyperplasia in perimenopause (Farquhar et al., 1999). About 10 % of women who have irregular bleeding in postmenopause will have a diagnosis of endometrial cancer, the same percentage will have hyperplasia, 60 % of women will be diagnosed with uterine atrophy, and 10 % will be diagnosed with polyps (Karlsson et al., 1995). Bleeding in postmenopausal women may increase the risk of developing endometrial cancer up to 64 times (Gull et al., 2003).

Oxidative stress may play an important role in individual risk of developing many diseases, including cancers. Cells developed an enzymatic antioxidant (AO) pathway against reactive oxygen species (ROS), which are generated in oxidative metabolism. Superoxide dismutase (SOD) catalyses dismutation of superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), which in the second step is converted to water by catalase (CAT) or glutathione peroxidase (GPx). GPx also reduces organic peroxides into alcohols, using glutathione as hydrogen donor (Halliwell, 2006). The activity of the first- and second-step AO enzymes has to be balanced to prevent potential oxidative damage in cells. Variations in AO capacity may influence individual susceptibility to pathological processes associated with the deleterious effects of oxidative reactions (Dalle-Donne et al., 2006; Pagliuso et al., 2008).

In our previous research, we have shown that the antioxidant status is altered in the blood and endometrium of women with endometrial hyperplasia and adenocarcinoma in comparison to those with polyps or leiomyomas. The specific changes were related to the enzyme type and diagnosis; however, the reduction in antioxidants and elevation of the lipid hydroperoxide level were observed in general (Pejić et al., 2006, 2009). In this study we sought to evaluate the association of age and reproductive factors such as parity, abortions and AUB with AO enzyme activities in the blood of these patients.

Material and Methods

Subjects

The material used in this study consisted of 88 blood samples of women admitted to the Department of Gynaecology and Obstetrics for gynaecological evaluation within routine checkups or for abnormal uterine bleeding (Metrorrhagia prolongata, Metrorrhagia recidivans, Metrorrhagia postmenopausi). The specimens were taken after obtaining the informed consent. The study was conducted prospectively and it was approved by the Human Studies Ethics Committee of the Clinical Centre. The protocol was consistent with the World Medical Association Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects). On the basis of diagnosis and histological examination, subjects were divided into the following groups: patients with polypus endometrii (PE), uterus myomatosus (UM), patients with hyperplasia simplex endometrii (SH), hyperplasia complex endometrii (CH), or adenocarcinoma endometrii, stage I (ACE). With regard to the age, parity (nulliparous, primiparous and multiparous) and abortions, patients were also classified in appropriate categories.

Samples

Venous blood samples were collected into heparinized tubes on the same day as endometrial biopsy and centrifuged at 2500 *g* for 5 min. Plasma was used for LOOH concentration measurement. For SOD assay (Oxis International, Inc., Portland, OR), the pellet was resuspended in four packed-cell volumes of ice-cold demineralized ultrapure water (MilliQ reagent grade water system, Millipore Corp., Bedford, MA). After addition of ethanol/chloroform extraction reagent (62.5/37.5 vol/vol) to remove haemoglobin interference, samples were centrifuged at 3000 *g* for 10 min (Eppendorf centrifuge 5417, Eppendorf AG, Hamburg, Germany). The upper aqueous layer was collected and kept at -70 °C until assay. The activities of CAT, GPx and GR were measured in blood lysates.

The enzyme activities and lipid hydroperoxide (LOOH) concentration were monitored spectrophotometrically (Perkin Elmer Spectrophotometer, Lambda 25, Perkin Elmer Instruments, Norwalk, CT). The specific enzyme activities were expressed as units (U) or mU per milligram of total cell protein (U or mU/mg protein). LOOH concentration was expressed as nmol/mg protein. Determination of protein concentration was performed by the method of Lowry et al. (1951) and expressed as mg/ml.

Assays

Assay of SOD activity. Determination of SOD activity was performed using Oxis Bioxytech® SOD-525™ Assay (Oxis International, Inc.). The method is based on a SOD-mediated increase of autoxidation of 5,6,6a11b-tetrahydro-3,9,10-tryhydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. The SOD activity is determined from the ratio of autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD. One SOD-525 activity unit is defined as the activity that doubles the autoxidation rate of the control blank.

Assay of CAT activity. CAT activity was determined by the method of Beutler (1982). The reaction is based on the rate of H_2O_2 degradation by catalase contained in the examined samples. The reaction was performed in an incubation mixture containing 1 M Tris-HCl, 5 mM EDTA, pH 8.0, and monitored spectrophotometrically at 230 nm. One unit of CAT activity is defined as 1 μmol of H_2O_2 decomposed per minute under the assay conditions.

Assay of GPx activity. GPx activity was assessed using the Oxis Bioxytech® GPx-340™ Assay (Oxis International, Inc.), based on the principle that oxidized glutathione (GSSG) produced upon reduction of an organic peroxide by GPx is immediately recycled to its reduced form (GSH) with concomitant oxidation of NADPH to $NADP^+$. The oxidation of NADPH was monitored spec-

Table 1. Score assigned to different factors of multifactorial analysis

Factor	Score assigned				
	1	2	3	4	5
Age (years) N =	25-35 (8)	36-46 (24)	47-57 (45)	58-68 (7)	69-79 (4)
Parity N =	0 (3)	1 (7)	2 (33)	3 (24)	> 3 (21)
Abortions N =	0 (0)	1 (35)	2 (18)	3 (17)	> 3 (18)
Abnormal uterine bleeding* N =	None (30)	MP (21)	MR (16)	MPM (21)	
Diagnosis N =	PE (18)	UM (12)	SH (31)	CH (22)	ACE (5)

* MP – Metrorrhagia prolongata, MR – Metrorrhagia recidivans, MPM – Metrorrhagia postmenopausi

trophotometrically as a decrease in absorbance at 340 nm. One GPx-340 unit is defined as 1 µmol of NADH oxidized per minute under the assay conditions.

Assay of GR activity. Activity of GR was measured using the Oxis Bioxytech® GR-340™ Assay (Oxis International, Inc.). The assay is based on the oxidation of NADPH to NADP+ during the reduction of oxidized glutathione (GSSG), catalysed by a limiting concentration of glutathione reductase. The oxidation of NADPH was monitored spectrophotometrically as a decrease in absorbance at 340 nm. One GR-340 unit is defined as 1 µmol of NADH oxidized per minute under the assay conditions.

Lipid hydroperoxides. The concentration of LOOH was measured by Oxis Bioxytech® LPO-560™ Assay (Oxis International, Inc.), which is based on the oxidation of ferrous (Fe^{2+}) ions to ferric (Fe^{3+}) ions by hydroperoxides under acidic conditions. Ferric ions then bind with the indicator dye, xylenol orange, and form a coloured complex. The absorbance of the complex was measured at 560 nm. Since hydrogen peroxide content in many biological samples is much higher than that of other hydroperoxides, samples were pre-treated with catalase to decompose the existing H_2O_2 and eliminate the interference.

Statistical analysis

Statistical analysis was conducted using the SPSS software package. The Pearson correlation method and multivariate regression analysis were used to test the association of age, parity, abortions and AUB with the activities of AO enzymes. The stepwise logistic regression model, as the most sophisticated one, was used to ensure the smallest possible set of predictor variables in the model. The principle was to enter each predictor in sequence and to assess its value. If adding the variable contributed to the model, then it was retained, and all other variables in the model were re-tested to see if they still contributed to the success of the model. To perform this analysis, variables were assigned certain numerical scores (Table 1). Statistical significance was set at $P < 0.05$.

Results

The Pearson correlation matrix obtained between 10 variables (5 predictors and 5 dependent variables) is depicted in Table 2. It is of interest to note that, except for GR activity, all other AO enzymes and LOOH level were correlated with different predictor factors. The ta-

Table 2. Variable correlation matrix in the blood

Variable	Age	Parity	Abortions	Bleeding	Diagnosis	CuZnSOD	CAT	GPx	GR	LOOH
Age	1.0	0.48***	0.25**	0.32**	0.26**	-0.22*	0.06	-0.07	-0.01	0.05
Parity		1.0	0.26**	0.28**	0.29**	-0.23*	0.08	-0.20*	-0.10	0.13
Abortions			1.0	0.12	0.11	-0.27**	0.17*	-0.07	-0.12	0.09
Bleeding				1.0	0.80***	-0.49***	0.30**	-0.55***	-0.02	0.35***
Diagnosis						-0.51***	0.34***	-0.65***	0.18*	0.39***
CuZnSOD						1.0	-0.09	0.22*	-0.08	-0.17
CAT							1.0	-0.39***	0.23**	0.42***
GPx								1.0	-0.02	-0.34***
GR									1.0	0.21*
LOOH										1.0

*P < 0.05, **P < 0.01, ***P < 0.001

Table 3. Multiple regression analysis (the strength of the model and independent contribution by significant predictor variables to the AO enzyme activities in the blood)

Activities of AO enzymes	Predictors	B	β	t	P value	$\beta \times r_{xy}$	F	Model		
								P value	r^2	Adjusted R^2
CuZnSOD	Diagnosis	-0.231	-0.483	-5.298	0.000	0.059	$F_{2,85} = 18.45$	0.000	0.303	0.286
	Abortions	-0.094	-0.218	-2.392	0.019	0.244				
CAT	Diagnosis	5.512	0.337	3.317	0.001	0.114	$F_{1,86} = 11.00$	0.001	0.113	0.103
GPx	Diagnosis	-3.210	-0.649	-7.912	0.000	0.421	$F_{1,86} = 62.59$	0.000	0.421	0.415
GR	No predictors met criteria									
LOOH	Diagnosis	0.028	0.386	3.878	0.000	0.149	$F_{1,86} = 15.04$	0.000	0.149	0.139

B = unstandardized regression coefficient, β = standardized regression coefficient, F = F statistics, which evaluates the model, r^2 = variance in enzyme activity accounted for by the predictors, t = t statistics, which evaluates the predictor

ble also shows a significant correlation between the predictor variables themselves as well as between the dependant variables. This points to possible interactions between them in the prediction of AO enzyme activities of an individual through multiple regression.

The final predictive model with multiple regression analysis for AO enzyme activities is shown in Table 3 and Fig. 1. These results show that two factors (diagnosis and abortions) fitted the best predictive model for CuZnSOD activity ($r^2 = 0.30$, $P < 0.001$). Diagnosis alone contributed with ~6 % and abortions with ~24 % to the total variations of the CuZnSOD activity.

One predictive factor alone (diagnosis) provided the best predictive model for CAT activity ($r^2 = 0.11$, $P = 0.001$) and GPx activity ($r^2 = 0.42$, $P < 0.001$). It explained ~11 % of total variations for the activity of CAT and 42 % of total variations for the activity of GPx. Diagnosis also scored alone for the predictive model of LOOH level ($r^2 = 0.15$, $P < 0.001$) and contributed with 15 %, while no predictors met criteria for the GR activity. A large part of variations of AO enzymes and LOOH level remained unexplained, which probably points to a role of many other factors that were not considered in this study or were unknown.

Discussion

The association of different clinical risk factors and various types of gynaecological pathologies is still not fully known, similarly as the influence they exert on the AO status in these patients. In this study, AO enzyme activities and the lipid peroxidation level in the blood of women with different gynaecological conditions and endometrial cancer were related to the diagnosis, AUB, age and reproductive factors (parity and abortions) to observe the strength of the relationship among them and independent association between AO enzymes and each independent variable.

The relationship between antioxidants and pathological changes found in this study points to a role of the AO defence mechanisms in the aetiology of various gynaecological disorders. A strong reverse relationship between SOD and GPx activities with diagnostic categories was recorded, as well as a positive one between diagnosis and CAT activity/LOOH concentration. These observations are in accordance with our previous findings showing a decreasing trend of SOD and GPx activities in women with endometrial hyperplasia or adenocarcinoma in comparison to those with endometrial polyp or leiomyoma (Pejić et al., 2006). Lowered SOD and GPx activities in the plasma of gynaecological patients were also reported in other studies (Chiou and Hu, 1999; Manoharan et al., 2004).

It is known that SOD, as primary scavenger of superoxide anions, along with GPx has a protective role against lipid peroxidation. Thus, the observed reverse relationship may be due to the increased endogenous production of ROS, as also evidenced by the recorded positive relationship of pathological changes in different diagnosis and LOOH concentration. It is also known that the levels of superoxide anion and hydrogen peroxide increase in various pathological conditions and that superoxide anion inactivates GPx (Blum and Fridovich, 1985). In support of these findings, a negative correlation between SOD/GPx activities and LOOH level was observed in gynaecological patients (Pejić et al., 2006). A positive relationship between diagnosis and CAT activity observed in this study indicate that CAT is less sensitive to the redox changes in the blood of the examined women. Some studies point to a greater role of this enzyme in protecting erythrocytes against peroxidative stress than GPx (Mueller et al., 1997). The positive correlation that we previously recorded between lipid hydroperoxides and CAT activity also supports this finding (Pejić et al., 2006).

When evaluating the influence of reproductive factors, we found a negative association of abortions with SOD activity only, while parity had no influence on AO enzymes or lipid peroxidation. Studies have consistently shown an inverse relation between the risk of endometrial cancer and the number of births (Cook et al., 2006). However, data about association between one or more incomplete pregnancies, differently defined in studies as miscarriages or induced abortions, and endometrial cancer are mixed (Xu et al., 2004; Pocobelli et al., 2011). Since benign gynaecologic diseases and hyperplasia may progress to cancer (Ricci et al., 2002; Brinton et al., 2005), the reproductive factors are con-

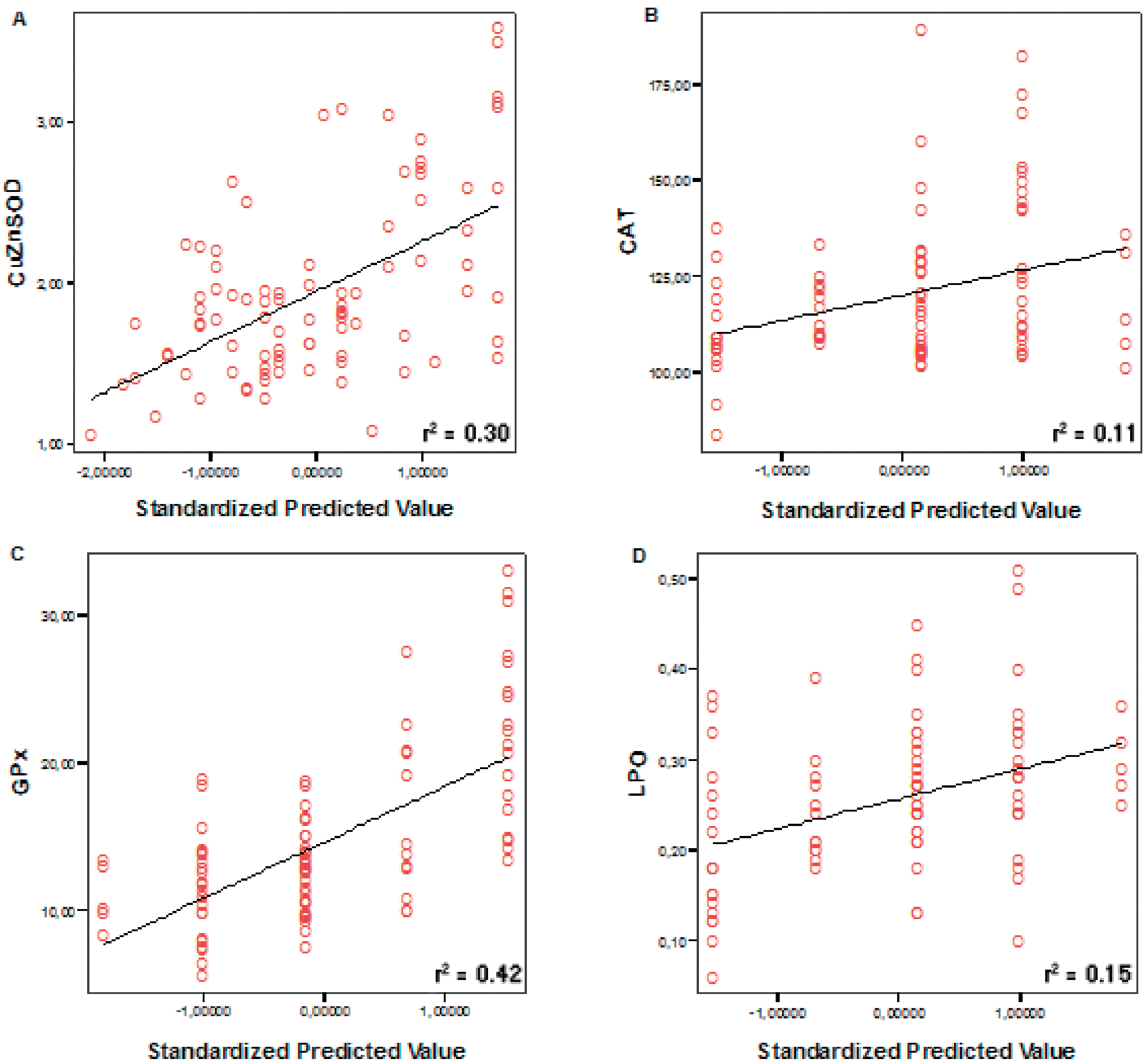

Fig. 1. Graphs showing the standard predictive value of the linear composite of predictors *vs*. CuZnSOD (**A**), CAT (**B**), GPx (**C**) activities and LOOH (**D**) level

sidered to be related with hyperplastic changes as well (Epplein et al., 2008).

Miscarriage and pregnancy appear to be associated with increased oxidative stress. During uncomplicated pregnancies, ROS levels are elevated at a certain timepoint and counterbalanced by the increased activity of antioxidants (Agarwal et al., 2012). In recurrent pregnancy loss, studies have pointed to a role of oxidative stress in its aetiology (Poston and Raijmakers, 2004; Agarwal et al., 2008). In these patients, significantly low levels of SOD, GPx and CAT were also found, in addition to an increased malondialdehyde level (El-Far et al., 2007). Spontaneous abortion is accompanied by a profound disruption of the pro-oxidant-antioxidant homeostasis towards oxidative stress (Lagod et al., 2001) and a first-trimester miscarriage was found to be associated with significantly reduced SOD levels (Jenkins et al., 2000). Thus, a negative relationship of the SOD activity and spontaneous or induced abortions observed in this study also supports the role which oxidative stress and AO defence may have in the aetiology of gynaecological disorders. Transformed tissues are known to produce high levels of ROS and are constantly under oxidative stress (Hileman et al., 2001). The increase of ROS, such as superoxide anion, is able to stimulate cell cycle progression and promote cell proliferation by molecular mechanisms that include oncogenic signals or respiratory chain malfunction (Pelicano et al., 2004). Cell damage caused by activated oxygen metabolites and altered AO capacity might be responsible for biological differences between transformed and normal tissues (Toyokuni, 2006). The negative relationship that indicates a lower SOD activity and increased superoxide concentrations, observed in our study, implies that patients with benign, premalignant and malignant gynaecological diseases are likely to be under oxidative stress.

A large part of the examined correlations remained unexplained, which probably points to a role of other factors that were not considered in this study or were unknown. However, this study shows that in gynaecological patients with various diagnoses, the reproductive and other factors may be associated with antioxidant capacity and ability to defend against oxidative damage. The correlations that were established between the predictor variables also indicate possible interactions in the prediction of antioxidant enzyme activities.

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