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ANTIOXIDANT ENZYMES IN WOMEN WITH HYPERPLASIA COMPLEX: RELATION WITH SEX HORMONES

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Abstract. *Endometrial hyperplasia complex is gynecological disorder characterized by morphological irregularities of glands shape and size. Antioxidant enzymes (AOE), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), have an essential role in preventing oxidative damage in cell caused by reactive oxygen species (ROS). In this study, we examined the AO status in hyperplastic tissue of patients in menstrual cycle (follicular and luteal phase) and in postmenopause, as well as the relationship between sex hormones and AO parameters. The phase-related activity of GPx and GR in examined patients was significantly different than in healthy women. A significant negative correlation between FSH/LH level and GPx activiy was observed. Endometrial hyperplasias are considered as precancerous lesions and are treated either conservatively or surgicaly, and also by radiation therapy. Since the effects of these therapies are associated with AO and hormonal changes, our results may contribute to the prediction of potential therapeutic efficacy and to selection of the most effective treatment for hyperplasia complex.*

Key words: endometrial hyperplasia, antioxidant enzymes, steroid hormones

Introduction

According to WHO (World Health Organization) classification, hyperplasia complex is a type of endometrial hyperplasia and could be with atypical cytology or without atypia (1). The main features of this condition, as in the other types of endometrial hyperplasia, are morphological irregularities in endometrium caused by changes in the glands shape and size. This leads to rise in the gland to stroma ratio when compared with normal endometrium (2). Beside the age, obesity, hormone replacement therapy and diabetes are among the risk factors significant for hyperplasia development (3). Endometrial tissue is under a strong influence of sexual hormones and endometrium exposure to an excessive amount of estrogen unopposed by progesterone leads to abnormal proliferation (4). Therefore, hormones are considered as one of the key factors in the development of endometrial hyperplasia and endometrial cancer.

Reactive oxygen species (ROS) are produced during cell physiological activity. When ROS production overcomes the antioxidant capacity of the cell, the result is oxidative stress, which may lead to different pathological conditions including cancer. Antioxidant enzymes (AOE) have an essential role in preventing oxidative damage in cell caused by reactive oxygen species. Superoxide dismutases (SODs) remove superoxide anion ($O_2^{\cdot-}$), catalyzing its reduction to hydrogen peroxide (H_2O_2). Hydrogen peroxide becomes a substrate for two enzymes, catalase (CAT) and glutathione peroxidase (GPx) that reduce H_2O_2 into water (5). For its catalytic activity, GPx requires glutathione (GSH) as a hydrogen donor, coupling its oxidation to GSSG with reduction of H_2O_2. Glutathione itself acts as antioxidant by scavenging reactive oxygen species. Glutathione reductase (GR) converts GSSG into GSH (6).

The AO status and hormone influence were studied during the menstrual cycle and postmenopause in healthy women and in some gynaecological disorders. We have shown that AO enzymes activity and lipid hydroperoxide (LOOH) level in patients with endometrial polyps are influenced by the changes in sex hormones during the menstrual cycle and in menopause (7). In this study, we examined the AO status in menstrual cycle and postmenopause of women with endometrial hyperplasia complex and a relationship between sex hormones and AO parameters.

Methods

Subjects. The material used in this study consisted of 31 endometrial tissue specimens of women admitted to the Department of Gynecology and Obstetrics for gynecological evaluation within routine checkups or for abnormal uterine bleeding. Study was conducted prospectively and it was approved by the Human Studies Ethics Committee of the Clinical Center. The protocol was consistent with the World Medical Association Declaration of Helsinki. None of them had undergone hormone therapy or any other medical treatment in the last 6 months. Women were divided in three groups: women in proliferative (follicular phase, F), in secretory (luteal phase, L), and women in postmenopause (PM).

Endometrial samples were washed in saline solution and homogenized in phosphate buffer containing 0.05M KH_2PO_4 and 1 mM EDTA, pH 7.8 and frozen at -70°C for 20 h in order to disrupt cell membranes. For SOD assay, thawed homogenates were vortexed 1 min and centrifuged at 8600 g, for 20 min at 4°C. According to manufacturer's recommendation, after addition of ethanol/chloroform extraction reagent (62.5/37.5 vol/vol) to completely remove hemoglobin interference, samples were centrifuged at 6000 g for 20 min, at 4°C. Upper aqueous layer was collected and kept at -70°C until assay. The enzyme activities and lipid hydroperoxide (LOOH) concentration were monitored spectrophotometrically using Oxis Bioxytech® Assays (Oxis International, Inc., Portland, OR, USA).

Assay of SOD activity. The method is based on 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 the autoxidation rates in the presence (Vs) and in the absence (Vc) of SOD. One SOD 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 (8). 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 1M Tris-HCl, 5mM EDTA, pH 8.0, and monitored 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 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 as a decrease in absorbance at 340 nm. One GPx unit is defined as 1 μmol of NADH oxidized per minute under the assay conditions.

Assay of GR activity. Assay is based on the oxidation of NADPH to NADP+ during the reduction of oxidized glutathione (GSSG), catalyzed by a limiting concentration of glutathione reductase. The oxidation of NADPH was monitored as a decrease in absorbance at 340 nm. One GR unit is defined as 1 μmol of NADH oxidized per minute under the assay conditions.

Lipid hydroperoxides. Concentration of LOOH measurement 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 colored 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 pretreated with catalase to decompose the existing H_2O_2 and eliminate the interference.
The specific enzyme activities were expressed as U or mU/mg protein. LOOH concentration was expressed as nmol/mg protein. Protein concentration was determined by the method of Lowry et al. (9) and expressed as mg/ml.

Serum hormone levels were analysed in the hormone analysis laboratory. Estradiol (E) and progesterone (P) were determined by the RIA-CT technique used for quantitative determination of E or P in human serum or plasma. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined by the IRMA FSH and LH test, which determines the quantity of immune-radiometric human FSH and LH in serum.

Statistics. Statistical analysis was carried out by use of the Kruskal-Wallis test and the Dunn's *post hoc test*, which considered the unequal and small sample sizes we used in this study. A linear regression model was used to evaluate associations between hormonal and antioxidant variables. Before plotting the data in the regression study, the normality test on the variables was performed and the values of estradiol and progesterone were log-transformed. Two-tailed p values are given throughout. All data were analyzed using GraphPad Prism software.

Results

Significant changes were observed for all hormone concentrations, FSH ($H=5.99$, $p<0.05$), LH ($H=7.11$, $p<0.05$), estradiol ($H=8.10$, $p<0.05$), and progesterone ($H=6.42$, $p<0.05$), (Table 1). We also found significant phase-related changes of GPx ($H=7.84$, $p<0.05$) and

GR activity (H=8.10, p<0.05). Both enzymes had similar activity pattern, which was higher in follicular phase compared to luteal phase and to postmenopause, ($p<0.05$). The phase-related activity of SOD and CAT as well as the LOOH level did not show any statistical difference (Table 2).

The linear regression analysis of individual hormonal variables against antioxidant parameters in endometrium showed a significant negative correlation between FSH/LH concentrations and GPx activity (r= -0.36; r= -0.47, $p<0.05$, respectively).

Table 1. Hormone levels during follicular phase, luteal phase and in postmenopause (Data are expressed as mean ± SEM; * p<0.05).

	F (n=10)	L (n=10)	PM (n=11)
FSH (U/L)*	5.97±1.42	18.06±4.87	25.59±5.62
LH (U/L)*	0.60±0.30	6.07±1.70	12.02±4.33
Estradiol (pg/ml)*	69.47±29.21	73.07±21.81	21.27±3.75
Progesterone (nmol/l)*	1.10±0.10	5.97±0.68	4.83±0.84

Table 2. LOOH concentrations and AO enzyme activities in follicular phase (F), luteal phase (L) and postmenopause (PM) in hyperplasia complex tissue. Data are shown as mean ± SEM. *P* values refer to the results of the Dunn test.

	F	L	PM
SOD (U/mg protein)	0.47 ± 0.05	0.54 ± 0.07	0.66 ± 0.07
CAT (U/mg protein)	70.18 ± 10.54	77.34 ± 10.73	67.52 ± 14.80
GPx (mU/mg protein)	37.44 ± 3.27*	21.48 ± 1.81	19.84 ± 1.60
GR (mU/mg protein)	5.96 ± 0.47*	3.41 ± 0.65	3.313 ± 0.42
LOOH (nm/mg protein)	0.32 ± 0.04	0.34 ± 0.03	0.35 ± 0.02

Discussion

Previous studies of AO enzymes in endometrium pointed to hormone-dependent expression and activity. Ota et al. (10) have shown the phase-dependent changes of both forms of SODs (copper, zinc SOD and manganese SOD) in glandular and surface epithelia of healthy fertile women during the menstrual cycle. Specifically, their expression was lowest during the early and midproliferative phases, then gradually increased and was most pronounced in the early and mid-secretory phases. In women with endometriosis and adenomyosis, the expression of both SODs was constantly elevated. Some data also indicated that SOD activity was not different in postmenopausal women compared to fertile ones (11). Our recent findings in women with endometrial polyp showed the opposite pattern of LOOH concentration and SOD activity in polyp tissue than in women with hyperplasia complex. Both parameters were higher in the proliferative phase compared to the secretory phase or to the women in postmenopause (7). The results of this study showed that women with endometrial hyperplasia complex had similar SOD activity pattern like the healthy ones. Although it was not statistically significant, SOD activity slightly increased in luteal (secretory) phase.

Regarding GPx, the studies shown that in normal human endometrium the amount of this enzyme was low in the early proliferative phase, gradually increased and reached a maximum in the late proliferative/early secretory phases, and decreased thereafter (12). In this study, however, the GPx activity in hyperplastic tissue had the opposite pattern than in healthy women. The same variation of activity was observed for GR. Ohwada et al. (13) also found that GPx activity is significantly higher in endometrial cancer tissue with atypical or mixed atypical adenomatous hyperplasia than in endometrial cancer without hyperplasia, pointing to the role of AO status and ROS in hyperplastic changes that may lead to endometrial cancer.

In this study we recorded a negative correlation between GPx and gonadotropins in women with endometrial hyperplasia complex, which may indicate the important role of these hormones in hyperplastic changes. Our previous research also showed a modulatory effect of gonadotropins on AO enzymes in women with endometrial polyp (7). Endometrial hyperplasias are generally considered as precancerous lesions and are treated either conservatively or surgicaly, and also by radiation therapy (14). The regression of hyperplastic to normal endometrium is the main purpose of any conservative treatment which is based on the administration of agents, like progestagens (15)

or gonadotropin-releasing hormone analogues (GnRHa) (16). Since the effects of these therapies are associated with AO and hormonal changes, our results may contribute to the prediction of potential therapeutic efficacy and to selection of the most effective treatment for hyperplasia complex.

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