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Research Paper

Sex hormones modulate circulating antioxidant enzymes: Impact of estrogen therapy

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ABSTRACT

Objective: Ovarian senescence affects many tissues and produces a variety of symptoms and signs. We hypothesized that estrogens may also influence circulating redox balance by regulating activity of the cellular antioxidative enzyme system. We aimed to explore the impact of surgical estrogen deprivation and replacement (ERT) on the glutathione balance and antioxidant enzymes expression in fertile women.
Study design: Nineteen healthy premenopausal women who underwent total hysterectomy with bilateral salpingo-oophorectomy were evaluated at baseline, 30 days after surgery without ERT and 30 days after ERT. Redox balance was determined by measuring blood reduced (GSH) and oxidized (GSSG) glutathione, as well as the GSSG/GSH ratio. Antioxidant status was evaluated by measuring serum estrogen (E2) levels and mRNA expression of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione S-transferase (GST) in peripheral blood mononuclear cells.
Results: Serum E2 significantly lowered after surgery, and increased in 12 out of 19 patients after 30 days of ERT (Responders). In such patients, an increase in oxidative stress was observed after surgery that resolved after ERT. Oxidative stress was sustained by reduction in the mRNA expression of both SOD and GSH-Px, that recovered after 30 days of therapy in responders. CAT and GST mRNA expression were not modified by surgery and replacement therapy.
Conclusions: Menopause is associated with significant change in antioxidant gene expression that in turn affects circulating redox state. Estrogens replacement therapy is able to prevent and counteract such modifications by acting as regulators of key antioxidant gene expression. These findings suggest that antioxidant genes are, almost in part, under the control of sex hormones, and that pathophysiology of the difference in gender disease may depend on the redox biology.

Introduction

Ovarian senescence occurs gradually during the fourth and fifth decades of life, leading to menopause. This process is associated with a progressive decline in estrogen level, which affects many tissues of the body and produces a variety of signs and symptoms [1]. In contrast, surgical removal of the ovaries interrupts abruptly and completely estrogen production, leading to more severe symptoms than those generated by natural menopause [2,3]. Bilateral oophorectomy at the time of hysterectomy for benign disease is commonly practiced in order to prevent the subsequent development of ovarian cancer [4]. However, premenopausal oophorectomy causes a rapid decline in circulating ovarian estrogens and androgens, since postmenopausal ovaries continue to produce significant amounts of testosterone and androstenedione which are converted to estrogen peripherally [5,6]. Estrogen depletion caused by oophorectomy is associated with coronary heart disease and hip fractures, as well as higher risk of cognitive impairment, depression and anxiety [7–9]. Symptoms and pathological manifestations associated with menopause may be, in part, related to oxidative stress [10]. Oxidative stress is defined as an imbalance between oxidants (superoxide anion radical, hydrogen peroxide, hydroxyl radical, peroxynitrite) and antioxidants (enzymatic and non enzymatic) in favor of the former [11].

Oxidative stress is higher in postmenopausal women, suggesting that antioxidant status may be related to estrogen deficit [12,13]. Surgical menopause is also associated with elevated production of oxidants, but estrogen counteracts oxidative stress,

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as observed during ovaries retention [14]. Estrogen exerts an antioxidant effect; in fact, estrogen level positively correlates with plasma antioxidant capacity and antioxidant enzymes expression throughout the menstrual cycle [15–17], and negatively with lipid peroxides, the product of oxidative damage [18]. Very interestingly, estrogen replacement therapy (ERT) restores total plasma antioxidant capacity and decreases lipid peroxides [18,19].

The antioxidant effect of estrogen may be related to a direct free radical scavenging activity [20]. However, the circulating level and the administered doses of estrogen are much lower than the necessary concentration of classical chemical antioxidants, indicating that its antioxidant effects is likely related to the upregulation of antioxidant enzymes [21]. Such hypothesis has been verified by in vitro and ex vivo animal studies [22,23]; nevertheless, the relationship between estrogen and the antioxidant enzymes system has been so far not addressed in humans. Thus, we aimed to investigate the impact of estrogen level change (depletion and replacement) and antioxidant gene expression and circulating redox balance in premenopausal women undergoing bilateral oophorectomy.

Materials and methods

Study design

Nineteen consecutive premenopausal women, referred the Department of Obstetrics and Gynecology of the University of Foggia for benign gynecological disease, who did not respond to standard medical treatments and with no indication for endometrial ablation, underwent hysterectomy and bilateral oophorectomy.

Exclusion criteria were Body Mass Index (BMI) ≥30 kg/m^2, a previous hospital admission related to cardiovascular disease, and a previous diagnosis of angina, hypercholesterolemia, diabetes, alcoholism, thyroid or any other endocrine disease. In addition, none of them had smoking habits or had taken medications, as well as contraceptive drugs, vitamin supplements and soy derivatives in the 6 months before the study. The subjects were asked to record any consumption of drugs not included in the experimental design. Study participants were assigned to a 60-day experimental period: 30 days after oophorectomy, they received 50 micrograms/day of continuous trans dermal in the form of patches (Dermestril, Rottapham S.p.A., Milan, Italy). The study protocol was approved by the institutional review board and the local Ethics Committee, and all patients gave written informed consent. Evaluation of treatment compliance was ascertained by weekly interview.

Blood sampling and analysis

Blood samples were collected the day before the surgical oophorectomy (Baseline time), 30 days after oophorectomy and before starting estrogen replacement therapy (Menopause time) and 30 days after starting therapy (ERT time) from an antecubital vein between 8:00 and 9:00 AM, with subjects in the supine position after an overnight fast. Blood samples for estradiol measurement were collected in 8 mL-Vacutainer tubes containing Z Serum Sep Clot Activator (Greiner Bio-one Gmbh, Frickenhausen, Germany) and centrifuged for 10 min at 1600 × *g* at room temperature to isolate serum. Blood samples for RNA isolation were collected into tubes containing K-ethylenediaminetetraacetic acid (EDTA) and processed within 30 min.

Oxidized (GSH) and reduced (GSSG) glutathione were determined in whole blood as previously described [25]. Briefly, blood samples were treated with an equal volume of 6% (v/v) perchloric acid containing 1 mM EDTA to determine GSH or with 6% perchloric acid containing 50 mM *N*-ethylmaleimide and 1 mM EDTA to determine GSSG by high-performance liquid chromatography (HPLC). Afterwards, samples were centrifuged for 10 min at 1500 × *g* and the acidic supernatants were neutralized and used for determination of metabolites.

Serum concentration of estrogen (E2), progesterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured using commercial chemiluminescence immunoassays (Vitros Estradiol, Johnson & Johnson Medical S.p.A., Milan, Italy).

RNA isolation and quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR)

Total cellular RNA was isolated from peripheral blood mononuclear cell (PBMC) samples using the RNAeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. To ensure minimum in vitro impact on the activation status of the cells, we employed a modified gradient separation. PBMC were immediately isolated by a rapid Ficoll–Hystopaque centrifugation for 30 min at 900 × *g*. Total cellular RNA was extracted with RNeasy kit and immediately stored at −80 °C. Samples were quantified by absorption spectrophotometry, and RNA integrity was confirmed using nondenaturing agarose gel electrophoresis. cDNA was obtained using a random hexamer primer and a SuperScript III Reverse Transcriptase kit as described by the manufacturer (Invitrogen, Frederick, MD, USA). A PCR master mix containing the specific primers (superoxide dismutase 1 (SOD1): forward, TGT GGG GAA GCA TTA AAG G; reverse, CCG TGT TTT CTG GAT AGA GG; catalase (CAT): forward, GCC ATT GCC ACA GGA AAG TA; reverse, CCA ACT GGG ATG AGA GGG TA; glutathione peroxidase (GSH-Px): forward, GGA GAC CTC ACC CTG TAC C; reverse, GTC ATT CAC CAT GTC CAC C; glutathione S-transferase (GST): forward, ACC TCC ACC GTA TAT TTG AG; reverse, TTG CCC CAG ACA GCC ATC TT; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward, CAA GGC TGA GAA CGG GAA; reverse: 59-GCA TCG CCC CAC TTG ATT TT-39) was added, along with AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA). Real-time quantification of mRNA was performed with a SYBR Green I assay, and evaluated using an iCycler detection system (Bio-Rad Laboratories). The threshold cycle (CT) was determined, and the relative genes expression was subsequently calculated as follows: fold change$=2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT_{target-CThousekeeping}$ and $\Delta(\Delta CT) = \Delta CT_{treated} - \Delta CT_{control}$.

Statistical analysis

Data were expressed as mean ± standard deviation of the mean (SDM). For biochemical and real-time RT-PCR data, the one way analysis of variance (ANOVA) for repeated measures was performed to compare results obtained before vs. after oophorectomy and ERT. Bonferroni's multiple comparison test was performed as post-hoc test. The student's t-test was used for paired data. A linear regression model was used to analyze the association between serum E2 levels and SOD, CAT, GSTP, GSTM levels, as well as GSH, GSSG and GSSG/GSH ratio, at all the times studied. A value of $p < 0.05$ was considered statistically significant. The Statistical Package for Social Sciences (SPSS v. 15) was used to perform all the statistical analysis.

Results

Baseline patients characteristics

Baseline characteristics of patients are shown in Table 1. Nine patients (47%) were overweight (BMI > 25 kg/m^2), four patients (21%) presented history of arterial hypertension (systolic blood