

Original Research

Antioxidant status and sex hormones in women with complex endometrial hyperplasia

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Abstract: Endometrial tissue is under a strong influence of sex hormones. These hormones are considered as developmental factors of endometrial hyperplasia and endometrial cancer. We examined the influence of gonadotropins (follicle-stimulating and luteinizing hormone) and sex hormones (estradiol, progesterone) on oxidant/antioxidant parameters in blood and endometrial tissue of women with complex endometrial hyperplasia. In blood, superoxide dismutase activity was significantly higher in luteal phase and postmenopause compared to the follicular phase. A significant phase-related difference of glutathione peroxidase and glutathione reductase activity was recorded in the endometrium. Both enzymes had lower activity in luteal phase and postmenopause compared to the follicular phase. The linear regression analysis of individual hormonal variables against antioxidant parameters showed negative correlation between glutathione peroxidase activity and gonadotropin concentrations in the endometrium. The regression of hyperplastic to normal endometrium is the purpose of conservative treatment based on administration of progestogens or gonadotropin-releasing hormone analogues. Our findings indicate that gonadotropins influence the antioxidant enzymes activity in women with complex endometrial hyperplasia, which may affect disease development. Further studies are needed to clarify the molecular basis of hormone action on antioxidant system that may potentially initiate a development of treatments based on redox-dependent mechanism.

Key words: Antioxidant enzymes, gonadotropins, estradiol, progesterone, hyperplasia complex.

Introduction

Hyperplasia complex is a non-physiological process encompassing endometrium, an inner layer of human uterus. According to WHO (World Health Organization) classification, hyperplasia complex could be presented with atypical cytology or without atypia (1). The main features of this condition, as in the other types of 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 in comparison to a normal endometrium (2). Hyperplasia complex is an age related disease. The incidence peaks were observed in women aged 50-54 and 60-64, respectively (3), indicating that hyperplasia complex is common among premenopausal woman and women in early postmenopause. Besides that, age, obesity, hormone replacement therapy (HRT) and diabetes are among the risk factors significant for hyperplasia development (4). Nulliparity, infertility and family history of colonic cancer have been noted as risk factors in premenopausal women with abnormal menstrual bleeding (5). There is a strong relationship between endometrial hyperplasia and endometrial carcinoma. The absence of cytological atypia in hyperplasia relates to a lower risk of malignant transformation (2).

It is well known that endometrial exposure to an excessive amount of estrogen, unopposed by progesterone leads to abnormal proliferation (6,7). Therefore, hormones are considered as one of the key factors in the development of endometrial hyperplasia and endometrial cancer. High concentration of progesterone receptors was found in hyperplasia without atypia (8). These findings are in accordance with its lower malignant potential and tendency to regress after progestogen treatment. Estrogens act as tumor promoters in endometrium. By their mitogen activity, they may stimulate hyperplasia development, which can further progress into well differentiated carcinoma (9,10). Malignant transforma-

tion could be more favored by the loss of estrogen and progesterone receptors expression, and by mutations of p53 gene. The result of these events would be a more aggressive and poorly differentiated carcinoma with worse prognosis (11).

When reactive oxygen species (ROS) production overcomes the antioxidant (AO) capacity of the cell, the result is oxidative stress. Oxidative stress may lead to different pathological conditions such as cancer (12). Antioxidant enzymes have an essential role in preventing oxidative damage in cell caused by reactive oxygen species. Superoxide dismutase (SOD) removes 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). Both enzymes reduce H_2O_2 into water. For its activity, glutathione peroxidase needs glutathione (GSH) to convert GSH to its oxidized form (GSSG). Glutathione reductase (GR) maintains GSH/GSSG ratio by reducing GSSG to GSH (13).

Antioxidant status varies according to different types of endometrial transformation (14). Role of AO enzymes and hormones during menstrual cycle and postmenopause was studied in healthy women and in those with gynecological disorders. For instance, the SOD has a role in maintaining luteal cell integrity and steroidogenic capacity in fertile women (15,16). It was found that GPx activity rises during menstrual cycle, which is related to the increased production of estrogen by ovaries from the late follicular to the early luteal phase (17). The lower GPx activity was observed in the blood and endometrium of the late menopausal women (18).

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It is known that hormone imbalance is the feature of menopause. Fall in estrogen serum level accompanied by rise in follicle-stimulating hormone (FSH) has been noticed in postmenopausal women compared with those in premenopause (19). Hormone replacement therapy showed antioxidant role by reduction of lipid peroxides (LOOH) serum level (20). Also, positive correlation between HRT and SOD activity was found in postmenopausal women (21).

We have previously shown that AO enzymes activity and lipid hydroperoxide level in patients with endometrial polyps are influenced by the differences in sex hormones during the menstrual cycle and in menopause (22). Following these findings, in this study we aimed to evaluate the AO status in menstrual cycle and in postmenopause of women with complex endometrial hyperplasia. Additionally, we wanted to examine the influence of gonadotropins and sex hormones on AO parameters in this gynecological disease.

Materials and Methods

The material used in this study consisted of 31 blood and endometrial specimens of women admitted to the Department of Gynecology and Obstetrics for gynecological evaluation within routine checkups, or women admitted for abnormal uterine bleeding (*Metrorrhagia prolongata*, *Metrorrhagia recidivans*, *Metrorrhagia postmenopausi*). On the basis of their diagnosis and histological examination, 26 subjects were diagnosed with complex hyperplasia without atypia, and 5 women had complex hyperplasia with atypia. The specimens were taken after obtaining their informed consent. The 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 (Ethical Principles for Medical Research Involving Human Subjects). Due to ethical reasons the normal endometrial tissue could not be obtained. This may be considered as a limitation of the study. None of subjects had undergone hormone therapy or any other medical treatment in the last 6 months. The menstrual cycle was standardized to 28 days to account for unequal cycle lengths and patients

were classified into their respective menstrual phase, and in postmenopause, using the date of the last menstrual period *i.e.* 10 patients in the proliferative (follicular phase, F), (age 42-52 yr; median 48 yr; 1 atypia case), 10 in the secretory (luteal phase, L) (age 29-51 yr; median 46 yr; 2 atypia cases), and 11 in the postmenopause (PM) (age 46-73 yr, median 51 yr; 2 atypia cases). The menstrual status was confirmed by endocrine data based on the cyclical fluctuations of gonadotropins and sex steroids documented in previous studies (23). Standard values for hormone concentrations in each phase are given in Table 1.

Endometrial tissue and venous blood were collected on the same day as the uterine biopsy and handled as described previously (22). 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. (24) and expressed in mg/ml.

Plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured by immunoradiometric assay (IRMA) on solid phase. The method is based on the two step interaction between antibodies and sample antigen. The first step includes interaction between monoclonal antibodies labeled with radioactive ^{125}I and the antigen in the sample, forming a complex. In the second step this complex interacts with immobilized antibodies and the result is a solid phase bound sandwich complex. After washing the excess of ^{125}I labeled antibodies, the intensity of radioactivity is proportional to the concentration of FSH or LH in the sample. Estradiol (E) and progesterone (P) levels were measured by radioimmunoassay (RIA). The method is based on competition between fixed amount of ^{125}I labeled steroid, and the steroid to be measured in the specimens, both of which compete for a fixed amount of antibody sites immobilized on the surface of a solid phase (the wall of a polystyrene tube). After incubation, aspiration step cancels the reaction, and free antigen is washed out with washing solution and aspirated. The amount of the steroid present in the sample and attached to antibody is inversely proportional to the concentration of the labeled steroid.

Table 1. Changes in hormone levels during follicular phase, luteal phase and in postmenopause (Data are expressed as mean \pm SEM; * $p < 0.05$).

| Hormones | Follicular phase | Luteal phase | Postmenopause |
|-------------------------------|-------------------|-------------------|------------------|
| FSH (U/L)* | 5.97 \pm 1.42 | 18.06 \pm 4.87 | 25.59 \pm 5.62 |
| Median | 6.2 | 14.5 | 20.7 |
| Normal values | (0-15) | (0-15) | (20-100) |
| LH (U/L)* | 0.60 \pm 0.30 | 6.07 \pm 1.70 | 12.02 \pm 4.33 |
| Median | 0.3 | 5 | 6.8 |
| Normal values | (1-10) | (0.4-15) | (10-50) |
| Estradiol (pg/ml)* | 69.47 \pm 29.21 | 73.07 \pm 21.81 | 21.27 \pm 3.75 |
| Median | 76.8 | 47.2 | 15.9 |
| Normal values | (25-100) | (50-220) | <40 |
| Progesterone (nmol/l)* | 1.10 \pm 0.10 | 5.97 \pm 0.68 | 2.83 \pm 0.34 |
| Median | 1.1 | 6.6 | 1.3 |
| Normal values | (1-9.5) | (9.5-75) | 0.1-2 |

Determination of SOD activity was performed using Oxis Bioxytech® SOD-525™ Assay (Oxis International, Inc., Portland, OR, USA). This method is based on SOD-mediated increase of autoxidation of 5,6,6a11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. One SOD-525 activity unit is defined as the activity that doubles the autoxidation rate of the controlled blank.

CAT activity was determined by the method of Beutler (25). The reaction is based on the rate of H₂O₂ 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 spectrophotometrically at 230 nm. One unit of CAT activity is defined as 1 μmol of H₂O₂ decomposed per minute under the assay conditions.

GPx activity was assessed using the Oxis Bioxytech® GPx-340™ Assay (Oxis International, Inc., Portland, OR, USA), 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 spectrophotometrically 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.

Activity of GR was measured using the Oxis Bioxytech® GR-340™ Assay (Oxis International, Inc., Portland, OR, USA). 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 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.

Concentration of LOOH was measured by Oxis Bioxytech® LPO-560™ Assay (Oxis International, Inc., Portland, OR, USA), which is based on the oxidation of ferrous (Fe²⁺) ions to ferric (Fe³⁺) 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.

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. The 95% confidence intervals (CIs) for the regression lines were calculated. The p value < 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism software.

Results

The phase-related concentrations of hormones are reported in Table 1. Significant difference among the phases was observed for gonadotropins concentration.

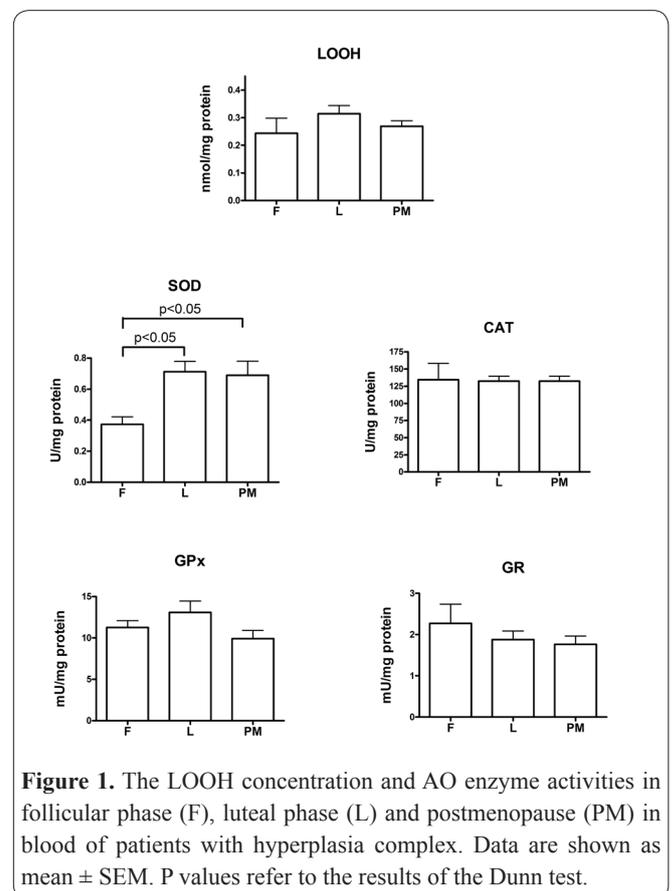


Figure 1. The LOOH concentration and AO enzyme activities in follicular phase (F), luteal phase (L) and postmenopause (PM) in blood of patients with hyperplasia complex. Data are shown as mean ± SEM. P values refer to the results of the Dunn test.

The FSH values in follicular phase and postmenopause were in normal range, while higher level was recorded in luteal phase ($H=5.99$, $p<0.05$, Kruskal-Wallis). The LH level was in a normal range in luteal phase and in postmenopause, but it was lower in follicular phase ($H=7.11$, $p<0.05$, Kruskal-Wallis).

Sex hormones concentrations also varied within phases. Although significantly different among phases ($H=8.10$, $p<0.05$, Kruskal-Wallis), the estradiol level was in a range of corresponding values for the menstrual cycle phases and for postmenopause. Women in this study had specific progesterone profile in the examined phases ($H=6.42$, $p<0.05$, Kruskal-Wallis). In the follicular phase progesterone level was normal, however, at the bottom limit of the normal values. In luteal phase it was below normal values and it seemed higher in postmenopause. This discrepancy is most probably the consequence of individual variations of hormone concentrations during perimenopausal to postmenopausal transition. With respect to their median age, those women could still ovulate and have higher hormone level.

Figure 1 shows the phase-related differences of LOOH concentrations and AO enzyme activities in the blood of examined patients. The significant difference with respect to the phase was observed for SOD activity ($H=5.98$, $p<0.05$, Kruskal-Wallis). In the follicular phase, it was significantly lower than in luteal phase and postmenopause ($p<0.05$, Dunn test). There were no significant differences of other parameters in the examined phases. No significant correlations were found between hormones and antioxidant variables.

The phase-related difference of the examined parameters in hyperplastic tissue is shown in Figure 2. Unlike blood, we found significant phase-related difference of GPx ($H=7.84$, $p<0.05$, Kruskal-Wallis) and GR activ-

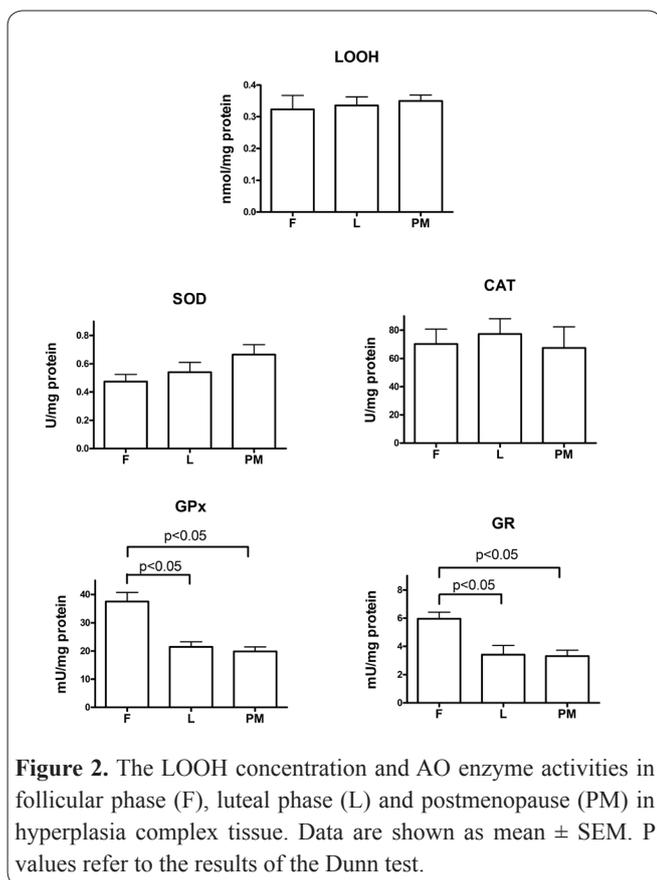


Figure 2. The LOOH concentration and AO enzyme activities in follicular phase (F), luteal phase (L) and postmenopause (PM) in hyperplasia complex tissue. Data are shown as mean \pm SEM. P values refer to the results of the Dunn test.

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

The linear regression analysis of individual hormonal variables against antioxidant parameters in endometrium (Figure 3) showed a significant negative correlation between FSH/LH concentrations and GPx activity ($r=-0.36$; $r=-0.47$, $p<0.05$, respectively).

Discussion

Studies highlight the role of oxidant/antioxidant status in physiological processes like ovarian folliculogenesis, early embryonic development and endometrial cyclic changes (26) but also in abortions, infertility and various gynecological diseases (27). Synchronized activity is needed to keep the AO system functional and aberrant expression of AO enzymes indicates the role of oxidative stress in etiology of many pathological conditions. Besides, hormonal regulation of AO enzymes has significant influence in modulating cellular redox status thus affecting the course of gynecological diseases.

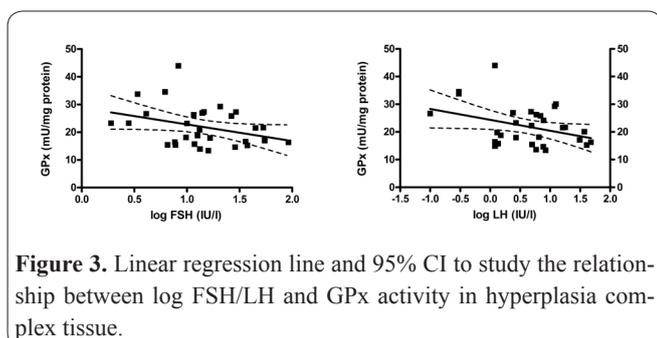


Figure 3. Linear regression line and 95% CI to study the relationship between log FSH/LH and GPx activity in hyperplasia complex tissue.

A significantly higher SOD activity in luteal phase and in postmenopause than in follicular phase was recorded in blood. In endometrial tissue, no significant difference of SOD activity was recorded in cycling women and in postmenopause, although slight increase may be observed in postmenopause. These results are similar to the previous findings of Gurdol et al. (18). They recorded the increased SOD activity in blood of healthy postmenopausal women, while no difference was found in endometrium of women in postmenopause and the fertile ones. However, there is also contrasting evidence regarding the effects of menopause. In the studies of Unfer et al. (21) and Shrivastava et al. (30) the SOD activity was lower in the blood of postmenopausal women than in cycling ones, while Bednarek-Tupikowska, et al. (19) have not observed any difference between these two groups of women. It is known that aging influences AO status (31) and that menopause is associated with significant difference of AO genes expression that in turn modify the circulating redox state (32). However, the results of this investigation may indicate that women with *hyperplasia complex endometrii* have elevated SOD activity due to hyperplastic changes and not in relation to age or fertility. Our recent study regarding the influence of age, diagnostic categories and reproductive factors also indicated that diagnosis is the most important predictive factor that affects the AO status in gynecological patients (33).

Previous studies have shown that GPx expression in normal human endometrium varied during the menstrual cycle. The GPx amount was low in the early follicular phase, gradually increased to the maximum in the early luteal phase, and decreased thereafter (29, 34). In this study, however, the endometrial GPx activity in hyperplastic tissue had the opposite pattern than in healthy women. It was highest in the follicular phase and decreased in luteal phase and in postmenopause. The same variation of activity was observed for GR. Altered GPx/GR activity in hyperplastic tissue found in this study is in accordance with the findings of Ohwada et al. (29). They observed a significantly higher GPx activity in endometrial cancer tissue with atypical/mixed atypical adenomatous hyperplasia than in endometrial cancer without hyperplasia. These findings point to the role of AO status and ROS in hyperplastic changes that may contribute to endometrial carcinogenesis.

Data confirmed the assumption that GPx activity is regulated by sex hormones in endometrial tissue (29, 34). GPx-3 is highly expressed in the luteal phase, while the expression levels are extremely low in the proliferative (follicular) phase. Within the promoter region of the GPx-3 gene, three possible progesterone response elements (PRE) and two possible estrogen response elements (ERE) have been registered, indicating that these steroids directly regulate GPx expression (35). Stimulatory effect of estradiol on human endometrium was shown in study of Serviddio et al. (28), in which a positive correlation between the estradiol levels and GPx activity was observed.

In this study we recorded a negative correlation between GPx and gonadotropin levels in women with endometrial hyperplasia complex. Our previous research also showed a modulatory effect of gonadotropins on AO enzymes in women with endometrial polyp, ob-

served as negative correlation between FSH/LH and GPx, and between LH and SOD (22). It is known that endometrium is under the influence of exogenous and endogenous hormones. However, the exact role of gonadotropins in pathological conditions, although still not clarified, may be very important in the etiology of various gynecologic disorders, including hyperplastic changes or endometrial cancer. Recent study on women with ovarian epithelial carcinoma revealed that beside estradiol, FSH and LH could induce ROS production and may therefore be involved in carcinogenesis (36). Also, modulating effect of gonadotropins found in our study could be a consequence of their action on cell signaling. For instance, FSH was shown to act through its membrane-bound receptor which activates the intracellular signaling cascade, starting with cyclic AMP/protein kinase A (cAMP/PKA). Activation is followed by phosphorylation of specific transcriptional factors, such as cAMP response element binding protein (CRE) or p38 MAPK that control other kinase cascades. The FSH receptor may also activate extracellular signal-regulated protein kinase (ERK) (37). These redox-regulated pathways have been reported to be activated in over 50% of acute myelogenous leukemia, acute lymphocytic leukemia and are frequently activated in breast, prostate and other cancer types (38). It was also shown that synthesis of antioxidants, such as glutathione, is regulated by gonadotropins but exact mechanism is still unknown (39). One of the possible FSH/antioxidants interactions involves the activation of nuclear factor erythroid 2 (Nrf2) expression, since gonadotropins were found to cause its upregulation (36). Nrf2 is a master regulator of antioxidant response elements (ARE)-driven genes and it was reported to be highly expressed in endometrial serous carcinoma and in some endometrial precursor lesions (40).

The pathogenesis of endometrial hyperplasias is still not fully understood. They are generally considered as precancerous lesions and are treated either surgically or conservatively. The regression of hyperplastic to normal endometrium is the main purpose of any conservative treatment and it is based on administration of progestagens (41) or gonadotropin-releasing hormone analogues (GnRHa) that influence gonadotropin levels and ovarian steroidogenesis (42). The results of our study indicate that gonadotropins, in certain degree, also modulate AO enzymes in women with complex endometrial hyperplasia. However, additional studies are needed to further clarify the molecular bases of hormone action on AO system and potentially initiate a development of improved treatments based on redox-dependent mechanism.

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