Modulation of Endometrial Redox Balance during the Menstrual Cycle: Relation with Sex Hormones

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This study aimed to evaluate the effects of changes in sex hormones occurring during the menstrual cycle on the redox balance and lipid peroxidation in normal human endometrial cells.

Forty women, ages 21–41 yr, who were admitted to the Department of Gynecology and Obstetrics of the University of Bari for routine checkups or were treated for benign uterine disease, underwent endometrial biopsy and venipuncture. On the basis of histological examination, patients were allocated as follows: 10 in the early proliferative phase, 8 in the early secretory phase, and 10 in the late secretory phase. LH, FSH (immunoradiometric essay), estradiol (E2), and progesterone (P4) (RIA) were determined in plasma samples.

On the endometrial specimens, total glutathione (GSH), oxidized GSH (GSSG), malondialdehyde, and GSH peroxidase activity (GSH-Px) were determined.

Significant cycle-dependent changes in endometrial GSH-Px (P < 0.0001), GSH (P < 0.001), and GSSG as a percentage of GSH (P < 0.0001) were observed. Malondialdehyde did not show significant differences. A linear regression model correlating sex hormone changes with redox indexes was performed. A significant positive correlation was observed between E2 and GSH-Px (r = 0.74; P = 0.0001), E2 and GSSG, as percentage of total (r = 0.84; P < 0.0001); a negative correlation was found between E2 and GSH (r = 0.57; P = 0.0001). No significant correlation was found between P4 or FSH and oxidative balance. LH was found to be correlated with GSH-Px (r = 0.66; P = 0.0001) and GSSG as percentage of GSH (r = 0.5; P < 0.001). We conclude that the hormonal pattern is involved in maintaining the optimal redox balance in endometrium, mainly through modulation of GSH level and metabolism.

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In the last few years, there has been a growing interest in the participation of oxidative reactions in human biological processes. The role played by the redox balance in regulating strategic cell functions, such as DNA expression (1) and mitochondrial energy metabolism, has been clearly shown (2). Several studies have reported a close relationship between human pathological conditions and tissue damage due to free radical overproduction (3–5).

In aerobic organisms, extensive protective mechanisms have evolved to prevent free radical damage. Human antioxidant defenses include natural molecules, such as urate, cystein, vitamins E and C, and enzymes, including superoxide dismutase, catalase, and glutathione peroxidase (GSH-Px). All cellular activities require oxygen for biosynthesis of their components, and oxygen consumption produces reactive oxygen species (ROS). The intracellular antioxidant system is based on glutathione (GSH), which plays a key role in cellular detoxification reactions and in regulating the thiol-disulfide status in the cell (6). Recently, elevated lipid peroxidation in erythrocytes and altered plasma redox balance have been associated to human cervicitis and uterine myoma (7); moreover, lipid peroxidation and GSH-Px in human endometrial cancer was found to be higher than in normal endometrium (8, 9). By contrast, very little is known about the levels of antioxidant systems in normal endometrial cells in vivo and their variation during the menstrual cycle.

The aim of the present study was to clarify whether the endometrium redox balance is influenced by changes in sex hormones during the menstrual cycle. To this purpose, GSH metabolism and levels were directly monitored in biotic specimens of uterine mucosa.

Subjects and Methods

Subjects and study design

The study population consisted of 40 women, ages 21–41 yr (median, 32 yr), admitted to the Department of Gynecology and Obstetrics of the University of Bari from January 2000 to August 2001 for gynecological evaluation within routine checkups or to be treated for benign uterine disease, usually subserous fibromyoma (Table 1).

After giving their written informed consent, patients were examined to verify their healthy status through medical history and physical and laboratory examinations. None of the subjects smoked, and all consumed a standard Mediterranean diet. None of them had undergone hormone therapy in the last year or any other medical treatment in the last 6 months. Cycle length ranged from 27–30 d. All patients underwent endometrial biopsy on different days of the cycle, ranging from d 5–25 after the beginning of the menstrual cycle. Tissue samples were taken from the anterior and/or posterior walls of the body of the uterus and immediately divided into two parts: one for histological examination, the other for oxidative determination. Endometrial specimens for histological study were fixed in 10% formalin, embedded in paraffin, cut into sections and stained with hematoxylin-eosin following routine procedures. On the basis of histological examination, patients were divided as follows: 10 in the early proliferative phase (age, 27–41 yr; median, 35 yr), 12 in the late proliferative phase (age, 25–39 yr; median, 31 yr), 8 in the early secretory phase (age, 27–36 yr; median, 30 yr), and 10 in the late secretory phase (age, 21–39 yr; median, 30 yr). On the basis of LH levels...
TABLE 1. Changes in hormone levels during follicular and luteal phases

<table>
<thead>
<tr>
<th></th>
<th>Early follicular phase</th>
<th>Late follicular phase</th>
<th>Early luteal phase</th>
<th>Late luteal phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrogen (ng/liter) (^a)</td>
<td>25 ± 2.6 (18–21)</td>
<td>113 ± 4.87 (75–132)</td>
<td>69 ± 3.2 (57–82)</td>
<td>36 ± 1.5 (28–44)</td>
</tr>
<tr>
<td>Median</td>
<td>21.5 (95% CI, 19–31)</td>
<td>116.5 (95% CI, 102–123)</td>
<td>70 (95% CI, 61–76-8)</td>
<td>35.5 (95% CI, 32.7–39.3)</td>
</tr>
<tr>
<td>Progesterone (pmol/liter) (^a)</td>
<td>3.2 ± 0.3 (2.1–5.1)</td>
<td>2.5 ± 0.39 (1–5.1)</td>
<td>22 ± 3.7 (9.7–36.9)</td>
<td>2.5 ± 0.09 (2–2.9)</td>
</tr>
<tr>
<td>Median</td>
<td>2.9 (95% CI, 2.5–3.8)</td>
<td>2.1 (95% CI, 1.6–5.4)</td>
<td>20.6 (95% CI, 13.3–36.9)</td>
<td>2.5 (95% CI, 2.3–2.7)</td>
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<tr>
<td>FSH (IU/liter)</td>
<td>7.6 ± 0.8 (3.6–11.2)</td>
<td>8.8 ± 0.59 (5.1–12)</td>
<td>6.2 ± 0.8 (4–9.8)</td>
<td>8.9 ± 1.2 (3.3–13.4)</td>
</tr>
<tr>
<td>Median</td>
<td>7.3 (95% CI, 5.8–9.4)</td>
<td>8.4 (95% CI, 7.43–10.08)</td>
<td>5.7 (95% CI, 4.3–8.1)</td>
<td>9 (95% CI, 5.91–11.9)</td>
</tr>
<tr>
<td>LH (IU/liter)</td>
<td>7.2 ± 0.69 (4.2–11.3)</td>
<td>35 ± 7.1 (4.7–71.7)</td>
<td>9.97 ± 0.66 (7.5–12.6)</td>
<td>6.3 ± 0.7 (2.7–9.8)</td>
</tr>
<tr>
<td>Median</td>
<td>6.9 (95% CI, 5.6–8.7)</td>
<td>35.6 (95% CI, 19.2–50.6)</td>
<td>9.6 (95% CI, 9.4–11.5)</td>
<td>6.1 (95% CI, 4.7–9.8)</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SEM. \(^a\) P < 0.0001.

Blood assays

Blood samples were taken on the same day of endometrial biopsy and were collected by venipuncture into heparinized tubes; after centrifugation, plasma was stored at −30°C until hormonal assay. LH and FSH were determined by immunoradiometric assay. E2 and P4 were determined by RIA. All biochemical hormonal analyses were carried out in the Central Laboratory of the University Hospital of Bari, accredited by Clinical Pathology Accreditation (UK) Ltd.

Endometrial oxidative balance

For reduced and oxidized GSH determination, endometrium samples were washed in isotonic saline solution for a few seconds and rapidly homogenized in ice-cold Krebs-Henseleit buffer (pH = 7.4). Homogenates were treated with 10% vol/vol concentrated perchloric acid containing 1 mM bathophenanthroline-disulfonic acid, and centrifuged at 15,000 × g for 3 min; the acidic supernatant was added to a tube containing γ-glutamyl-glutamate, used as internal standard, iodoacetic acid, and m-cresol purple. GSH and oxidized GSH (GSSG) were measured by high-pressure liquid chromatography (HPLC) following the method of Fariss and Reed (10) using a 3-amino propyl-spherisorb 4.6 × 200 mm column. The results were calculated relative to peak areas of freshly prepared standards. The recovery of GSH and GSSG standards added to homogenate was 96 ± 5 and 92 ± 4% (sd), respectively.

Endometrial malondialdehyde (MDA) was determined by HPLC using the thiobarbituric acid reaction according to modification of the method of Bird et al. (11) as previously described. Briefly, the spontaneous auto-oxidation of lipids was inhibited or minimized by adding butylated hydroxytoluene to the system, which was heated at 200°C for 45 min. All the chromatophore was then extracted with butanol and butanol and centrifuged at 15,000 × g. The recovery of MDA added to homogenate was 93 ± 6% (sd). For standard solutions, 1,3,5,6-tetraethoxypropane was used.

GSH-Px activity in the endometrial cells was assessed by the method of Paglia and Valentine (12).

Total protein concentration in endometrium homogenate was determined by the Peterson method (13).

Statistical analysis

Data were expressed as mean ± SEM. Because the data were not paired, differences between cycle phases were analyzed by one-way ANOVA after Gaussian distribution evaluation. The Tukey-Kramer multiple comparisons test for all pairs of columns was also applied as post test.

A linear regression model was used to evaluate associations between hormonal and oxidative variables. Before plotting the data in the regression study, we performed the Kolgomorov-Smirnov test on the variables, and we log-transformed LH progesterone and GSSG as percentage of total GSH. We also calculated 95% confidence intervals (CIs) for these linear regression lines. A multiple linear regression model was also applied to explore the capacity of the sex hormonal pattern to predict the variations of oxidative balance values. In all instances, P less than 0.05 was taken as the lowest level of significance. The SPSS software package (SPSS, Inc., Chicago, IL) was used to perform all of the statistical analyses.

Results

Redox balance in endometrial cells

Histological evaluation excluded any endometrial pathology. On the basis of histological findings, each endometrium was allocated in one of the following four groups: early follicular phase (EF), late follicular phase (LF), early luteal phase (EL), and late luteal phase (LL). Circulating values of hormonal levels were consistent with the day of the cycle and with histological findings.

The cycle phase-related changes in sex hormones during the menstrual cycle are reported in Table 1. Figure 1 shows the cycle-dependent changes in endometrial redox pattern. Significant changes in GSH-Px values were observed (ANOVA, F = 17.7; P < 0.0001; df = 3); the level in the LF was significantly higher than in the EF (P < 0.001), EL (P < 0.01), and LL (P < 0.001). The levels of GSSG increased in the LF compared with the EF (P < 0.05), LL and EL (P < 0.01). The difference was more evident when GSSG was expressed as percentage of total GSH (ANOVA, F = 24.382; P = 0.0001; df = 3). The levels of GSH showed a statistically significant difference when LF was compared with EF and LL (P < 0.01) (ANOVA, F = 7.096; P = 0.0007; df = 3). On the contrary, MDA did not show any significant difference.

Correlation study between sex hormones and GSH

To assess the effects of the sex hormone changes occurring during the menstrual cycle on the oxidative balance, we analyzed a linear regression model that correlated sex hormones with redox indexes. The results are shown in Fig. 2. A significant positive correlation was observed between E2 and GSH-Px (r = 0.74; P < 0.0001), E2 and GSSG (r = 0.51; P = 0.0007), and between E2 and log of GSSG % of total (r = 0.85; P < 0.0001); a negative correlation was found between E2 and GSH total (r = 0.57; P = 0.0001). No significant correlations were found between Log Pro or FSH and redox balance patterns. Log LH was shown to be correlated with GSH-Px (r = 0.66; P < 0.0001), GSSG (r = 0.47; P < 0.0005), and log %GSSG (r = 0.5; P = 0.001).

The Simple Linear Regression study does not allow evaluation of the influences exerted by hormonal variables, taken as a whole, on the redox pattern changes, because it only explores the relationship into a hormone and a single redox
parameter; to evaluate whether the hormonal pattern could explain the variance of each redox variable, we have performed a Multiple Linear Regression model in which the hormonal status and the redox pattern were the independent variable and the dependent variable, respectively. The model applied explains 61% of variance of GSH-Px (F: 13.8; \( P < 0.0001 \)), 37% of total GSH (F: 5; \( P < 0.005 \)), 36% of GSSG (F: 4.89; \( P < 0.005 \)), and 80% of the log-GSSG as percentage of

**FIG. 1.** Changes in oxidative pattern during follicular and luteal menstrual phases. Only significant differences are reported. *P* values refer to the results of the Tukey-Kramer multiple comparisons test. Data are expressed as mean ± SEM.
FIG. 2. Linear regression line and 95% CI to study the relationship between E2 or log LH levels and oxidative pattern. Only significant relationships are reported.
total GSH (F: 34; P < 0.0001). To better characterize the influence of LH in the peri-ovulatory phase, in the regression study we have performed an additional analysis in which data from peri-ovulatory patients were excluded. Again, the model well describes variations in the oxidative parameters; specifically, it explains 45% of variance of GSH-Px (F: 5.5; P < 0.0005), 47.5% of GSH (F: 6.1; P < 0.001), 37% of GSSG (F: 4; P < 0.005), and 89% of log-GSSG as percentage of total (F: 56; P < 0.0001).

Discussion

This study shows the changes in endometrial redox balance during the menstrual cycle. Although other authors have investigated the possible relation between sex hormonal pattern and redox balance (14–18), to our knowledge no previous studies have evaluated this change in endometrial cells in vivo, and none has demonstrated the possible existence of an oxidative cycle in endometrial cells.

In the present study, the levels of GSH-Px, an enzyme that catalyzes the chemical reduction of hydrogen peroxide and lipid hydroperoxides by transforming GSH to GSSG, were increased in the follicular phases and decreased during the luteal phases. GSSG levels exhibited a similar trend, increasing in the LF and decreasing during the luteal phases. By contrast, the differences in MDA levels during the estrous phases did not reach statistical significance. When a linear regression model was applied to evaluate possible correlations between fluctuations of sex hormones and GSH-related indices, a significant relationship was found between estrogen changes and all of the considered parameters, except for MDA. LH also showed a positive correlation with GSH-Px and GSSG as a percentage of total GSH.

To assess whether the cyclic changes in hormonal pattern may explain the modulation of the major redox indices in oxidative balance, we used a multiple linear regression model to describe the overall change in all oxidative parameters related to hormonal pattern; the results clearly showed that the majority of variations in oxidative balance could be explained by the sex hormonal cycle. Our data also show that the estrogen cycle has the greatest influence over redox changes. The influences exerted by LH deserve particular attention; in fact, although the role played by estrogens, FSH, and P, is clearly defined, LH showed a direct correlation only with GSH-Px and GSSG but not with the other redox parameters. The real weight of LH in modulating the redox balance was explored by performing two different multiple regression models, with and without peri-ovulatory patients, and the results showed no significant differences between the two models.

At present, the existence of a relationship between ovarian steroids and antioxidant activity has been demonstrated in vitro (19–21), GSH-dependent enzyme activities have been measured in rat cells (14, 22), and the only available data correlating GSH-Px activity and estrogen cycle levels come from studies in erythrocytes (15). In rat, hepatic GSH-Px activity has been shown to be higher in the estrous phase than in the diestrous phase of the menstrual cycle (23); in addition, two different studies have recently demonstrated the influence of steroid hormones on endometrial cells. Diaz-Flores et al. (14) have shown that in rats subjected to bilateral ovariectomy, the activity of GSH reductase is modulated by estrogen and/or P administration, and Maruyama et al. (24) have demonstrated that thioredoxin, an important redox-active protein that catalyzes protein disulfide/dithiol reactions, is induced by ovarian steroids. These results suggest that the ovarian cycle could be an important redox controller in sex tissues; this hypothesis is also supported by the finding of lower levels of lipoperoxidation in fertile women than in menopausal women, but studies demonstrating significant changes in redox equilibrium during the cycle are lacking.

Our data show that major variations of GSH metabolism and levels occur in endometrial cells during the estrous phase; these seem to be closely related to the hormonal changes probably because the oxygen demand and synthesis activity increase in this phase. Up to now, the phase during which lipid peroxides are formed is a matter of debate. In fact, although several studies suggest a free radical overproduction in the high estrogogenic phase, due to increased oxygen consumption as a result of cell growth and DNA synthesis (25, 26), a condition of marked oxidative stress does not seem to occur during the menstrual cycle. The absence of MDA variations supports this point.

It is of interest that, based on our results, the total GSH (GSH plus GSSG) level does not change significantly during the menstrual cycle, although the maintenance of the redox homeostasis occurs at the expense of enhanced GSH consumption by increasing GSH-Px activity. These data show that the hormonal pattern (and specifically the estrogen) plays an important role in preserving the endometrium redox equilibrium, by increasing the antioxidant enzyme activity, i.e. GSH-Px. Many reports have suggested that estrogens might protect against various diseases (27, 28) and in the last few years several studies have proposed that the beneficial effects of E2 could be related to its antioxidant effects (29, 30). However, the question of a direct antioxidant capacity of estrogens is still controversial (31); several reports have shown, in fact, the capability of estrogens to induce the release of myeloperoxidase, peroxidase, and other enzymes involved in oxidative reactions (32–34). Furthermore, the induction of lipid peroxidation by estrogens is widely documented (35). These findings may lead to the hypothesis that estrogens themselves may induce oxidative stress. A possible explanation for this apparent biological paradox is that the beneficial effects of E2 could be related to its prooxidant effects (31); a number of recent studies have documented the induction of antioxidant enzymes by oxidants (36, 37). Our study indicates that the estrogen cycle changes are closely related to GSH metabolism and suggests that the hormonal pattern is involved in maintaining the optimal oxidative balance in the endometrium, stimulating antioxidant enzyme activity, and regulating GSH metabolism. Because human endometrium is under strict hormonal control and GSH is required for the optimal redox state of thiol groups and detoxification reactions, the regulation of GSH metabolism appears to play a critical role in the maintenance of endometrial cell functions.

Proliferation of endometrium is stimulated by estrogens and, in the LL, a withdrawal of hormone activates a chain of events known as menses. At present, the cellular signals...
inducing this complex of events are still unknown. Recently, the growing understanding about apoptosis signaling pathway has revealed a close relationship between hormone balance and apoptosis system.

Apoptosis is a physiological mechanism to eliminate dysfunctional cells and represents thereby an important regulator of cell homeostasis in many hormone-dependent organs (38); in human endometrium, apoptosis occurs predominantly in the late secretory and menstrual phases (39). Furthermore, a role for oxidative stress in apoptosis has been indicated by several observations (40, 41) indicating that depletion of GSH pools may induce the activation of apoptosis cell signaling, which in turn is accompanied by ROS production.

In conclusion, our study suggests that, in endometrial cells, GSH metabolism is regulated by hormonal changes, which in turn may, through modulation of the redox balance, control the cellular modifications during menstrual cycle.

Whether the relationship between endometrial redox state and sex hormonal changes during the menstrual cycle produces biological modulations of the intracellular metabolism or whether these changes play a biologically protective role remains to be evaluated. The importance of ROS in regulating endometrial cell apoptosis during menstrual phases also deserves further investigation.

Acknowledgments

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