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ORIGINAL RESEARCH

Impact of Serum Estradiol on Biomarkers of Oxidative Stress in Polycystic Ovary Syndrome and Ovulatory Women

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Abstract

Background: Estrogens are thought to possess antioxidant properties in vivo, with estradiol being the most biologically active and available. Unlike ovulatory women, those with polycystic ovary syndrome (PCOS) have a relative steady-state serum estradiol concentration across a typical month. To better understand the antioxidant role of serum estradiol in premenopausal women, we evaluated biomarkers of oxidative stress at two time points in both ovulatory and anovulatory cycles (ie, women with PCOS).

Methods: A total of 16 women (7 PCOS, 9 ovulatory) completed this study. Ovulatory women were tested on cycle day 3, and again on cycle day 21. Women with PCOS were tested at a random time and returned to the clinic 14 days later. At each visit, blood was collected for determination of malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and Trolox Equivalent Antioxidant Capacity (TEAC). Estradiol, progesterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were also measured.

Results: There were no significant differences observed in any oxidative stress biomarker between ovulatory and PCOS women. Estradiol levels were positively correlated with TEAC in women with PCOS ($r = 0.57$; $P = 0.03$), but not in ovulatory women. While not statistically significant, negative correlations were noted between estradiol and MDA and estradiol and H_2O_2 in women with PCOS but not in ovulatory subjects.

Conclusions: Our data indicate that oxidative stress biomarkers do not differ between PCOS and ovulatory women. The changing estrogen level that occurs throughout ovulatory cycles does not appear to impact overall oxidative status when compared to the relative steady-state estradiol levels in PCOS subjects in our study. Furthermore, estradiol may be associated with antioxidant status and biomarkers of oxidative stress in women with PCOS but not in those with regular menstrual cycles.

Keywords: oxidative stress, lipid peroxidation, estradiol, PCOS

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Introduction

Oxidative stress is an imbalance of the body's innate homeostasis of protective anti-oxidant defenses and the formation of potentially damaging reactive oxygen species (ROS), particularly superoxide (O_2–), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). ROS, in turn, affect cells and tissues through a variety of mechanisms including DNA damage, protein alterations, and lipid peroxidation.[1–3] The cumulative effects of these oxidative modifications have been implicated in several disease states, from cardiovascular disease to cancer, as well as in the normal aging process.[4–7] Specific to reproductive biology, oxidative stress is postulated to play a role in endometriosis,[8–9] uterine environment alterations,[2] hydrosalpinges,[10] conception,[1] and pregnancy.[11]

Estrogen has been shown to possess direct free radical scavenging properties at high concentrations, to enhance the activity of some naturally occurring antioxidant enzymes (particularly glutathione peroxidase), and to function as a chain-breaking antioxidant in vivo.[12–20] In women who have regular menses, estrogen levels peak twice throughout the cycle, once in the late follicular phase and to a lesser degree in the luteal phase.[21] On the contrary, anovulatory women demonstrate a relatively steady-state estradiol level and often have oligo- or amenorrhea.[21] These anovulatory women frequently have polycystic ovary syndrome (PCOS), which is associated with numerous co-morbidities including abdominal obesity, hyperlipidemia, cardiovascular disease, endometrial cancer, and diabetes mellitus in insulin-resistant patients.[21,22]

The oxidative balance in eumenorrheic patients, as well as in patients with PCOS, has been investigated. Collectively, these data are mixed. Some studies have noted increased oxidative stress and/or impaired antioxidant capacity in women with PCOS compared to controls,[2,3,23–30] whereas at least one has demonstrated opposite findings.[31] Two studies to our knowledge have indicated no differences between the two groups.[32,33] Many authors have suggested vacillating antioxidant status across the normal menstrual cycle and postulate that fluctuating levels of estrogen contribute to these menstrual phase differences.[14,34–36] It is possible that the relatively steady state of monthly serum estradiol concentration in PCOS women is partially responsible for the findings of increased oxidative stress or decreased antioxidant capacity in these patients, given the proposed in vivo antioxidant properties of estradiol.[14,34,35]

To better understand the antioxidant role of serum estradiol in premenopausal women, we evaluated biomarkers of oxidative stress at two time points in both ovulatory and anovulatory cycles (ie, women with PCOS). We hypothesized that cyclic variations in estradiol level during ovulatory cycles could lower oxidative stress biomarkers when compared to the relative steady state of estradiol in anovulatory women.

Methods

Subjects and screening

A total of 22 women were recruited into the study. PCOS and ovulatory subjects were matched to within 1.5 $kg \cdot m^{-2}$ in body mass index (BMI). Two of the 11 subjects in the PCOS group did not meet the definition of PCOS (as indicated below), and two more in this group failed to return for the second blood draw. Of the 11 ovulatory women, two failed to complete the second blood draw. Sixteen subjects completed the study (7 PCOS [2 African American, 3 Caucasian, 1 Hispanic, 1 Indian]; 9 ovulatory [4 African American, 4 Caucasian, 1 mixed Hispanic/Caucasian]). The diagnosis of PCOS was defined according to the Rotterdam Consensus Statement modified, in that all PCOS subjects exhibited chronic anovulation within 45 days of enrollment.[22] Ovulatory women experienced regular, predictable menses 25–34 days apart and/or had a measured luteal phase progesterone > 5 $ng \cdot mL^{-1}$. All women had follicle stimulating hormone (FSH) levels of ≤ 12 $mIU \cdot mL^{-1}$, had thyroid stimulating hormone and prolactin levels within normal range, were aged between 20 and 35 years, and had a BMI of 18–40 $kg \cdot m^{-2}$. The following exclusion criteria were used in enrolling subjects in this study: pregnancy; use of birth control pills/patches/injections; current cigarette smoker; use of diabetic medications such as oral hypoglycemic agents; presence of diabetes (in particular due to the common use of diabetic medications) or cardiovascular disease; vitamin therapy; resting blood pressure $>$ 160/100 mmHg. Although we understand that certain women with PCOS may present with some of the above exclusion criteria, these conditions may have impacted our outcome measures and hence, needed to be controlled for in the present design. Descriptive characteristics are presented in Table 1.

Table 1. Descriptive data of ovulatory and PCOS women.

Variable	PCOS (n = 7)	Ovulatory (n = 9)	*P* value
Age (yrs)	29.4 ± 2.1	33.2 ± 1.1	0.11
Height (cm)	159.7 ± 2.2	166.5 ± 1.3	0.01
Weight (kg)	74.2 ± 9.1	89.8 ± 6.8	0.18
BMI ($kg \cdot m^{-2}$)	29.2 ± 3.6	32.3 ± 2.2	0.45
Systolic blood pressure (mmHg)	118.9 ± 3.3	128.2 ± 3.5	0.08
Diastolic blood pressure (mmHg)	75.1 ± 2.1	78.7 ± 1.9	0.24

Note: Values are mean ± SEM.

Based on the above information, women were considered relatively young and healthy. Height was measured using a stadiometer and body weight was measured using a calibrated medical scale. Body mass index was calculated as weight (kg) divided by height (m^2). Blood pressure was monitored using standard automated procedures. Women were informed of all procedures, potential risks, and benefits associated with the study through both verbal and written form in accordance with the approved procedures of the University Institutional Review Board for Human Subjects Research (H09-28). All women signed an informed consent form prior to participating.

Procedures

Women reported to the clinic on two different days over the course of one month. Ovulatory women first reported on day 3 of their menstrual cycle and then home tested for the urinary luteinizing hormone (LH) surge. Seven days after the LH surge, at approximately cycle day 21, they reported to the clinic a second time. Women with PCOS reported to the clinic at a random time and then returned 14 days later for a second blood draw. At each visit, a blood sample was collected for determination of ovarian hormones and oxidative stress biomarkers.

Blood collection and biochemical variables

Venous blood samples (~15 mL) were taken from a forearm vein via needle and Vacutainer® tubes. Following collection, blood was immediately processed for collection of plasma or serum. A portion was analyzed within one day for: estradiol, progesterone, LH, and FSH by automated chemiluminescent assay (Vitros® 5600, Ortho Clinical Diagnostics, Raritan, NJ).

Remaining samples were immediately frozen at –20 °C in multiple aliquots until analyzed for biomarkers of oxidative stress. Malondialdehyde (MDA) was analyzed in plasma following the procedures of Jentzsch et al[37] using reagents purchased from Northwest Life Science Specialists (Vancouver, WA). Hydrogen peroxide (H_2O_2) was analyzed in plasma using the Amplex Red reagent method as described by the manufacturer (Molecular Probes, Invitrogen Detection Technologies, Eugene, OR). Total antioxidant status was analyzed in plasma using the Trolox Equivalent Antioxidant Capacity (TEAC) assay using procedures outlined by the reagent provider (Sigma Chemical, St. Louis, MO).

Statistical analysis

Measures of ovarian hormones and oxidative stress biomarkers were compared between PCOS and ovulating women using a 2 (group) × 2 (time) analysis of variance (ANOVA). Pairwise correlations were made between ovarian hormones and oxidative stress biomarkers. Descriptive variables were analyzed between groups using a *t*-test. Analyses were performed using JMP statistical software (version 4.0.3, SAS Institute, Cary, NC). Statistical significance was set at $P \leq 0.05$. The data are presented as mean ± SEM.

Results

No differences were noted between groups for anthropometric variables or for resting blood pressure, with the exception of height, with ovulatory women being taller ($P = 0.01$). However, ovulatory women were generally older and heavier than PCOS women, with slightly higher blood pressure. Data are presented in Table 1.

There were no significant differences observed in any oxidative stress biomarker between ovulatory and PCOS women ($P > 0.05$). A significant positive effect of estradiol on TEAC was noted in women with PCOS ($r = 0.57$; $P = 0.03$), but not in ovulatory women. While not statistically significant, a negative correlation was noted between estradiol and MDA and H_2O_2 in women with PCOS. No such correlations were noted for ovulatory women. No other correlations were noted for ovulatory or anovulatory women for any ovarian hormone or oxidative stress biomarker

except for LH and H_2O_2 in ovulatory women (r = 0.52; $P = 0.03$).

In our evaluation of ovarian hormones, a group effect was noted for LH ($P = 0.02$), which was higher in women with PCOS, as physiologically expected. A group effect was also noted for FSH ($P = 0.007$), which was higher in women with PCOS. No group effect was noted for estradiol or progesterone, although these hormones were generally higher for ovulatory women. It should be noted that upon further review of the raw data, two PCOS subjects may have ovulated, although they did not do so prior to enrollment in the study. These subjects had progesterone levels of ~6 and ~17 $ng \cdot mL^{-1}$, which led to the higher mean progesterone level in the group. These two subjects also resulted in the higher estradiol level in PCOS subjects at Time 2 (Table 2). Because of the fact that they met the PCOS inclusion criteria at enrollment, they remained in the analyses. An expected physiologic time effect was noted for estradiol ($P = 0.005$), progesterone ($P = 0.004$), and FSH ($P = 0.0005$), with estradiol and progesterone higher and FSH lower at collection 2 compared to collection 1. No other effects were noted for any variable. Data for oxidative stress biomarkers and ovarian hormones are presented in Table 2.

Discussion

Results from this study indicate that oxidative stress biomarkers do not differ between PCOS and ovulatory women. Moreover, estradiol may have a stronger association with antioxidant capacity and markers of oxidative stress in women with PCOS compared to ovulatory women. These findings are in opposition to our original hypothesis, as we believed that the cyclic variations in estradiol concentration in ovulatory women would be associated with higher antioxidant capacity and lower levels of oxidative stress biomarkers, as has been reported in some prior investigations. However, our findings were in agreement with the work of Karadeniz et al[32] and Lutoslawska et al[33] who reported no differences in oxidative stress biomarkers between these groups of women, highlighting the inclusion of "polycystic ovary syndrome patients and age-matched healthy controls" and "regularly menstruating women with ovulatory and anovulatory menstrual cycles," respectively. As women in our study were generally overweight or obese based on BMI status (Table 1), it is unknown if these results will directly translate to women who are of normal weight, as obesity is known to be associated with increased oxidative stress.[38]

Although the mean estradiol concentrations were generally higher for ovulatory women compared to PCOS women in our study (Table 2), this difference failed to reach statistical significance. A larger sample size, or measurements over more than one cycle, may have resulted in a significant difference between groups for this measure. We obtained blood samples from ovulatory women on approximately cycle days 3 and 21, and in doing so may have missed both their follicular and luteal phase estradiol peaks. However, it should be noted that our intent was not necessarily to obtain "peak" measures of estradiol during these times. Rather, we wanted to obtain two different blood samples demonstrating both "low" and "high" estradiol values. The data presented in Table 2 indicates success in achieving this goal. Measuring estradiol in

Table 2. Hormonal and oxidative stress biomarker data for ovulatory and PCOS women.

Variable	PCOS		Ovulatory	
	Time 1	Time 2	Time 1	Time 2
Estradiol ($pg \cdot mL^{-1}$)	36.29 ± 5.35	54.00 ± 14.34	37.11 ± 5.13	88.50 ± 12.31
Progesterone ($ng \cdot mL^{-1}$)	0.57 ± 0.16	3.91 ± 2.45	0.50 ± 0.12	9.21 ± 2.15
Luteinizing hormone ($IU \cdot L^{-1}$)	12.30 ± 2.33	9.71 ± 2.48	5.65 ± 0.91	5.12 ± 1.16
Follicle stimulating hormone ($IU \cdot L^{-1}$)	7.88 ± 0.51	6.48 ± 0.55	6.86 ± 0.74	3.20 ± 0.46
Malondialdehyde ($\mu mol \cdot L^{-1}$)	2.23 ± 0.45	1.77 ± 0.30	1.63 ± 0.17	2.20 ± 0.45
Hydrogen peroxide ($\mu mol \cdot L^{-1}$)	3.91 ± 1.29	3.06 ± 0.67	3.07 ± 0.45	3.63 ± 0.91
TEAC ($mmol \cdot L^{-1}$)	0.83 ± 0.04	0.94 ± 0.04	0.90 ± 0.03	0.91 ± 0.04

Notes: Values are mean ± SEM. Group effect noted for leutinizing hormone ($P = 0.02$) and follicle stimulating hormone ($P = 0.007$). No other group effects noted ($P > 0.05$). Time effect noted for estradiol ($P = 0.005$), progesterone ($P = 0.004$), and follicle stimulating hormone ($P = 0.0005$). No other time effects noted ($P > 0.05$). No group x time interaction effects noted ($P > 0.05$).

the late follicular phase and later luteal phase may have resulted in higher serum concentrations of the hormone in our ovulatory subjects and therefore a larger discrepancy for this measure between our two study groups. It is unknown whether this potential group difference in estradiol concentrations would have translated into a difference in our measured oxidative stress biomarkers.

The antioxidant effect of various estrogens is thought to be dose-dependent, and estradiol appears to be the most biologically available and active of the estrogens.[13,39–41] Other members of the estrogen family, including estrone and estriol, are thought to possess less potent antioxidant properties.[13,15,19,40] Many of the studies demonstrating the in vivo antioxidant effects of estrogens have been performed on postmenopausal women taking different formulations of hormone replacement therapy.[42–45] Massafra et al[34] described an increase in the erythrocyte antioxidant enzyme glutathione peroxidase in the late follicular phase of ovulatory women's menstrual cycles, a time when serum estradiol peaks. Similarly, Michos et al[14] studied serum hormone and antioxidant levels in 13 eumenorrheic, ovulatory women and discovered a positive correlation between estradiol levels and total antioxidant plasma status, as well as ascorbic acid levels, in all menstrual phases but particularly around the time of ovulation. Browne et al[36] demonstrated a day-to-day antioxidant status fluctuation in 9 healthy, ovulatory women over the course of one menstrual cycle. However, no statistically significant differences or temporal effects were noted for several components of the antioxidant system when evaluating the menstrual cycle as a whole in the study by Browne et al.[36] We attempted to account for some of this described day-to-day difference in antioxidant status by obtaining two separate blood draws from both our ovulatory and anovulatory subjects. Similar to the findings of Browne et al,[36] mean values for all oxidative stress biomarkers across the month were relatively alike between groups of women in our study. Thus, the changing estrogen level that occurs throughout ovulatory cycles does not appear to impact overall oxidative status when compared to the relative steady-state estradiol levels in PCOS patients in our study.

Measurements of oxidative stress biomarkers in PCOS patients have been assessed by several investigators, with mixed results. Superoxide dismutase (SOD) reacts with various free radicals to protect tissues from oxidative damage, and increased SOD activity suggestive of higher oxidative stress has been described in PCOS patients.[3,24] Elevations in MDA, a byproduct of lipid peroxidation, have been previously reported in PCOS patients when compared to controls,[2,3,24,25,27,28] as have higher levels of MDA-modified proteins.[2] However, other reports refute these findings and agree with the results obtained in the present investigation. For example, Karadeniz et al[32] reported no difference in MDA between PCOS patients and controls. Moreover, Sova et al[31] reported lower 8-hydroxydeoxyguanosine (8-OHdG, formed when ROS react with DNA) in PCOS women as compared to controls, indicating lower oxidative stress in PCOS patients in their study.

Similar to oxidative stress biomarkers, studies of antioxidant status in PCOS patients have provided mixed results. Total antioxidant status (TAS) is meant to reflect the body's multifaceted protective mechanism against molecular damage to cells by ROS. Lower antioxidant capacity in PCOS patients, whether measured by individual antioxidant markers or by TAS, has been reported by several investigators.[24,25,27–29] Our study showed no difference in TAS, measured using the TEAC assay procedure between ovulatory women and those with PCOS. Lutoslawska et al[33] likewise showed no difference in the antioxidant enzymes glutathione, glutathione peroxidase, and glutathione reductase in anovulatory patients when compared to controls. Increased TAS in PCOS women was found by Verit and Erel,[26] who suggest that this finding may reflect a compensatory mechanism in PCOS patients to balance oxidative stress (also supported by Sova et al[31]). If such a compensatory mechanism indeed exists, it may help explain why we found no difference in total antioxidant capacity among our study groups (as oxidative stress levels were similar between groups). Though estrone is a less potent antioxidant than estradiol,[13,15,19,40] the elevated circulating levels of estrone present in PCOS patients[21] may also account for some of this compensation and lack of difference in antioxidant capacity in our study. Our failure to measure other biologically active estrogens is a limitation of this work.

Estradiol may impact biomarkers of oxidative stress and antioxidant capacity differently in women with PCOS and those with ovulatory cycles. Though our study showed relatively similar mean values for all biomarkers across the month for both groups of

women, TEAC was increased and MDA and H_2O_2 were both decreased as estradiol concentrations rose from measurement time 1 to time 2 in PCOS women. The same finding was not observed in women with normal menstrual cycles. In fact, we noted a slight increase in both MDA and H_2O_2 from measurement Time 1 to Time 2 as serum estradiol increased in ovulatory women. A some what similar finding occurred in the study by Verit and Erel,[26] where a positive correlation between TAS and LH, free androgen index, and ovarian volume was described in the PCOS group but no correlations between these or other variables were seen in the control group. While not statistically significant, we noted a negative correlation between estradiol and MDA and H_2O_2 in women with PCOS. Due to our small sample size and the fact that other confounding variables may have influenced our results, further study with the inclusion of a larger sample of women in both groups, is needed to extend these findings.

The clinical importance of continuing research in the area of oxidative stress, particularly in PCOS patients, is evident. Oxidative status in PCOS is thought to contribute to its comorbidities, including abdominal obesity, endothelial dysfunction, dyslipidemia, elevated insulin levels, and insulin resistance, and may vary between lean and obese PCOS patients.[23–25,30] Targeted antioxidant therapies may become future treatment adjuncts for PCOS patients as more is discovered and clarified in this area of investigation.

Conclusion

We report that oxidative stress biomarkers do not differ significantly between PCOS and ovulatory women. Serum estradiol may be associated with antioxidant capacity and markers of oxidative stress in women with PCOS, but our study suggests this is not the case for ovulatory women. This data adds to the growing body of literature focused on the difference between the oxidative stress markers of ovulatory and anovulatory women, while providing new insight into the potential in vivo antioxidant role of serum estradiol in women with PCOS. Additional work, inclusive of a larger sample size, is needed to extend these findings.

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Competing Interests

RWK's institution has received a grant from Merck. RJB has received consulting fees from Bergstrom Nutrition, OmniActive Health, CE-Bio, Sigma-tau Healthscience, Purity Products. And his institution has received grants from USPlabs, Kaneka Nutrients, Miami Research Associates, Sigma-tau HealthScience, Mannatech, Advanced Oral Technologies, Purity Products, Life Extension Clinical Research, Danisco, and he has received speaking fees from Bergstrom Nutrition, manuscript preparation fees from Miami Research Associates and royalties from Formulife. Other authors disclose no competing interests.

Authors Contributions

RWK and RJB were responsible for the study design. DBA and RWK were responsible for subject recruitment, screening and retention, and data collection. RJB was responsible for analysis of oxidative stress biomarkers and statistical analyses. DBA and RJB were responsible for manuscript preparation. All authors assisted in reviewing/editing the manuscript.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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