Reproductive Sciences
2017, Vol. 24(4) 619-626

DOI: 10.1177/1933719116667225
journals.sagepub.com/home/rsx
SAGE

Myeloperoxidase as a Potential Target in Women With Endometriosis Undergoing IVF

Nalini Santanam, PhD, MPH, FAHA[1,2], Nathaniel Zoneraich, MD[1,3], and Sampath Parthasarathy, PhD, MBA[1,4]

Abstract

As infertility is intimately associated with endometriosis, the levels of myeloperoxidase (MPO), a leukocyte enzyme and an oxidative stress marker, were determined in a case–control prospective study of 68 women with and without endometriosis undergoing in vitro fertilization in the outpatient fertility center within a tertiary care academic medical center. Measured values included plasma and follicular fluid (FF) concentrations of MPO, plasma estradiol, as well as oocyte quality, fertilization, implantation, and pregnancy rates in these women. In FF (mean $\pm$ standard error of mean [SEM]), the MPO concentrations (ng/mL) for controls were 4.3 $\pm$ 0.37, mild endometriosis (stages I-II) 3.9 $\pm$ 0.17, and moderate/severe endometriosis (stages III-IV) 16.6 $\pm$ 12.5 ($P < 0.0143$). In FF, among patients supplemented with vitamins E and C, the MPO levels decreased significantly only in moderate/severe endometriosis from 25.3 $\pm$ 22.0 ng/mL to 4.9 $\pm$ 1.61 ng/mL, respectively. Plasma levels of MPO between groups did not change. Outcome data revealed a trend toward decreased percentage of mature oocytes, implantation rate, and clinical pregnancy rate with severity of endometriosis and MPO levels. Myeloperoxidase may be a potential oxidative stress target for endometriosis-associated infertility.

Keywords

oxidative stress, antioxidants, infertility

Introduction

Infiltration of leukocytes in the preovulatory follicle after exposure to luteinizing hormone (LH) is a hallmark of luteolysis.[1-4] The lack of functional deoxyribonucleic acid (DNA) repair mechanisms in the mammalian predictyate oocyte makes them highly vulnerable to oxygen radical attack.[5-7] Reactive oxygen species may play a role in follicular atresia, ovulation, and in overall oocyte function. Production of physiologic levels of oxygen radicals at ovulation, in response to LH, may signal differentiation of the oocyte, but overproduction may cause injury such as that seen in atresia.[8-11] Recent studies showed a positive association between total antioxidant capacity and follicular fluid (FF) estradiol levels.[12,13] The FF total antioxidant capacity was higher in oocytes that successfully fertilized, but lower where the resultant embryo survived to transfer.[14-16] These studies reiterate the importance of oxidative stress in female reproduction.

Endometriosis is a disease associated with marked subfertility.[17-20] We have earlier hypothesized that endometriosis is a disease of oxidative stress and have provided evidence in its support.[21-23] We hypothesize that women with endometriosis undergoing in vitro fertilization (IVF) may have an increase in myeloperoxidase (MPO) levels and subsequent decrease in oocyte quality and fertility.

Human MPO is a hemoprotein with a molecular weight of 140 kDa, which is stored in primary azurophilic granules of neutrophils. Upon its release, MPO has the capacity to catalyze the production of hypochlorus acid (HOCl), a powerful oxidant derived from chloride ion and hydrogen peroxide. In a number of inflammatory situations, MPO is released into the phagolysosome or extracellular medium where its measurement can be used as an index of neutrophil activation and a marker of oxidation.[24] Traditionally, MPO has been known for its role in microbicidal activity; however, recent reports implicate a role for MPO in processes unrelated to host defense against

[1] Department of Gynecology & Obstetrics, Emory University School of Medicine, Atlanta, GA, USA
[2] Department of Pharmacology, Physiology & Toxicology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, USA
[3] Advanced Fertility Care, Scottsdale, AZ, USA
[4] Burnett School of Biomedical Sciences, University of Central Florida, Orlando, FL, USA

Corresponding Author:
Nalini Santanam, Department of Pharmacology, Physiology & Toxicology, Joan C. Edwards School of Medicine, Marshall University, 1700 3rd Ave, 435S Byrd Biotech Building, Huntington, WV 25755, USA.
Email: santanam@marshall.edu

bacteria, including carcinogenesis, atherosclerosis, and degenerative diseases of the central nervous system.[24,25] Furthermore, structurally related proteins in invertebrates suggest that MPO may serve additional important and unanticipated cellular functions such as cellular adhesion. Our work supports a role for oxidative stress and especially MPO in gynecologic cancer.[26] Chun et al examined the involvement of leukocytes in follicular rupture of the rat by quantifying MPO activity in ovarian tissue at given time intervals after human chorionic gonadotropin (hCG) administration. This data showed significant increase in ovarian MPO activity during the periovulatory period, which reflected an influx of neutrophils into the ovaries.[27] Santanam et al provided the first evidence in humans for an increase in plasma MPO in association with elevated levels of estradiol in women undergoing ovarian hyperstimulation.[28,29] Estradiol has also been shown to increase neutrophil activation during phagocytosis, thereby increasing MPO release.[28]

In this study of women undergoing controlled ovarian hyperstimulation (COH) and IVF, we chose to examine the role of MPO which may have significant involvement in altering the follicular environment of the developing oocyte and subsequently impacting fertility.

Materials and Methods

Patients

One hundred and seventeen patients, aged 21-42 years were recruited into this study and met our inclusion criteria. This group was composed of patients undergoing IVF at the Emory Center for Reproductive Endocrinology and Infertility at Emory University/Crawford Long Hospital, Atlanta, Georgia. Oocyte donors were included in the control (non-endometriosis) group. After informed consent was obtained, we collected baseline (day 2 or 3 of stimulation) and retrieval day (day of transvaginal oocyte retrieval) blood samples, in addition to retrieval day FF samples from patients undergoing IVF. Of the 117 participants enrolled in the study, we proceeded to analyze data for MPO from 68 women for whom we were able to collect and obtain a complete set of data points. Biochemical analysis of MPO concentrations was performed on all samples. There was no deviation in IVF stimulation protocols from otherwise standard treatment as dictated by the patients' physicians. For every patient enrolled in the study, the presence of endometriosis was clearly documented with staging of disease determined by review of previous operative notes and scored according to the revised American Fertility Society classification of endometriosis.[30] By convention, the staging of endometriosis at the time of initial surgical diagnosis was utilized since the patient's classification of endometriosis should not change despite any subsequent surgical or medical treatment. Therefore, in order to minimize the potential inherent variability of operator staging, patients were grouped into broader categories of endometriosis. The 3 groups were controls (no endometriosis), mild endometriosis (stages I + II), and moderate/severe endometriosis (stages III + IV). Patients who were excluded from this study included those with a reported history of endometriosis without operative documentation. Patients with no previously documented history of endometriosis were placed in the control group. For majority of the patients recruited in the study, the date of surgical diagnosis of endometriosis was approximately within 1 to 3 years. The minimum duration from last surgery to IVF was at least 3 months, regardless of timing of original surgery. Any patients who received gonadotropin releasing hormone (GnRH) agonist therapy within 6 months of IVF treatment were excluded due to the possibility of interference with the outcome measurements. Although not an exclusion criteria, none of the patients recruited were on oral contraceptive therapy.

Those patients grouped as taking high-dose vitamins were ingesting a prescribed dose of 800 IU of vitamin E and 1000 IU of vitamin C for a minimum of 8 weeks prior to the start of their IVF cycle. The use of the high-dose vitamins was not subject to randomization and was prescribed independently by our physicians. The Emory University Human Investigations Committee approved this study and the study was performed with Health Insurance Portability and Accountability Act (HIPAA) compliance. Informed consent was obtained from all participating patients.

There were several outcome measures analyzed in this study. Oocyte maturity was graded by 1 of the 2 embryologists after transvaginal oocyte retrieval or at the time of intracytoplasmic cell injection (ICSI), if applicable to the patient. Oocytes were considered mature if they met the following criteria: expanded cumulus, extruded first polar body, appropriate cytoplasmic maturation, and arrest in metaphase II. Percentage fertilization was calculated by quantifying the number of mature oocytes that underwent fertilization either spontaneously or through ICSI. Implantation rate was defined as the ratio of number of gestational sacs over the number of embryos transferred. The number of patients with fetal viability at 7 weeks' gestation is designated as the pregnancy rate.

Initial Patient Evaluation

All patients included in the study underwent a standard initial evaluation including complete history and physical examination, initial IVF consultation, semen analysis of male partner, hysterosalpingogram, and/or sonohysterogram. Blood evaluation included cycle day 3 estradiol and follicle-stimulating hormone (FSH), prolactin, thyroid-stimulating hormone, HIV, hepatitis B virus surface antigen and hepatitis C antibody, Rapid Plasma Reagin test (RPR) (syphilis) as well as a Pap smear, and cervical cultures.

In Vitro Fertilization Controlled Ovarian Hyperstimulation Protocol

Stimulation protocols were individualized and included (1) leuprolide acetate long protocol or downregulation, (2) leuprolide acetate coflare or short protocol, and (3) GnRH antagonist protocol. Human chorionic gonadotropin (hCG) 10,000 IU

intramuscularly was administered at the appropriate time in follicular development. Transvaginal oocyte retrieval was performed 35 to 36 hours after hCG administration using intravenous sedation with Fentanyl and Versed.

Collection of FF

Standard medical protocol for all physicians involved in the study was that all visible follicles were aspirated, regardless of size. Any follicle that had a suspicious appearance for possible endometrioma was aspirated last and quarantined in separate tubes from the aspirated ''normal'' follicles. At the time of oocyte retrieval, oocytes were microscopically removed from the aspirated ovarian FF. The discarded FF, excluding samples contaminated by culture media, was centrifuged for 10 minutes first at 1000 rpm and then at 3000 rpm for an additional 10 minutes. Samples with either blood contamination or suspected endometrioma were excluded from analysis. The pellet was discarded and the FF sample was frozen at -80° C.

Collection of Blood Plasma

Ten milliliters of blood were obtained in a heparinized tube, from each woman at baseline/on days 2 to 3 of the stimulation and on the day of oocyte retrieval, prior to induction of any anesthetics. After centrifugation for 10 minutes at 1000 rpm, blood plasma samples were stored at -80°C until analysis.

Determination of MPO Protein in Plasma and FF

An enzyme-linked immunosorbent assay (ELISA) kit (Bioxytech MPO-EIA Assay Kit; Oxis Research, Portland, Oregon) was used on 100 μL of baseline and retrieval day plasma as well as FF to measure the MPO levels.[26] The MPO-EIA assay system is a ''sandwich'' ELISA in which antigen is captured by a solid phase monoclonal antibody that is detected with a biotin-labeled goat polyclonal anti-MPO. An avidin–alkaline phosphatase conjugate then binds to the biotinylated antibody. The alkaline phosphatase substrate *p*-nitro phenyl phosphate is added and the yellow product (*p*-nitrophenol) is measured at 405 nm using a spectrophotometer. Each sample was run in duplicate. According to the manufacturer, within-run precision determined by 20 replicate determinations of 4 different serum samples in 1 assay had a coefficient of variation that ranged from 1.7% to 4.9%.

Determination of Estradiol in Plasma

Plasma estradiol levels were measured using an automated DPC-Immulite analyzer (DPC Cirrus; Diagnostic Products Corporation, Randolf, New Jersey). The Immulite System utilizes assay-specific antibody-coated plastic beads as the solid phase test unit. The test unit serves as the reaction vessel for the immune reaction, incubation, wash, and signal development. Light emission from the chemiluminescent substrate reacting with enzyme conjugate bound to the bead is proportional to the amount of analyte originally present in the patient sample. A commercial control serum pool (Con 6; DPC, Los Angeles, California) with 3 estradiol concentrations (low, medium, and high) was used as quality control with each run. The Immulite Estradiol assay has a broad working range of 20 to 2000 pg/mL. Specimens with estradiol concentrations greater than 2000 pg/mL were diluted with commercial diluent (DPC, Los Angeles, California) and the analysis was repeated. Serum estradiol levels correlate well with levels present in FF.

Statistical Analysis

Graphpad Prism (GraphPad Software Inc, La Jolla, CA, USA) was used for all statistical analyses. Continuous variables were compared between groups using a multivariate analysis of variance (MANOVA), looking at the 3 dependent variables of day 3 plasma (D3-p), retrieval day plasma (RT-p), and FF. Fixed effects including stages of endometriosis, high-dose vitamins E and C usage, as well as, age were considered. A P value of <0.05 was used as significant difference between groups. When significance was demonstrated using ANOVA, Tukey post hoc analysis was performed. Correlation of outcome data with MPO levels, severity of disease, and vitamins E and C usage was performed using a t test with Bonferroni correction. Pearson's correlation was used to determine correlation between outcome measures and severity of endometriosis and MPO levels.

Results

A total of 68 women were included for evaluation. Forty-nine women from the initial number recruited were not included due to incomplete data sets due to cancelled IVF cycles (n = 28), inadequate amount of analyzable sample (n = 10), or procedural error in collecting samples (n = 11). The characteristics of the patient groups are summarized in Table 1. The age ranges were similar within the groups. The ovarian reserve (FSH levels) and the amount of gonadotropins administered (FSH + LH IU) were similar between the groups.

Patients in this study were grouped by severity of endometriosis. The control group consisted of those women with no objective history of endometriosis. In order to strengthen the value of the analysis, patients with stage I and stage II endometriosis (*mild endo*) were grouped together as were patients with stage III and stage IV endometriosis (*moderate/severe endo*). This grouping schema for endometriosis is well supported and documented in the endometriosis literature.[30]

Overall, 21% of patients were on the high-dose vitamins E and C regimen. The breakdown for those on high-dose vitamins in each endometriosis grouping was control (5/41), mild endo group (5/20), and severe endo group (4/7).

Myeloperoxidase Concentration and Stage of Endometriosis

Analysis of the data for plasma MPO concentrations by endometriosis classification revealed no difference between D3-p or RT-p MPO values across all 3 groups (day 3: control/stage 0:

Table 1. Patients' Characteristics Grouped by Endometriosis Grouping.[a]

	All Participants (n = 68)	Control (no endo), n = 41	Mild Endo (Stages I and II), n = 20	Severe Endo (Stages III and IV), n = 7
Age (years)	33.4 ± 3.88	33.6 ± 3.01	33.6 ± 2.10	31.6 ± 1.24
Age range (years)	23-41	24-41	23-41	29-36
Plasma FSH (U/L)	6.05 ± 1.35	5.08 ± 2.08	6.87 ± 1.24	5.71 ± 1.09
FSH (IU) +LH (IU)	2840 ± 554.42	2464 ± 759.07	2749 ± 317.4	3120 ± 689.12

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; SD, standard deviation.
[a]Values are mean ± SD. No statistical difference in age between groups.

16.4 ± 2.2 ng/mL, mild endo/stage I-II: 11.3 ± 1.7 ng/mL, severe endo/stage III-IV: 10.8 ± 1.5 ng/mL, P <0.27; RT-p: control/stage 0: 9.8 ± 2.4 ng/mL, mild endo/stage I-II: 11.6 ± 3.4 ng/mL, severe endo/stage III-IV: 6.1 ± 1.4 ng/mL, $P < 0.76$).

As illustrated in Figure 1, when analyzing just the data for FF, ANOVA showed statistical significance indicating that at least 1 endo group mean was different from the others. Post hoc analysis showed the mean MPO concentrations in the FF of the severe endo group (16.6 ± 12.5 ng/mL) were significantly higher than that of the control (4.3 ± 0.37 ng/mL) and mild endo groups (3.9 ± 0.17 ng/mL; $P < 0.0143$). There was no significant difference between the control and the mild endo groups. When the control group is grouped with the mild endo group and compared to the severe endo group, a more highly significant difference in MPO concentration ($P = 0.004$) was seen. There is a positive correlation between the MPO levels and severity of endometriosis.

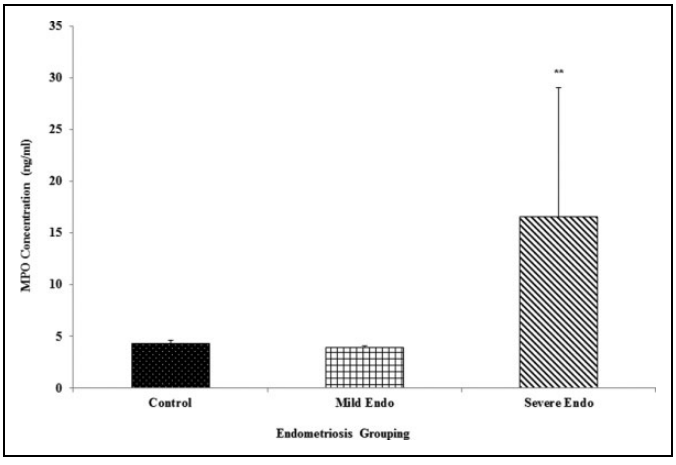

Figure 1. The MPO concentration by endometriosis grouping in follicular fluid. The MPO levels were determined in the follicular fluid obtained from women with various stages of endometriosis. The MPO was determined using the MPO ELISA kit obtained from Oxis Research. The figure represents the MPO levels in nanogram per milliliter (ng/mL) ± SEM, according to the severity of the endometriosis. **$P < 0.0143$. ELISA indicates enzyme-linked immunosorbent assay; MPO, myeloperoxidase; SEM, standard error of mean.

Role of Vitamins E and C

There is no difference in plasma MPO concentrations in D3-p or RT-p for those patients taking high-dose vitamins E and C compared to those not taking it. There is also no measurable difference in the interaction of vitamins E and C on plasma MPO levels when analyzed by endometriosis grouping.

Myeloperoxidase concentrations are increased in FF in women with severe endo (Figure 1). When we adjust for high-dose vitamins E and C intake, there is still a significant difference between the severe endo group and the other groups. Post hoc analysis showed the mean MPO concentration for the severe endo group not on vitamins E and C (25.3 ± 22.0 ng/mL) was significantly higher than that of the control (3.40 ± 0.23 ng/mL) or mild endo groups (3.86 ± 0.16 ng/mL). As shown in the Figure 2, vitamins E and C intake did not significantly reduce the MPO levels in the control and mild endo groups. However, the mean MPO level within the severe endo group between those on high-dose vitamins E and C (4.9 ± 1.61 ng/mL) and those not taking the vitamins (25.3 ± 22.0 ng/mL) was different.

Estradiol Concentration and Stage of Endometriosis

Figure 3 illustrates the peak plasma estradiol levels by endometriosis grouping. The difference in mean estradiol concentration between the severe endo group (1257.1 ± 156.6 pg/mL) and control group (2582.6 ± 182.6 pg/mL) was statistically significant ($P < 0.01$). There was no significant difference in mean peak estradiol levels between the control group and mild endo group (2119.9 ± 228.2 pg/mL). Pearson's correlation showed no significant correlation between plasma estradiol levels and FF-MPO ($r^2 = .009$, $P < 0.43$), RT-P MPO ($r^2 = .003$, $P < 0.64$), or D3-p-MPO ($r^2 = .04$, $P < 0.08$).

Outcome Measures by Stage of Endometriosis

Analysis of the data for outcome measures (percentage mature oocytes, clinical pregnancy rate, and percentage implantation) by endometriosis grouping (control vs mild vs severe) revealed a trend toward decreased percentage of mature oocytes, decreased implantation rate, and decreased clinical pregnancy rate in the severe group (Figure 4). Due to the lower patient numbers, correlation between MPO levels and oocyte maturity ($P < 0.12$), clinical pregnancy rate ($P < 0.28$), and percentage implantation ($P < 0.11$), respectively, did not reach significance.

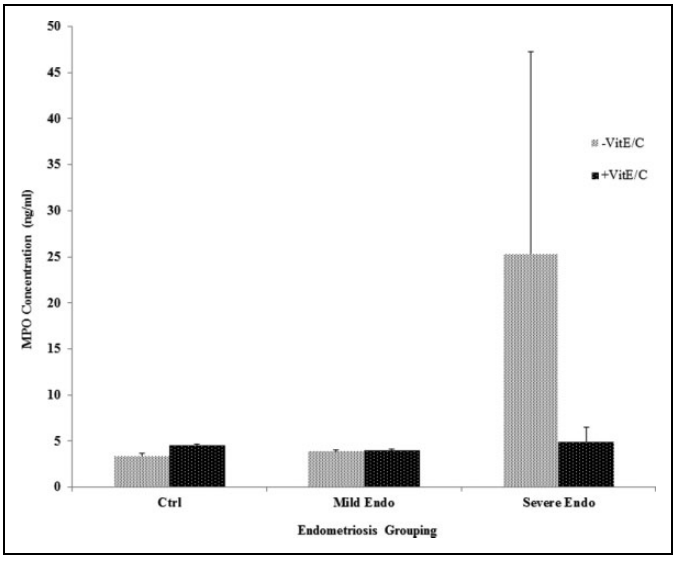

Figure 2. The MPO concentration in follicular fluid after vitamins E and C supplementation: MPO levels were determined in the follicular fluid of women with or without supplementation of 800 IU of vitamin E and 1000 IU of vitamin C for 8 weeks prior to IVF. The MPO was determined using the MPO ELISA kit obtained from Oxis Research. The figure represents the MPO levels in nanogram per milliliter (ng/mL) $\pm$ SEM, according to the severity of the endometriosis. ELISA indicates enzyme-linked immunosorbent assay; MPO, myeloperoxidase; SEM, standard error of mean.

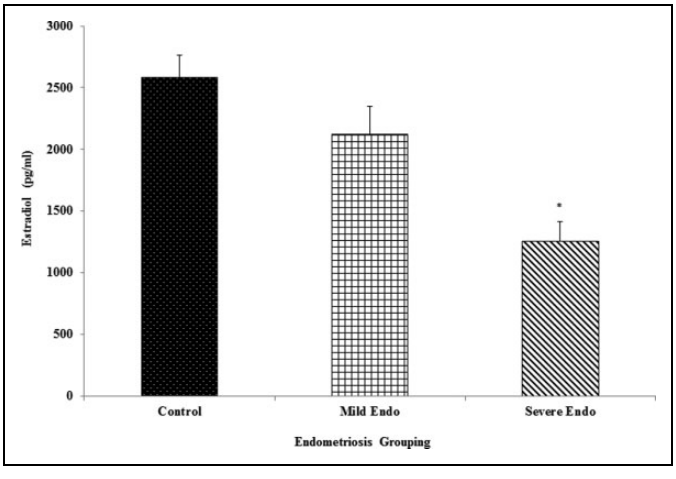

Figure 3. Peak estradiol levels by endometriosis grouping. Peak estradiol levels (pg/mL) were determined in the plasma of patients with or without endometriosis at different stages of endometriosis, using the Enzyme immunoassay Immulite method. *$P < .05$ for severe endo versus control.

Discussion

Oocyte quality is one of the most important factors associated with successful pregnancy outcomes during IVF. The FF serves as the microenvironment for the oocyte and is believed to be vital for normal oocyte development, folliculogenesis, and timely ovulation.[31,32]

Increased oxidative stress plays a very important role in female fertility.[33,34] Oxidative stress plays a role in various

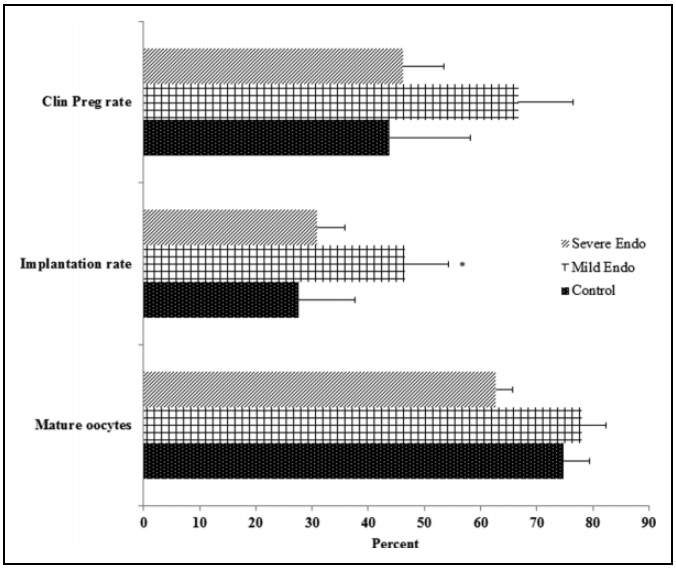

Figure 4. Outcome measures by stage of endometriosis: percentage mature oocytes, clinical pregnancy rate, and percentage implantation was determined for each group (control vs mild vs severe). A trend toward decreased percentage of mature oocytes, decreased implantation rate, and decreased clinical pregnancy rate in the severe group (not statistically significant) was observed. *$P < .05$ for mild endo versus control.

stages during pregnancy, including oocyte maturation, ovulation, implantation, blastocyst formation, and luteogenesis.[16,34-36] The age-mediated decline in fertility is also associated with oxidative stress.[37,38] Due to its constant interactions with exogenous substances, the reproductive system has a well-developed defense mechanism against extraneous stress. An example is to provide a low oxygen environment for the developing germ cells.[39] Besides structural defenses, inhibition of production, detoxification, and repair of damaged cell products serve as the main means of defense against oxygen-free radicals. Several antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase, and catalase, have been identified in the granulosa and thecal cells of ovarian follicles.[40] Oxidative stress plays a role in preeclampsia, endometriosis, and infertility.[39,41-43] We and others have observed increased oxidative stress markers in the peritoneal fluid and endometrial tissues obtained from women with endometriosis.[22,44-49]

In this study, we examined the levels of MPO (a neutrophil marker that is increased during oxidative stress) in plasma and the follicular environment in patients undergoing IVF. Using severity of endometriosis as our determinant of degree of oxidative stress, our data show a significant increase ($P < 0.01$) in MPO concentration within the FF in patients diagnosed with severe endometriosis (stage III or IV) when compared to those with no endometriosis or mild endometriosis (stage I or II). We were unable to show this difference in blood plasma. The MPO is a potent pro-oxidant in the follicular milieu. Its presence in high amounts in the preovulatory follicle of women with severe endometriosis may contribute to a decrease in oocyte quality and subsequently decreased fertilization and implantation.

In vivo, oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers present in follicular and oviduct fluids. Antioxidant vitamins such as vitamin E have a protective action in lipid-enriched membranes and vitamin C in aqueous compartments. Vitamin E serves a primary role in terminating peroxidation chain reactions of unsaturated lipids and protects cells in vivo and in vitro. Significantly measurable amounts of vitamins C and E are found in the ovary and FF.[50-52] Vitamin C serves to recycle oxidized vitamin E back to the reduced state and the oxidized vitamin C is then reduced by transhydrogenases or replaced from extracellular stores. Ascorbic acid also functions by reducing sulfhydryls, scavenging free radicals, and protecting against endogenous oxidative DNA damage. Thus, depletion of vitamin C is directly related to the production of oxygen radicals.

We analyzed our data about the effect of high-dose vitamins E and C usage on MPO levels. Post hoc analysis of the data showed that patients with severe endometriosis who were not on supplemental high-dose vitamins E and C had a significant ($P < 0.009$) increase in MPO concentration in the FF than those with severe endometriosis but taking supplemental vitamins. These results support previous data which suggest that high-dose antioxidant use can decrease the levels of oxidative stress, in this case elevations of MPO levels, in the follicular environment.[45,53-56] This in turn may preserve oocyte quality and promote better fertility rates. The implication of this finding may be that the antioxidant protection of endogenous vitamins is overcome in severe endometriosis, thus requiring supplementation in order to maintain a more suitable ovarian/follicular environment.

We have previously shown that estradiol induces MPO and becomes a pro-oxidant.[28] This would also imply that estradiol becomes a radical and thus would not be measured in an estradiol assay. Women taking vitamins E and C would be less likely to generate an estradiol radical because antioxidants quench free radical generation. Therefore, it is more likely to be maintained as estradiol rather than an estradiol radical. We hypothesize that it is the estradiol radical that induces MPO and results in lower MPO levels in women with stages III-IV endometriosis who are taking vitamins E and C. It is well known that vitamin E recycles phenolic antioxidants (estradiol has a phenyl group) and vitamin C serves to recycle vitamin E. The difference in response to vitamins E and C supplementation in plasma versus FF is difficult to interpret. High-density lipoprotein (HDL) is the predominant lipoprotein in the FF, and MPO has been suggested to bind to HDL. Depending on the oxidizability of HDL, it is likely that MPO which is considered harmful might have different association between unoxidized and oxidized HDL (Ox-HDL). On the other hand, plasma MPO might be both HDL associated and unassociated with total amounts comparable. We have not analyzed FF HDL levels or their oxidation status. To date, no methodology is available for the determination of Ox-HDL.

Although our analysis of outcome such as oocyte quality, implantation rate, fertilization rate, and pregnancy rate pointed to the trends in the data (Figure 4), the small number of participants precluded statistical significance for the findings.

The major limitation of this study is the overall small number of patients, especially in the severe endometriosis group. A significant portion of patients who come to our fertility center have endometriosis. Our data may be skewed, given that 28 patients who were originally enrolled in this study were not analyzed due to cycle cancellation and 20 of these would have been classified as having severe endometriosis.

Although the sample size in this pilot study was relatively small, we were able to show that human MPO levels are significantly elevated in the FF of patients with severe endometriosis. Furthermore, it appears that antioxidant vitamin use can decrease FF MPO levels. These findings open the door to further studies which will elucidate the role of antioxidants as well as MPO inhibitors[57] on oocyte quality and the significance of elevated MPO levels on overall fertility for women with endometriosis, undergoing IVF.

Acknowledgments

The authors would like to express their special thanks to Drs Ana Murphy and John Rock for providing us with the patient samples. The authors also acknowledge the following people for their significant contribution to this study: Drs Spencer Richlin, Aida Shanthi, and Nino Kavtradze for help with recruiting patients and Steve Voelkel, Paul Weiss, and Sharon Wicker for their technical contributions.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by funding obtained from National Institutes of Health grant, P01-HD35276 to SP.

References

1. Shirasuna K, Shimizu T, Matsui M, Miyamoto A. Emerging roles of immune cells in luteal angiogenesis. *Reprod Fertil Dev*. 2013; 25(2):351-361.
2. Brannstrom M, Norman RJ. Involvement of leukocytes and cytokines in the ovulatory process and corpus luteum function. *Hum Reprod*. 1993;8(10):1762-1775.
3. Brannstrom M, Pascoe V, Norman RJ, McClure N. Localization of leukocyte subsets in the follicle wall and in the corpus luteum throughout the human menstrual cycle. *Fertil Steril*. 1994;61(3): 488-495.
4. Suzuki T, Sasano H, Takaya R, et al. Leukocytes in normal-cycling human ovaries: immunohistochemical distribution and characterization. *Hum Reprod*. 1998;13(8):2186-2191.
5. Brannstrom M, Enskog A. Leukocyte networks and ovulation. *J Reprod Immunol*. 2002;57(1-2):47-60.
6. Makinoda S, Hirosaki N, Waseda T, Tomizawa H, Fujii R. Granulocyte colony-stimulating factor (g-csf) in the mechanism of human ovulation and its clinical usefulness. *Curr Med Chem*. 2008;15(6):604-613.

7. Yen S, Jaffe Rb, Barbieri RL. Reproductive endocrinology-physiology, pathophysiology and clinical management. 2015, 7th Edition.
8. Jana SK, NB K, Chattopadhyay R, Chakravarty B, Chaudhury K. Upper control limit of reactive oxygen species in follicular fluid beyond which viable embryo formation is not favorable. *Reprod Toxicol*. 2010;29(4):447-451.
9. Agarwal A, Gupta S, Sharma R. Oxidative stress and its implications in female infertility—a clinician's perspective. *Reprod Biomed Online*. 2005;11(5):641-650.
10. Tilly JL, Tilly KI. Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology*. 1995;136(1):242-252.
11. Zhang X, Li XH, Ma X, Wang ZH, Lu S, Guo YL. Redox-induced apoptosis of human oocytes in resting follicles in vitro. *J Soc Gynecol Investig*. 2006;13(6):451-458.
12. Velthut A, Zilmer M, Zilmer K, Kaart T, Karro H, Salumets A. Elevated blood plasma antioxidant status is favourable for achieving IVF/ICSI pregnancy. *Reprod Biomed Online*. 2013;26(4): 345-352.
13. Showell MG, Brown J, Clarke J, Hart RJ. Antioxidants for female subfertility. *Cochrane Database of Systematic Reviews*. 2013;(8): CD007807. DOI: 10.1002/14651858.CD007807.pub2.
14. Huang B, Li Z, Ai J, et al. Antioxidant capacity of follicular fluid from patients undergoing in vitro fertilization. *Int J Clin Exp Pathol*. 2014;7(5):2273-2282.
15. Oyawoye O, Abdel Gadir A, Garner A, Constantinovici N, Perrett C, Hardiman P. Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: relationship to outcome. *Hum Reprod*. 2003;18(11):2270-2274.
16. Appasamy M, Jauniaux E, Serhal P, Al-Qahtani A, Groome NP, Muttukrishna S. Evaluation of the relationship between follicular fluid oxidative stress, ovarian hormones, and response to gonadotropin stimulation. *Fertil Steril*. 2008;89(4):912-921.
17. Fadhlaoui A, Bouquet de la Joliniere J, Feki A. Endometriosis and infertility: how and when to treat? *Front Surg*. 2014;1:24.
18. Lessey BA, Lebovic DI, Taylor RN. Eutopic endometrium in women with endometriosis: ground zero for the study of implantation defects. *Semin Reprod Med*. 2013;31(2):109-124.
19. Gupta S, Goldberg JM, Aziz N, Goldberg E, Krajcir N, Agarwal A. Pathogenic mechanisms in endometriosis-associated infertility. *Fertil Steril*. 2008;90(2):247-257.
20. Khawaja UB, Khawaja AA, Gowani SA, et al. Frequency of endometriosis among infertile women and association of clinical signs and symptoms with the laparoscopic staging of endometriosis. *J Pak Med Assoc*. 2009;59(1):30-34.
21. Murphy AA, Santanam N, Parthasarathy S. Endometriosis: a disease of oxidative stress? *Semin Reprod Endocrinol*. 1998;16(4): 263-273.
22. Shanti A, Santanam N, Morales AJ, Parthasarathy S, Murphy AA. Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. *Fertil Steril*. 1999;71(6): 1115-1118.
23. Santanam N, Song M, Rong R, Murphy AA, Parthasarathy S. Atherosclerosis, oxidation and endometriosis. *Free Radic Res*. 2002;36(12):1315-1321.
24. Klebanoff SJ.Myeloperoxidase: friend and foe. *J Leukoc Biol*. 2005;77(5):598-625.
25. Suzuki K, Muso E, Nauseef WM. Contribution of peroxidases in host-defense, diseases and cellular functions. *Jpn J Infect Dis*. 2004;57(5):S1-S2.
26. Song M, Santanam N. Increased myeloperoxidase and lipid peroxide-modified protein in gynecological malignancies. *Antioxid Redox Signal*. 2001;3(6):1139-1146.
27. Chun SY, Daphna-Iken D, Calman D, Tsafriri A. Severe leukocyte depletion does not affect follicular rupture in the rat. *Biol Reprod*. 1993;48(4):905-909.
28. Chiang K, Parthasarathy S, Santanam N. Estrogen, neutrophils and oxidation. *Life Sci*. 2004;75(20):2425-2438.
29. Santanam N, Shern-Brewer R, McClatchey R, et al. Estradiol as an antioxidant: incompatible with its physiological concentrations and function. *J Lipid Res*. 1998;39(11):2111-2118.
30. ASRM. Revised American society for reproductive medicine classification of endometriosis: 1996. *Fertil Steril*. 1997;67(5): 817-821.
31. Pellicer A, Albert C, Garrido N, Navarro J, Remohi J, Simon C. The pathophysiology of endometriosis-associated infertility: follicular environment and embryo quality. *J Reprod Fertil Suppl*. 2000;55:109-119.
32. Goud PT, Goud AP, Joshi N, Puscheck E, Diamond MP, Abu-Soud HM. Dynamics of nitric oxide, altered follicular microenvironment, and oocyte quality in women with endometriosis. *Fertil Steril*. 2014;102(1):151-159. e5.
33. Augoulea A, Mastorakos G, Lambrinoudaki I, Christodoulakos G, Creatsas G. The role of the oxidative-stress in the endometriosis-related infertility. *Gynecol Endocrinol*. 2009; 25(2):75-81.
34. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. *Curr Opin Obstet Gynecol*. 2006;18(3): 325-332.
35. Tamura H, Takasaki A, Miwa I, et al. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res*. 2008;44(3): 280-287.
36. Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. *Antioxid Redox Signal*. 2008;10(8):1375-1403.
37. Carbone MC, Tatone C, Delle Monache S, et al. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. *Mol Hum Reprod*. 2003;9(11): 639-643.
38. Alpay Z, Saed GM, Diamond MP. Female infertility and free radicals: potential role in adhesions and endometriosis. *J Soc Gynecol Investig*. 2006;13(6):390-398.
39. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. *Reprod Biol Endocrinol*. 2012;10:49.
40. Younis A, Clower C, Nelsen D, et al. The relationship between pregnancy and oxidative stress markers on patients undergoing ovarian stimulations. *J Assist Reprod Genet*. 2012;29(10): 1083-1089.

41. Gupta S, Ghulmiyyah J, Sharma R, Halabi J, Agarwal A. Power of proteomics in linking oxidative stress and female infertility. *BioMed Res Int*. 2014;2014:916212.
42. Carvalho LF, Samadder AN, Agarwal A, Fernandes LF, Abrao MS. Oxidative stress biomarkers in patients with endometriosis: systematic review. *Arch Gynecol Obstet*. 2012;286(4):1033-1040.
43. Santanam N, Murphy AA, Parthasarathy S. Macrophages, oxidation, and endometriosis. *Ann N Y Acad Sci*. 2002;955:183-198; discussion 119-200, 396-406.
44. Murphy AA, Santanam N, Morales AJ, Parthasarathy S. Lysophosphatidyl choline, a chemotactic factor for monocytes/t- lymphocytes is elevated in endometriosis. *J Clin Endocrinol Metab*. 1998;83(6):2110-2113.
45. Santanam N, Kavtaradze N, Murphy A, Dominguez C, Parthasarathy S. Antioxidant supplementation reduces endometriosis-related pelvic pain in humans. *Transl Res*. 2013;161(3):189-195.
46. Ray K, Fahrmann J, Mitchell B, et al. Oxidation-sensitive nociception involved in endometriosis-associated pain. *Pain*. 2015; 156(3):528-539.
47. Santulli P, Chouzenoux S, Fiorese M, et al. Protein oxidative stress markers in peritoneal fluids of women with deep infiltrating endometriosis are increased. *Hum Reprod*. 2015;30(1):49-60.
48. Polak G, Wertel I, Barczynski B, Kwasniewski W, Bednarek W, Kotarski J. Increased levels of oxidative stress markers in the peritoneal fluid of women with endometriosis. *Eur J Obstet Gynecol Reprod Biol*. 2013;168(2):187-190.
49. Defrere S, Lousse JC, Gonzalez-Ramos R, Colette S, Donnez J, Van Langendonckt A. Potential involvement of iron in the pathogenesis of peritoneal endometriosis. *Mol Hum Reprod*. 2008;14(7):377-385.
50. Tiboni GM, Bucciarelli T, Giampietro F, Sulpizio M, Di Ilio C. Influence of cigarette smoking on vitamin e, vitamin a, beta-carotene and lycopene concentrations in human pre-ovulatory follicular fluid. *Int J Immunopathol Pharmacol*. 2004;17(3):389-393.
51. Campos Petean C, Ferriani RA, dos Reis RM, de Moura MD, Jordao AA Jr, Navarro PA. Lipid peroxidation and vitamin e in serum and follicular fluid of infertile women with peritoneal endometriosis submitted to controlled ovarian hyperstimulation: a pilot study. *Fertil Steril*. 2008;90(6):2080-2085.
52. Prieto L, Quesada JF, Cambero O, et al. Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis. *Fertil Steril*. 2012;98(1):126-130.
53. Ozkaya MO, Naziroglu M. Multivitamin and mineral supplementation modulates oxidative stress and antioxidant vitamin levels in serum and follicular fluid of women undergoing in vitro fertilization. *Fertil Steril*. 2010;94(6):2465-2466.
54. Kashanian M, Lakeh MM, Ghasemi A, Noori S. Evaluation of the effect of vitamin E on pelvic pain reduction in women suffering from primary dysmenorrhea. *J Reprod Med*. 2013;58(1-2):34-38.
55. Hovdenak N, Haram K. Influence of mineral and vitamin supplements on pregnancy outcome. *Eur J Obstet Gynecol Reprod Biol*. 2012;164(2):127-132.
56. Hovdenak N, Haram K. Authors' response. *Eur J Obstet Gynecol Reprod Biol*. 2013;171(1):193.
57. Lazarevic-Pasti T, Leskovac A, Vasic V. Myeloperoxidase inhibitors as potential drugs. *Curr Drug Metab*. 2015;16(3):168-190.