Female Infertility and Antioxidants

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Abstract: *Aim*: Many studies have implicated oxidative stress in the pathogenesis of infertility causing diseases of the female reproductive tract. The aim of this study was to review the current literature on the effects of antioxidant therapy and to elucidate whether antioxidant supplementation is useful to prevent and/or treat infertility and poor pregnancy outcomes related to various obstetric and gynecologic conditions.

Methods: Review of recent publications through Pubmed and the Cochrane data base.

Results: Antioxidant supplementation has been shown to improve insulin sensitivity and restore redox balance in patients with PCOS. Supplementation with RU486, Curcuma longa, melatonin, caffeic acid phenethyl ester (CAPE) and catechins may induce remission and halt disease progression in endometriosis. Selenium therapy may improve pregnancy rates in unexplained infertility. Currently there is no evidence to substantiate the use of antioxidants to prevent or treat preeclampsia. Up to 50-60% of recurrent pregnancy loss may be attributable to oxidative stress. Observational studies have confirmed a link between antioxidant-poor diet and recurrent pregnancy loss.

Conclusion: Although many advances are being made in the field of antioxidants therapy, there is a need for further investigation using randomized controlled trials within a larger population to determine the efficacy and safety of antioxidant supplementation.

Keywords: Oxidative stress, antioxidants, polycystic ovarian syndrome (PCOS), endometriosis, unexplained infertility, preeclampsia, spontaneous abortion.

INTRODUCTION

Reactive oxygen species (ROS) can modulate cellular functions, and oxidative stress (OS) can impair the intracellular milieu, resulting in diseased cells or endangered cell survival. Reproductive cells and tissues remain stable when free radical production and the scavenging antioxidants remain in balance. The role of ROS in various diseases of the female reproductive tract has been investigated. ROS can affect a variety of physiological functions in the reproductive tract, and excessive levels can result in precipitous pathologies affecting female reproduction. The oxidant status can influence early embryo development by modifying the key transcription factors, hence modifying gene expression.

The review will focus on ROS homeostasis and generation of OS in the female reproductive processes. Our review elucidates the role of ROS in physiological processes such as folliculogenesis, oocyte maturation, endometrial cycle, luteolysis, implantation, and embryogenesis and the role of antioxidants in various reproductive pathologies. This review encapsulates the role of OS, which is becoming increasingly important as new evidence of its role in conditions such as polycystic ovarian disease and abortions is discovered. The review highlights how OS modulates natural and assisted fertility and the importance of antioxidant strategies to intercept OS to overcome its adverse effects.

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WHAT IS OXIDATIVE STRESS?

Oxidative stress arises from an imbalance between pro-oxidant molecules generated from aerobic metabolism and protective antioxidants. OS influences the entire reproductive lifespan of a woman. Reactive oxygen species may act as key signalling molecules in physiological processes but at excess, uncontrolled levels they may also mediate pathological processes involving the female reproductive tract. There is a body of literature providing clinical evidence that substantiates the link between OS and female infertility.

Pro-Oxidants

Under physiological conditions, biomolecules are comprised of stable bonds formed by paired electrons. Weakened, disrupted bonds allow for the generation of free radicals- unstable and highly reactive species with one or more unpaired electrons. They gain stability by acquiring electrons from nearby nucleic acids, lipids, proteins, and carbohydrates, initiating a cascade of chain reactions that may result in cellular damage and disease [1-4].

Reactive oxygen species are formed endogenously during aerobic metabolism and as a result of various metabolic pathways of oocytes and embryos or as part of the body's defense mechanisms. ROS also can arise from exogenous sources, such as alcohol, tobacco, and various environmental

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pollutants. ROS include hydroxyl radicals, superoxide anion, hydrogen peroxide, and nitric oxide (NO) [5]. Several biomarkers indicative of redox status, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), lipid peroxides, and nitric oxide, have been identified within the ovary, endometrium, fallopian tubes, embryo, placenta, and the peritoneal fluid of women. At controlled levels, free radicals are capable of exerting physiological effects and mediating processes such as tissue remodelling, hormone signalling, oocyte maturation, folliculogenesis, tubal function, ovarian steroidogenesis, cyclical endometrial changes, and germ cell function [6, 7]. However, when ROS increase to pathological levels they are capable of inflicting significant damage to cell structures.

Antioxidants

Under normal conditions, antioxidants act to oppose ROS production, scavenge existing free radicals, and promote the repair of ROS-induced damage to cell structures [8]. Non-enzymatic antioxidants include vitamin C, vitamin E, selenium, zinc, beta carotene, carotene, taurine, hypotaurine, cysteamine, and glutathione. Enzymatic antioxidants include SOD, catalase, GSH-Px, glutaredoxin and glutathione reductase [5]. The degree of antioxidant defense present is often expressed as total antioxidant capacity (TAC) [6].

A disruption in the delicate balance between antioxidants and pro-oxidant molecules can result in OS. OS arises when the generation of reactive oxygen species and other radical species overrides the scavenging capacity by antioxidants, either due to the excessive production of ROS or an inadequate availability of antioxidants. Thus, oral antioxidant supplementation may serve to prevent and alleviate OS and its contribution to the pathogenesis of obstetrical disease such as preeclampsia and recurrent pregnancy loss and gynecological disorders such as polycystic ovarian syndrome (PCOS) and endometriosis.

OS IN THE FEMALE REPRODUCTIVE TRACT – PHYSIOLOGICAL ROLE OF OS

Follicle

The expression of various markers of OS has been demonstrated in normally cycling ovaries [9, 10]. The follicular fluid microenvironment contains leukocytes, macrophages, and cytokines, all of which are known sources of ROS. ROS within the follicular fluid plays a role in modulating oocyte maturation, folliculogenesis, ovarian steroidogenesis, and luteolysis [11]. Follicular development involves the progression of small primordial follicles into large pre-ovulatory follicles. Studies have implicated the nitric oxide radical in the follicular growth and programmed follicular cell death that occur during folliculogenesis [12, 13]. Moderate OS levels are required for ovulation. The final stages of oocyte maturation are associated with fluctuations in cytokines, prostaglandins, proteolytic enzymes, nitric oxide, and steroids, which increase the level of ROS, influencing ovarian blood flow and eventually facilitating follicle rupture [14]. A degree of oxidative enzyme activity is exhibited by thecal cells, granulosa lutein cells, and hilus cells, illustrating the role of OS in ovarian steroidogenesis [11].

ROS is controlled and kept at physiological levels within the ovary by various antioxidant systems, including catalase, vitamin E, glutathione and carotenoids [4]. SOD, a metal-containing enzymatic antioxidant that catalyzes the decomposition of superoxide into hydrogen peroxide and oxygen, has been characterized in the theca interna cells in the antral follicles. Therefore, the theca interna cells may protect the oocyte from excess ROS during its maturation [15]. Another antioxidant factor important for healthy follicle development is transferrin, an iron-chelating glycoprotein that suppresses ROS generation [16]. Vitamin C also is known to have a protective effect within the follicle as vitamin C deficiency has been reported to result in ovarian atrophy, extensive follicular atresia, and premature resumption of meiosis [15].

The overall ROS scavenging ability of antioxidants within the follicular fluid microenvironment may diminish with reproductive aging. Carbone *et al.* demonstrated decreased levels of follicular fluid catalase and SOD in older women, whose oocytes were seen to exhibit lower fertilization rates and decreased blastocyst development compared with oocytes of younger women [17]. Therefore, the redox status of the follicle is closely related to oocyte quality and fertilization capacity.

Endometrium

The relationship between OS and cyclical changes in the endometrium is well-established. OS-promoting alterations in ROS and SOD levels have been demonstrated just prior to menstruation, during the late-secretory phase [18]. Estrogen and progesterone withdrawal in endometrial cells *in vitro* has been associated with a decrease in SOD activity, resulting in the unopposed activity of ROS [18]. Elevated lipid peroxide and decreased SOD in the endometrium during the late-secretory phase may modulate endometrial breakdown, leading to menstruation. NO is known to regulate the endometrial microvasculature and is produced by endothelial NO synthase (NOS), which is distributed in the glandular surface epithelial cells of the endometrium [19]. NO is thought to mediate endometrial decidualization and menstruation as endothelial NOS mRNA expression has been detected in the mid-secretory and late-secretory phase. Endothelial NOS is also implicated in the changes seen in the endometrium in preparation for implantation [20]. ROS may mediate the physiological processes of shedding and implantation by its activation of nuclear factor K B within the endometrium, leading to increased cyclooxygenase-2 mRNA and prostaglandin F2α synthesis [18].

Infertility

Approximately 1.3 million American couples receive medical advice or treatment for infertility every year [21]. Infertility is a disease defined as the inability to conceive following 12 or more months of unprotected sex [22]. In general, an estimated 84% of couples conceive after 1 year of intercourse, and 92% of the couples conceive after 2 years

[23]. A primary diagnosis of male factor infertility is made in 30% of infertile couples. High levels of ROS biomarkers have been detected in semen samples of 25-40% of infertile men [5]. Although ROS have a physiological role in normal sperm function, mediating the acrosome reaction, hyperactivation, motility, and capacitation of spermatozoa, excessive levels of ROS may arise from immotile or morphologically abnormal spermatozoa and leukocytes. Spermatozoa lack the necessary cytoplasmic antioxidant enzymes and are vulnerable to OS-induced DNA damage and apoptosis [5, 24]. Substantial evidence exists that implicates OS in many causes of male infertility. Oral antioxidant supplementation has become standard practice for male infertility [5].

Combined female and male factor infertility is responsible for 20%–30% of cases. If the results of a standard infertility examination are normal, a diagnosis of unexplained or idiopathic infertility is assigned [25]. OS has a well-established role in pathogenesis of unexplained infertility, which is seen to affect 15% of couples [25]. Although the frequency and origin of different forms of infertility varies, 40%–50% of the etiology of infertility studied is due to female causes [26].

OS induces infertility in women through a variety of mechanisms. Excess ROS in the follicle may overwhelm follicular fluid antioxidant defense and directly damage oocytes. The DNA of oocytes and spermatozoa may be damaged, leading to defective fertilization when the peritoneal cavity microenvironment is plagued with severe OS. Even when fertilization is achieved, OS-induced apoptosis may result in embryo fragmentation, implantation failure, abortion, impaired placentation, and congenital abnormalities [27]. Excess ROS may hinder the endometrium, which normally functions to support the embryo and its development [28]. OS may induce luteal regression and insufficient luteal hormonal support for the continuation of a pregnancy [8]. The association of OS with various gynecologic and obstetric conditions related to infertility suggests a potential role for oral antioxidant supplementation (Fig. **1**). Additional research is needed to determine whether such supplementation can ensure successful fertilization and pregnancy by controlling the OS experienced by patients with endometriosis, PCOS, unexplained infertility, preeclampsia, and recurrent pregnancy loss.

THE USE OF ANTIOXIDANTS IN TREATMENT OF GYNECOLOGICAL CONDITIONS

Polycystic Ovarian Syndrome

PCOS is an anovulatory cause of infertility affecting 6-10% of premenopausal women [29-32]. PCOS often can be characterized by hyperandrogenism, hirsutism, and oligomenorrhea or amenorrhea. Metabolic, endocrinologic, and cardiovascular disorders may also coexist. Oxidative stress has been implicated in mediating the insulin resistance and increase in androgens seen in these patients [33].

A recent study by Kuscu *et al.* demonstrated increased MDA levels and upregulated SOD activity in patients with PCOS compared to controls. MDA levels were highest in patients who exhibited insulin resistance [34]. Insulin resistance and hyperglycemia are established as factors that increase oxidative stress. Fulghesu *et al*. evaluated the effect of N-acetyl-cysteine (NAC), known to replenish stores of the anti-oxidant glutathione, on insulin secretion and peripheral insulin resistance in subjects with PCOS. Patients were treated for 5-6 weeks with a 1.8g oral NAC per day. Massively obese patients were given a higher dose of 3g per day. NAC treatment was found to improve parameters of glucose control in hyperinsulinemic patients. Insulin levels were reduced, with increased peripheral insulin sensitivity. Therefore, the anti-oxidant effects of NAC may serve as a therapeutic strategy to improve the level of circulating insulin and insulin sensitivity in PCOS patients with hyperinsulinemia [35].

Non-obese PCOS patients without insulin resistance also have been reported to have elevated total oxidant and anti-

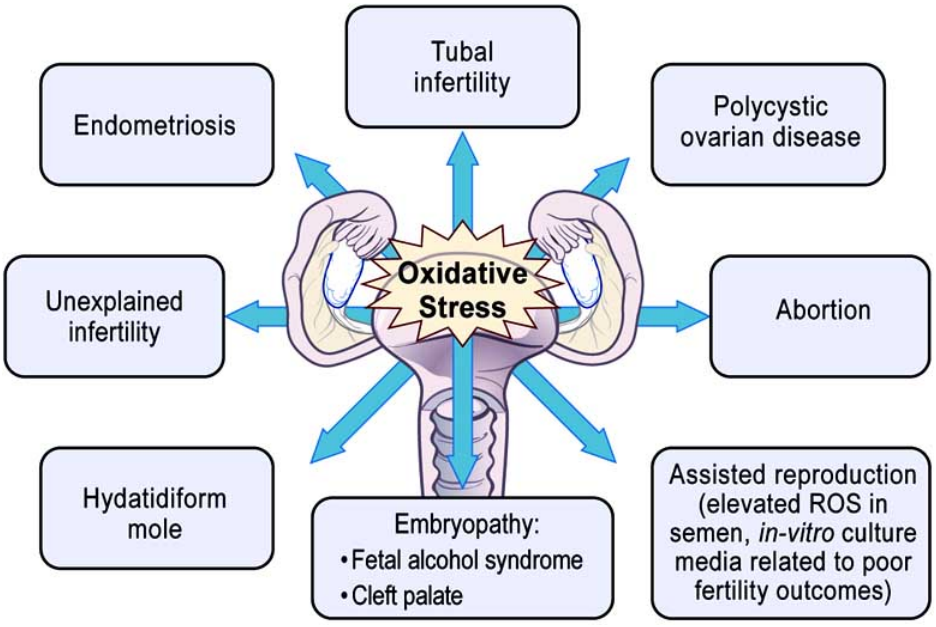

Fig. (1). The role of oxidative stress in obstetric and gynecologic conditions that contribute to infertility.

oxidant status [36]. Verit *et al.* demonstrated that total antioxidant status in these types of PCOS patients was correlated with raised luteinizing hormone levels and free androgen and dehydroepiandrosterone (DHEAS) levels [36]. Yilmaz *et al.* studied the effects of 12 weeks of treatment with oral hypoglycemic agents on OS in lean patients with PCOS [37]. Before treatment, PCOS patients exhibited OS with significantly raised serum MDA and homocysteine and significantly decreased serum TAS. PCOS patients treated with rosiglitazone showed an increase in TAS and a decrease in MDA levels, compared with a metformin-administered patient group in which these parameters did not change [37]. Therefore, rosiglitazone may be useful in combating OS in hyperinsulinemic PCOS patients.

Zhang *et al.* used methods of chemicalorimetry to measure and compare levels of serum lipid peroxides (LPO), MDA, SOD, vitamin E, and vitamin C in patients with PCOS and normal women [38]. Levels of serum LPO and MDA in patients with PCOS were significantly higher than those found in normal women. Levels of vitamin E, vitamin C, and SOD were lower in patients with PCOS than in the control group. After 3 months of therapy with oral ethinylestradiol and cyproterone acetate tablets (Diane-35®, Merck, Whitehouse Station, N.J.), an anti-androgenic oral contraceptive often used to treat hirsutism associated with PCOS, MDA and LPO levels decreased, while vitamin E, vitamin C, and SOD levels increased in patients with PCOS [38]. Therefore, this therapy may alleviate the symptoms of PCOS through both its anti-androgenic and anti-oxidant actions.

Endometriosis

Severe cases of endometriosis are thought to render a woman infertile by mechanical hindrance of the sperm-egg union by adhesions, endometriomata, and pelvic anatomy malformations. However, in women with mild-to-moderate forms of endometriosis and no pelvic anatomical distortion, the mechanism by which their fertility is reduced is poorly understood.

ROS production may be amplified in the setting of endometriosis due to menstrual reflux, which subjects the peritoneal cavity to pro-inflammatory hemoglobin and heme molecules released from transplanted erythrocyte debris. Peritoneal fluid containing ROS-generating iron, macrophages, and environmental contaminants such as polychlorinated biphenyls may disrupt the prooxidant/antioxidant balance, resulting in increased proliferation of tissue and adhesions [39-42]. ROS are thought to promote the growth and adhesion of endometrial cells in the peritoneal cavity, contributing to the pelvic anatomical distortion known to cause infertility in endometriosis [43]. OS may have a role in promoting angiogenesis in ectopic endometrial implants by increasing vascular endothelial growth factor (VEGF) production [44]. This effect is partly mediated by glycodelin, a glycoprotein whose expression is stimulated by OS. Glycodelin may act as an autocrine factor within ectopic endometrial tissue by augmenting VEGF expression [44].

Altered molecular genetic pathways may contribute to the effects of OS in the pathogenesis of endometriosis and endometriosis-associated infertility. Differential gene expression of ectopic and normal endometrial tissue has been identified, including differential gene expression of glutathione-S-transferase, an enzyme in the metabolism of the potent antioxidant glutathione [45]. This suggests that altered molecular genetic pathways may determine the development of OS and its ability to induce cellular proliferation and angiogenesis in women with endometriosis.

Peritoneal fluid of women with endometriosis has been reported to exhibit increased ROS generation by activated peritoneal macrophages [46]. Increased macrophage activity is accompanied by the release of cytokines and other immune mediators such as NO. NO is a pro-inflammatory free radical that exerts deleterious effects on fertility by increasing the amount of OS in the peritoneal fluid, an environment that hosts the processes of ovulation, gamete transportation, sperm-oocyte interaction, fertilization, and early embryonic development [2, 47, 48]. However, the results of further studies with large patient numbers failed to confirm an antioxidant or oxidant imbalance as ROS levels in peritoneal fluid of patients with endometriosis were not reported to be significantly higher than controls [49, 50].

After adjusting for confounding factors such as age, BMI, gravidity, serum vitamin E, and serum lipid levels, Jackson *et al.* reported a weak relationship of elevated levels of thiobarbituric acid reactive substances (TBARS), an overall measure of OS, in women with endometriosis [51]. Increased NO production and lipid peroxidation have been reported in the endometrium of women with endometriosis [2, 52]. However, several studies failed to find significant differences in the peritoneal fluid levels of NO, lipid peroxide, and ROS in women with and without endometriosis-associated infertility.

The failure of some studies to confirm alterations in peritoneal fluid NO, lipid peroxide and antioxidant status in women with endometriosis may be explained by the fact that OS may occur locally, without affecting total peritoneal fluid ROS concentration. Also, markers of OS may be transient and not detected at the time endometriosis is diagnosed.

An imbalance between ROS and antioxidant levels may play an important role in the pathogenesis of endometriosis-associated infertility. Increased concentrations of oviductal fluid ROS may adversely affect oocyte and spermatozoa viability and the process of fertilization and embryo implantation. Also, pro-inflammatory macrophages and activated neutrophils in the oviductal fluid may significantly amplify ROS production by endometriotic foci [43]. Increased ROS production may inflict oxidative damage to the sperm plasma and acrosomal membranes, resulting in a loss of motility and decreased spermatozoal ability to bind and penetrate the oocyte. The various possible consequences of OS-induced DNA damage include failed fertilization, reduced embryo quality, pregnancy failure, and spontaneous abortion.

Modest levels of OS have been shown to induce the proliferation of endometrial stromal cells *in vitro*, which has been shown to be inhibited by antioxidants [53]. Several studies have shown that the peritoneal fluid of women with endometriosis-associated infertility have insufficient antioxi-

dant defense, with lower total antioxidant capacity (TAC) and significantly reduced SOD levels [2, 47, 54].

An early study used a simple rabbit model to demonstrate the beneficial effect of antioxidant therapy in halting progression of the disease [55]. SOD and catalase were instilled in the rabbit peritoneal cavity and were shown to significantly reduce the formation of intraperitoneal adhesions at endometriosis sites by blocking the toxic effects of the superoxide anion and hydrogen peroxide radicals [55]. More recently, RU486- a potent antiprogestational agent with antioxidant activity, has been shown to decrease the proliferation of epithelial and stromal cells in endometriosis [56].

Another drug being investigated for its potential use in the treatment of endometriosis-associated infertility is pentoxifylline, a 3',5'-nucleotide phosphodiesterase inhibitor. Pentoxifylline has potent immunomodulatory properties and has been shown to significantly reduce the embryotoxic effects of hydrogen peroxide [57]. Zhang *et al.* conducted a recent randomized control trial in which pentoxifylline treatment failed to demonstrate significant reduction in endometriosis-associated symptoms such as pain. Furthermore, there was no evidence of an increase in the clinical pregnancy rates in the pentoxifylline group compared with placebo [58]. Currently, there is not enough evidence to warrant the use of pentoxifylline in the management of premenopausal women with endometriosis-associated pain and infertility.

Curcumin is a polyphenol derived from turmeric (Curcuma longa) with antioxidant, anti-inflammatory, and antiproliferative properties. This compound has been shown to have an anti-endometriotic effect by targeting aberrant matrix remodelling in a mouse model. Matrix metalloproteinase-9 (MMP-9) has been shown to correlate with severity of endometriosis. In randomized controlled trials, curcumin treatment was seen to reverse MMP-9 activity in endometriotic implants near to control values. Furthermore, the anti-inflammatory property of curcumin was evidenced by the fact that the attenuation of MMP-9 was accompanied by a reduction in cytokine release. Decreased expression of tumor necrosis factor alpha (TNF-α) was demonstrated during regression and healing of endometriotic lesions within the mouse model. Pretreatment of endometriotic lesions with curcumin was shown to prevent lipid peroxidation and protein oxidation within the experimental tissue, attesting to its therapeutic potential to provide antioxidant defense against OS-mediated infertility in endometriosis [59].

MMP-9 also was identified as a therapeutic target in the treatment of OS-mediated endometriosis in another study evaluating the effectiveness of melatonin in treating experimental endometriosis in a mouse model [60]. Melatonin is a major secretory product of the pineal gland with anti-oxidant properties. Melatonin was shown to arrest lipid peroxidation and protein oxidation, while downregulating MMP-9 activity and expression in a time- and dose-dependent manner. Tissue inhibitors of metalloproteinase (TIMP)-1 were found to be elevated. Regression of peritoneal endometriotic lesions was seen to accompany the alteration in metalloproteinase expression [60]. Guney *et al.* confirmed these findings in that treatment with melatonin was also shown to cause regression and atrophy of endometriotic lesions in rats [61]. Endometrial lesions treated with melatonin demonstrated lower MDA levels and significantly increased SOD and catalase activity [61], corroborating the usefulness of this hormone in neutralizing OS.

Guney *et al.* conducted another study that evaluated the effects of antioxidant and anti-inflammatory caffeic acid phenethyl ester (CAPE) on experimental endometriosis in a rat model, and the levels of peritoneal SOD and catalase activity, and MDA [62]. Treatment with CAPE was seen to decrease peritoneal MDA levels and antioxidant enzyme activity in rats. Endometriotic lesions treated with CAPE were histologically demonstrated to undergo atrophy and regression, compared with untreated controls [62].

As previously mentioned, OS stimulates factors that increase VEGF expression and promote angiogenesis of endometriotic lesions. The green tea-containing compound, epigallocatechin gallate (EGCG) has been evaluated as a treatment for endometriosis due to its powerful antioxidant and anti-angiogenic properties. Xu *et al.* conducted a study in which eutopic endometrium transplanted subcutaneously into a mouse model was used to compare the effects of EGCG treatment on endometriotic implants to the effects seen with vitamin E treatment or untreated controls [63]. Lesions treated with EGCG exhibited significantly down-regulated VEGF-A mRNA. While the control endometrial implants exhibited newly developed blood vessels with proliferating glandular epithelium, the EGCG group demonstrated significantly smaller endometriotic lesions and smaller and more eccentrically distributed glandular epithelium. Despite its widely studied benefits as a potent antioxidant in the treatment of female infertility, vitamin E was not shown to control or decrease angiogenesis compared with baseline controls [63]. As EGCG was shown to significantly inhibit the development of experimental endometriotic lesions in a mouse model, its effectiveness as an oral supplement in female patients to limit progression and induce remission of their endometriosis should be further investigated.

A recent study by Mier-Cabrera *et al.* utilized questionnaires to compare the dietary intake of antioxidant vitamins and minerals by women with and without endometriosis. Relative to healthy control subjects, women with endometriosis were found to have a significantly lower intake of vitamins A, C, E, zinc, and copper. However, intake of selenium was not significantly different between the two groups studied [64]. A randomized control trial in which the effect of antioxidant supplementation was studied revealed a significant increase in the concentrations of serum retinol, alpha-tocopherol, leukocytes, and plasma ascorbate after 2 months of treatment. Antioxidant supplementation was also observed to increase the activity of antioxidant enzymes (SOD and GPx), while decreasing markers of oxidative stress such as malondialdehyde and lipid peroxides [64]. These effects were not observed in the control group, suggesting a role for antioxidant supplementation in decreasing the levels of oxidative stress afflicting patients with endometriosis-associated infertility.

Unexplained Infertility

Elevated levels of ROS that disturb the redox balance within the body may be the root cause of infertility in women who do not have any other obvious cause. The ovum released from the ovary, the zygote or embryo, and spermatozoa are very vulnerable to damage inflicted by OS [8]. Wang *et al.* compared ROS levels in peritoneal fluid between women undergoing laparoscopy for infertility evaluation and fertile women undergoing tubal ligation and demonstrated that higher levels of ROS exist in the peritoneal fluid aspirated from patients with unexplained infertility compared to that measured within the peritoneal fluid of fertile women [65]. Polak *et al.* analyzed peritoneal fluid samples obtained at laparoscopy and found that women with unexplained infertility had increased MDA concentrations and TAS, implicating the role of redox imbalance in its pathogenesis [66].

Elevated ROS levels in patients with unexplained infertility implies exhausted antioxidant defense, resulting in the inability to scavenge ROS and neutralize their toxic effects [65]. This was substantiated by the results of a study in which antioxidant concentrations were seen to be significantly lower in the peritoneal fluid of patients with unexplained infertility compared with antioxidant levels in fertile patients [47]. The link between decreased antioxidant status and lowered fecundity suggests a potential use for antioxidant supplementation to treat the high levels of ROS seen in patients with idiopathic infertility.

Howard *et al.* described a group of patients with a history of unexplained infertility and abnormal red blood cell magnesium (RBC-Mg) levels. These patients' RBC-Mg levels were unresponsive to oral magnesium supplementation and shown to be associated with deficient red blood cell glutathione peroxidase (RBC-GSH-Px) activity [67]. Treatment with 200 μg of oral selenium as selenomethionine and oral magnesium for a period of 2 months was shown to normalize RBC-Mg and RBC-GPx levels. This correlated with 100% of previously infertile women in the treatment group successfully achieving clinical pregnancies within 8 months of normalizing their RBC-Mg [67].

Badawy *et al.* conducted a prospective, randomized, double-blind, controlled trial comparing the effects of using clomiphene citrate combined with glutathione-replenishing N-acetyl cysteine versus clomiphene citrate alone in inducing ovulation in women with unexplained infertility [68]. Despite the proposed benefits of strengthening the antioxidant defense of women with unexplained infertility, no difference was seen in the rates of successful pregnancy between both groups [69]. Therefore, the use of N-acetylcysteine to improve outcome during ovulation induction in women with unexplained infertility is not justified.

THE USE OF ANTIOXIDANTS TO PROMOTE HEALTHY PREGNANCY

Preeclampsia

Preeclampsia complicates 5% of all pregnancies and 11% of first pregnancies and is associated with high maternal and fetal morbidity and mortality [70]. Although the exact mechanism by which preeclampsia develops is not known, there is increasing evidence that corroborates the role of OS in its etiopathogenesis. Reduced antioxidant response [71, 72], reduced levels of antioxidant nutrients [73], and increased lipid peroxidation [72, 73] have been observed in patients with preeclampsia.

Preeclampsia is associated with defective placentation, in which the dislodging of extravillous trophoblast plugs in the maternal spiral arteries leads to the onset of blood flow into the intervillous space, causing an oxidative burst that generates ROS. Abnormal placentation leads to reduced fetoplacental circulation secondary to decreased NO-mediated vascular relaxation. Placental ischemia and hypoxia leads to ischemic reperfusion injury to the placenta in which there is release of cytokines and prostaglandins, which triggers the endothelial cell dysfunction seen in preeclampsia. Hypoxia and reperfusion injury leads to increased expression of xanthine oxidase and NADPH oxidase and increased generation of SOD.

Poorly perfused placental tissue, abnormal lipid metabolism, and resultant lipid peroxidation and redox imbalance are established factors that promote the development of preeclampsia. Numerous studies have demonstrated insufficient antioxidant defenses and overwhelming degrees of ROS in women with preeclampsia [73]. Oxidative stress has been evaluated by measuring elevated lipid peroxidation in patients with preeclampsia, as well as elevated protein carbonyl concentrations, plasma MDA levels, and SOD activity. Placental oxidative stress has been proposed as a promoter of lipid peroxidation and endothelial cell dysfunction [74-78]. Increased lipid peroxidation may result in the consumption of antioxidants and depletion of vitamin A, C, and E, erythrocyte thiol, glutathione, and SOD.

There currently is no accepted method of preventing the development of preeclampsia. Some trials have evaluated the use of supplementation with antioxidants vitamin C and vitamin E for prevention. Early intervention at 16–22 weeks of pregnancy with supplementation of vitamin E and C resulted in significant reduction of preeclampsia in the treatment group [79]. However, supplementation in women with established preeclampsia did not yield any benefit [80]. A recent randomized trial failed to demonstrate any beneficial effects of vitamin C and E supplementation in preventing preeclampsia [81]. Poston *et al.* investigated the use of vitamin C and E supplementation to reduce OS, limit the injury of endothelial cells, and prevent or reduce disease severity of preeclampsia. In this placebo-controlled trial in a diverse group of high-risk women, antioxidant supplementation was not associated with a reduction in the preeclampsia risk. Instead, treatment was associated with a significantly higher incidence of complications, including low birth weight, gestational hypertension, and increased need for intravenous antihypertensive and magnesium sulphate therapy [82].

Although a causal relationship between OS and preeclampsia is well-established, trials have failed to detect any risk reduction for preeclampsia with antioxidant supplementation. Trials powered to detect any smaller, more subtle benefits of antioxidant therapy in preventing placental pathology must be conducted before the routine use of anti-

oxidant vitamins by nulliparous, pregnant women can be recommended.

Recurrent Pregnancy Loss

Abnormal placentation has been implicated in the pathogenesis of both preeclampsia and miscarriage [71]. Recurrent pregnancy loss is a condition in which three or more consecutive, spontaneous abortions occur [83]. It affects 0.5% to 3% of women of reproductive age. Recurrent pregnancy loss has been associated with several factors, including maternal age, genetic factors, endocrinologic factors, anatomic problems, and environmental toxins [83]. Moreover, the etiology of recurrent pregnancy loss may be linked to chromosomal abnormalities, uterine anatomic anomalies, immunologic disorders such as antiphospholipid antibody syndrome, clotting disorders, and sperm DNA fragmentation [6]. However, 50%-60% of recurrent pregnancy losses are considered idiopathic [84]. Oxidative stress may be implicated in this subgroup as placental oxidative stress can lead to recurrent abortions by impairing placental development and causing syncytiotrophoblast degeneration [85]. During pregnancy both extracellular and intracellular ROS production increases sharply, originating from the developing embryo [84]. Thus, the demand for enzymatic antioxidant defense is increased in embryos and oocytes and their tubal and follicular fluid microenvironments to successfully support a pregnancy and the heightened OS it produces.

The increase in peripheral white blood cell count consisting of polymorphonuclear leukocytes (PMNL) accounts for the normal and natural rise in OS seen with normal pregnancy [86]. Fait *et al.* compared the changes in peripheral PMNL counts during early pregnancy with the non-pregnant state and found that in an uncomplicated early pregnancy, peripheral PMNL and neutrophilia counts were elevated [86]. The priming of the PMNL is known to cause an increased release of ROS and OS, which occur in early pregnancy [86]. This conclusion is also supported by a study by Safronova *et al.* that explored the changes in regulation of oxidase activity of peripheral blood granulocytes in women with habitual abortion. Researchers found that in the early stages of pregnancy, the peripheral polymorphonuclear leukocyte count increases [84].

A successful pregnancy requires a successful embryo implantation and adequate uteroplacental circulation for materno-fetal exchange [84,87]. The sharp peak in the expression of OS markers in the trophoblast in normal pregnancy may result in damage to protein, lipids, and DNA, which may ultimately lead to cell death if this oxidative burst becomes excessive. Joanne *et al.* confirmed the contribution of placental oxidative stress to early pregnancy failure, in demonstrating significant increases in both morphological and immunohistochemical evidence of syncytial oxidative stress and damage in miscarried placental tissues. In a miscarriage, disorganized placental blood flow may lead to hypoxia and reperfusion injury with a resultant increase in the oxygen tension within the early placenta [88].

Moreover, the increase in oxygen concentration seen during normal early pregnancy renders the body more vulnerable to ROS formation, particularly within the mitochondria where electron leakage from the enzymes of the respiratory chain occurs. This increase in oxygen concentration may also lead to acute stress in the syncytiotrophoblast, with loss of function and extensive degeneration [88]. The syncytiotrophoblast is susceptible to OS because of its location on the villous surface, which makes this tissue first to experience the increase in intervillous PO2. Also, the syncytiotrophoblast possesses much lower concentrations of the antioxidant enzymes than other villous tissues during early gestation [87].

The connection between recurrent pregnancy loss and OS is not only corroborated by the increase in ROS generation seen in early pregnancy but also can be related to increased levels of antioxidants needed to neutralize and scavenge excessive ROS present in women affected by habitual abortion. Wang *et al.* has reported that levels of plasma vitamin E and lipid peroxides are increased in pregnant women versus non-pregnant controls [89]. Lipid peroxidation is an oxidative process that normally occurs at low levels, and antioxidant function has the ability to control the amount of oxidative stress it induces. However, when there is a deterioration of the antioxidants' capacity to neutralize ROS, peroxidative activity occurs at the expense of polyunsaturated fatty acids.

Simsek *et al.* evaluated the outcome of deficient antioxidant defense in women with habitual abortion and demonstrated elevated lipoperoxides and significantly decreased vitamin A, E, and beta carotene in this population compared with the control group. Their findings confirm that OS may be involved in the pathogenesis of recurrent pregnancy loss [90]. Sane *et al.* found that women undergoing induced or spontaneous abortions exhibited a maximum rise in serum lipid peroxidase levels immediately before the onset of abortion and significantly depressed levels of serum lipid peroxidase after the abortion was complete [91]. Jenkins *et al.* studied changes in antioxidant levels by measuring SOD levels, which measure the amount of oxygen ion scavenger that may result in increased ROS production [92]. This study found that SOD levels were significantly lower in women whose pregnancies ended in miscarriage than in healthy pregnant women [92].

The glutathione and glutathione transferase family of enzymes has been investigated in patients who experience recurrent pregnancy loss [70, 71]. The glutathione peroxidase reductase antioxidant system is an ROS scavenger, preventing lipid peroxidation in cells and maintaining intracellular homeostasis and redox balance [84]. Studies have shown glutathione concentration and activity to be significantly higher in women with recurrent miscarriage compared with the glutathione concentration seen in women with normal pregnancies or in healthy, non-pregnant woman [93]. Red blood cell GSH-Px activity was not seen to differ between pregnant women and the control group, but were seen to be significantly deficient in women that had a miscarriage [93].

The bioavailability of selenium is directly related to the activity of the GSH-Px system. GSH-Px catalyzes the reduction of hydrogen peroxide and hydroxyperoxides, acting as a free radical scavenger and preventing the lipid peroxidation of cell membranes and [93]. And because GSH-Px is an enzyme that is essential in cells to neutralize the effects of free

radicals, selenium concentrations may decrease in those patients at risk of recurrent miscarriage because selenium is incorporated into the active site of GSH-Px [94,95]. Al-Kunani *et al.* reported significantly lower concentrations of selenium in the hair samples of women with recurrent pregnancy loss compared with controls [96]. Although this study failed to confirm a difference in the overall blood plasma selenium concentrations in women who had a miscarriage compared with those with viable pregnancies, selenium levels were found to be significantly higher in non-pregnant women [96], confirming that pregnancy in general is accompanied by a state of increased OS.

In addition to the various female factors related to recurrent pregnancy loss, several male factors can contribute to OS in these patients. A recent study by Gil-Villa *et al.* assessed sperm factors possibly involved in early recurrent pregnancy loss by evaluating and comparing standard sperm parameters, lipid peroxidation of sperm plasma membranes, antioxidant capacity of seminal plasma, and sperm chromatin integrity in ejaculates from partners who have a history of recurrent pregnancy loss with those from a control group [97]. Reactive oxygen species in semen from sources such as seminal leukocytes and morphologically abnormal spermatozoa is harmful to the functionality and structure of the sperm. An increase in sperm DNA damage has been associated with increased risk of undergoing embryo loss and augmented time to reach pregnancy [98]. This study successfully showed that a larger number of individuals of the recurrent pregnancy loss group presented alterations in sperm concentration, motility, morphology, and thiobarbituric acid-reactive substance production and lower antioxidant capacity of seminal plasma than did the individuals in the control group [97]. These findings may justify the use of antioxidant therapy in the male partner in couples experiencing recurrent pregnancy loss [97].

Given the array of evidence implicating OS in the pathogenesis of recurrent abortion, many studies have focused on the role of antioxidant supplementation in women affected by recurrent pregnancy loss [99]. Poor dietary intake of vitamins has been associated with an increased risk of miscarriage [100]. For instance, there are a variety of non-enzymatic antioxidants, including vitamins C, E, and A, lycopenes, selenium compounds, lipoic acid, and ubiquinones [101] that have the ability to scavenge ROS and ultimately prevent OS and cellular damage [102]. An observational study demonstrated an association between the risk of spontaneous early miscarriage and intake of green vegetables, fruit, and dairy products [103]. Some evidence suggests that reduced intake of micronutrients during pregnancy exposes women to nutritional deficiencies and may affect fetal growth [104]. Thus, adequate maternal nutrition, particularly vitamin intake, may be an important factor in preventing miscarriage [100]. Although the evidence in regards to exactly what vitamin combinations, type, and amount are optimal for a pregnant woman is insufficient, the use of any vitamin supplements in pregnancy needs to be carefully monitored and evaluated [100].

Vitamin C and E are two popular vitamins that may have a potential role in alleviating the effects of OS in women affected with miscarriages. Vitamin E's principal function is to protect against OS-related damage and thereby serve as an antioxidant. In a normal pregnancy, vitamin E level naturally increases, while in an abnormal pregnancy, vitamin E concentrations are lower [102]. Moreover, vitamin C levels increase physiologically during pregnancy [102]. These occurrences suggest that perhaps vitamins C and E may play a role in compensating for the oxidative burst during early pregnancy, reducing the risk of pregnancy loss [102]. However, it is necessary to perform an accurate assessment of the appropriate type and dosage of vitamins that can be tolerated without causing deleterious side-effects to the mother and baby [100].

Homocysteine is a thiol-containing amino acid that is involved in the sulfurylation and methylation metabolic pathways and has been proposed to have pro-oxidant effects [84]. Normally, plasma homocysteine levels fall during a normal pregnancy. However, Del Banco *et al.* demonstrated significantly elevated homocysteine levels in women with a history of at least two consecutive miscarriages [105].

Quere *et al.* conducted a study evaluating the effect of vitamin supplementation on pregnancy outcome in 25 women with recurrent early pregnancy loss and hyperhomocysteinemia in the absence of any folate supplementation during pregnancy [106]. This study involved hyperhomocysteinemic patients. Folic acid supplements are believed to reduce a woman's risk for having a baby with a neural-tube defect. The potential for folic acid to prevent elevated homocysteine levels and OS- induced miscarriage has been the focus of many investigations. Szymanski *et al.* found that women receiving folic acid supplements had better quality oocytes and a higher degree of mature oocytes compared with those who did not receive folic acid supplementation [107]. However, the results of a study by Gindler *et al.* failed to confirm that the consumption of folic acid decrease a woman's risk for miscarriage [108]. Thus, the role of folic acid supplementation to prevent recurrent pregnancy loss is inconclusive and requires further analysis.

Antiphospholipid (aPL) antibody syndrome is a known autoimmune cause of recurrent pregnancy loss [84]. The pathophysiology of antibody formation is still not clear; however, OS has been proposed to have a role in the formation of these antibodies [84]. Omega-3 supplements have been used in prevention of recurrent miscarriage with antiphospholipid syndrome [109]. Del Bianco *et al.* studied the effects of omega-3 fatty acid supplementation in women with three or more miscarriages associated with antiphospholipid syndrome [105]. All of the subjects in this trial achieved successful pregnancy with no further miscarriages [105]. Although the results of this study are promising, the safety and efficacy of omega-3 supplementation must be confirmed by follow-up trials.

Melatonin, a hormone that acts as a powerful agent against ROS, has been hypothesized to have properties essential for successful pregnancy and prevention of spontaneous abortion [110]. Tamura *et al.* propose that deficient melatonin production in early pregnancy may be related to the development of spontaneous abortion as melatonin is a free-radical scavenger and antioxidant that is known to physiologically increase to compensate for the oxidative burst during normal pregnancy [110]. Therefore, the safety

and efficacy of melatonin supplementation to combat OS and prevent recurrent pregnancy loss should be further investigated.

EXPERT COMMENTARY

The purpose of this article was to discuss the role of antioxidant supplementation in the treatment of various gynecological and obstetric conditions known to contribute to female infertility and poor pregnancy outcome. The association between high levels of uncontrolled oxidative stress and polycystic ovarian syndrome, endometriosis, unexplained infertility, preeclampsia, and recurrent pregnancy loss has been well established by numerous studies that have measured various biomarkers of redox status. As high degrees of reactive oxygen species and low antioxidant status has been implicated in these diseases, treatment based on strategies to boost the exhausted antioxidant defense of the female reproductive microenvironment are intuitive. This approach seems especially plausible in light of the fact that oral antioxidant supplementation in males has been proven effective to treat male infertility and is widely employed in current clinical practice. However, reports regarding the safety and efficacy of oral antioxidant supplementation in the treatment of female infertility are conflicting. Additional studies using double-blinded, randomized, controlled trials are needed to further evaluate the potential use of antioxidants to treat female reproductive disease.

FIVE YEAR VIEW

There is a need for continued investigation of the efficacy and safety profiles of various oral antioxidant supplements before this modality of treatment can be relied upon to modulate the levels of oxidative stress that contribute to infertility and abnormal pregnancy in women with obstetrical and gynecologic disease. Observational studies and randomized control trials have identified several antioxidant therapies of interest which have demonstrated striking promise in the prevention and treatment of female reproductive disease. However, it is still unclear as to what types or combinations of therapy as well as the amounts and dosing that are optimal for women, particularly during pregnancy. It is necessary to conduct further studies to identify any possibility of deleterious side effects of antioxidants on mothers and their unborn baby. Despite the fact that oxidative stress is strongly implicated in the etiopathogenesis of preeclampsia, the literature fails to show strong evidence to support the efficacy of antioxidant supplementation in preventing or treating preeclampsia. Further investigation with randomized controlled trials powered to detect subtler effects may reveal any previously hidden benefits of antioxidant therapy for preeclampsia. In addition to analyzing the effect of antioxidant supplementation in women to improve fertility and pregnancy outcome, the benefits of antioxidant supplementation in male partners of couples with infertility and recurrent pregnancy loss should be studied.

KEY ISSUES

- Oxidative stress (OS) occurs when the generation of reactive oxygen species (ROS) and other radical species overrides the scavenging capacity of antioxidants, either due to excessive ROS production or an inadequate availability of antioxidants.
- At controlled levels, OS facilitates the following physiological female reproductive functions: oocyte maturation, folliculogenesis, ovarian steroidogenesis, luteolysis, ovulation, cyclical endometrial changes, and menstruation.
- At higher levels, OS is implicated in pathological processes of the female reproductive tract that contribute to infertility and poor pregnancy outcome, such as polycystic ovarian syndrome (PCOS), endometriosis, unexplained infertility, preeclampsia, and recurrent pregnancy loss.
- Antioxidant treatment of PCOS: N-acetyl cysteine may improve glucose control and peripheral insulin sensitivity in hyperinsulinemic patients. Oral hypoglycaemic agent rosiglitazone and anti-androgenic oral contraceptives have been shown to reduce parameters of OS in hyperinsulinemic patients.
- Endometriosis: The antioxidants catalase, RU486, curcumin, melatonin, and catechins may have anti-proliferative and anti-angiogenic effects capable of halting disease progression.
- Selenium supplementation of women with unexplained infertility has been shown to normalize patient's RBC-Mg levels and result in clinical pregnancy after 8 months of treatment.
- Despite the well-established causal relationship between OS and preeclampsia, studies have failed to detect any risk reduction for preeclampsia with vitamin C and vitamin E supplementation.
- The antioxidants folic acid, melatonin, and omega-3 fatty acids (particularly in women with antiphospholid antibody syndrome) have been investigated for their use in preventing recurrent pregnancy loss. Further studies to confirm the safety and efficacy of these compounds are needed.

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