

The Role of Free Radicals and Antioxidants in Female Infertility and Assisted Reproduction

a report by

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There is a complex interaction of oxidants and antioxidants that modulates the generation of oxidative stress. Under normal conditions, paired electrons create stable bonds in biomolecules; however, if the bond is weak, it may break, leaving unpaired electrons in the outer shell of the atom and leading to the formation of free radicals that are very avid to react in order to regain stability.¹ This phenomenon of erratic binding initiates a cascade of reactions of more free radicals leading to uncontrolled chain reactions.

Oxygen-free radicals may be produced normally, as a part of cellular metabolism, or as a requirement for body defense. Relentless formation of free radicals with the absence of proper antioxidant balance produces pathological changes in cells. However, as previously indicated, free radical generation under controlled conditions plays an important role in cell homeostasis. Increased oxidant formation, and even the presence of oxidative stress, is considered normal during the second trimester of pregnancy.^{2,3}

While an increasing amount of research is carried out focusing on the reactive oxygen species (ROS), there is new light being shed on the role of these radicals in physiological functions, and whenever there is an excess of the free radicals, they precipitate pathologies in the female reproductive tract. The pathologic effects are exerted by various mechanisms, including lipid damage, inhibition of protein synthesis, and depletion of adenosine triphosphate (ATP). There are some studies that provide an understanding of how the free radicals affect a gamut of physiologic functions in female reproduction. These are oocyte maturation, ovarian steroidogenesis, ovulation, implantation, and formation of fluid-filled cavity, blastocyst, luteolysis, and luteal maintenance in pregnancy.

Oxidative destruction of polyunsaturated fatty acids by lipid peroxidation is damaging because it may alter the integrity of cell membranes. There are two major types of free radical species—ROS and reactive nitrogen species (NOS). In a healthy body, ROS and

antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, OS occurs. In most cases, OS appears to be a result of increased generation of ROS, rather than a depletion of antioxidants. Cells have developed a wide range of antioxidant systems to limit production of ROS, inactivate them and repair cell damage.

Oxidative stress (OS) affects multiple physiological processes, from oocyte maturation to fertilization, embryo development and pregnancy. It has been suggested that the age-related decline in fertility is modulated by OS. OS plays a role during pregnancy and normal parturition and in initiation of pre-term labor. OS can affect sperm and oocyte quality, the fertilization process, and the embryo. This article highlights the adverse effects of free radicals and how they can affect female fertility. It also addresses how OS can adversely affect assisted reproduction, a technique which is utilized to help infertile couples conceive their own biological child. The article further summarizes information on how OS can affect both natural and assisted fertility. OS has been recognized to play a role in female reproductive diseases such as endometriosis, polycystic ovarian disease, tubal factor infertility, and unexplained infertility. The authors have also discussed the management of OS with oral antioxidant supplementation, and supplementation of the *in vitro* culture media with antioxidants.

Role of OS in Female Infertility

Infertility affects approximately six million American women and their partners, or approximately 10% of the US population. There has been a marked increase in the absolute number of couples seeking infertility services. Each year, approximately 1.3 million American couples receive medical advice or treatment.⁴ Assisted reproductive techniques (ARTs) have been widely employed and allowed many infertile couples to realize their dream of having biological offspring. The role of OS has been demonstrated in many of the causes of infertility, such as endometriosis, polycystic ovarian disease, unexplained infertility, tubal infertility, and recurrent pregnancy loss (see *Figure 1*).

Role of OS in Endometriosis

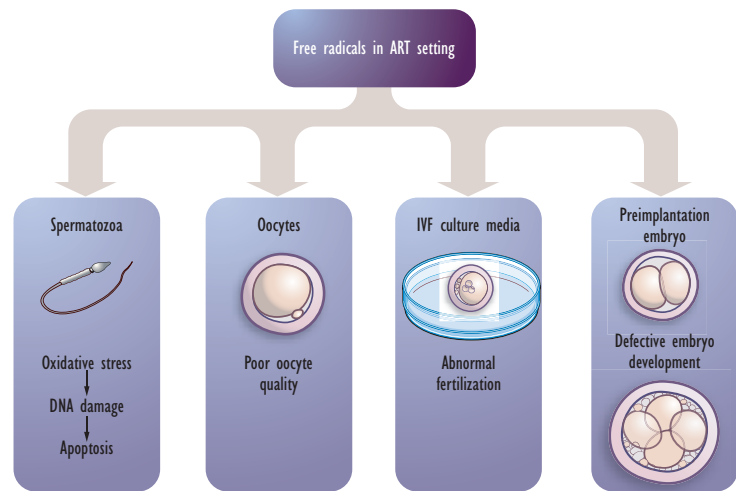
Endometriosis is a common gynecological disorder characterized by the growth of endometrial glands and stroma outside the uterus. Endometriosis has been reported to have an incidence varying from 2–50% amongst reproductive age women in the US. Deleterious effects of OS on gametes, gamete interaction, embryos, fertilization, and implantation may explain the infertility associated with endometriosis. Oxidative modification has been reported in peritoneal fluid (PF), endometrium, and endometriosis.^{5,6}

OS has been investigated as one of the many causative factors in the etiopathogenesis of endometriosis. Markers of OS have been studied in the PF and the serum to investigate OS locally and systemically. Increased generation of ROS by PF macrophages with increased lipid peroxidation in patients with endometriosis has been demonstrated, whereas others have reported contrary findings.⁷ Liu et al. reported elevated levels of lipid peroxidation in PF of patients with endometriosis. Antioxidant superoxide dismutase (SOD) levels were reported to be significantly lower in the PF of women with endometriosis.^{8,9} Jackson et al. reported a weak association between the thiobarbituric acid reactive substances (TBARS) and endometriosis after having adjusted for confounding factors such as age, body mass index (BMI), gravidity, serum vitamin E and serum lipid levels.¹⁰ Bedaiwy et al. found evidence that serum interleukin (IL)-6 and peritoneal fluid tumor necrosis factor (TNF)- α levels could be used to distinguish patients with endometriosis from those without, with a high degree of sensitivity and specificity.¹¹ ROS levels, on the other hand, were similar in peritoneal fluid of patients with endometriosis and disease-free controls.¹²

OS and Unexplained Infertility

The pathophysiology of unexplained infertility remains debatable. ROS have been demonstrated in the PF of patients with endometriosis, and even in those patients undergoing tubal ligation. Localized OS in the peritoneal cavity may have a role in the etiology of unexplained infertility.¹³ In a prospective study to determine whether ROS in peritoneal fluid is a factor in infertility, Wang et al. compared levels of peritoneal fluid ROS in women with endometriosis and idiopathic infertility who underwent laparoscopy for infertility with levels in patients who underwent tubal ligation as controls.¹⁴ ROS levels were demonstrated to be significantly elevated in patients with idiopathic infertility compared with the controls. Polak et al.¹⁵ found that the levels of antioxidants in patients with unexplained infertility were significantly lower than

Figure 1: The Role of Free Radicals in Female Infertility



those in fertile patients, and the levels of malondialdehyde (MDA), a lipid peroxidation end-product, in PF were higher in patients with unexplained infertility than in fertile women. Reduced levels of antioxidants and increased ROS-induced lipid peroxidation damage may have a role in pathophysiology of idiopathic infertility.

Role of OS in Polycystic Ovarian Syndrome

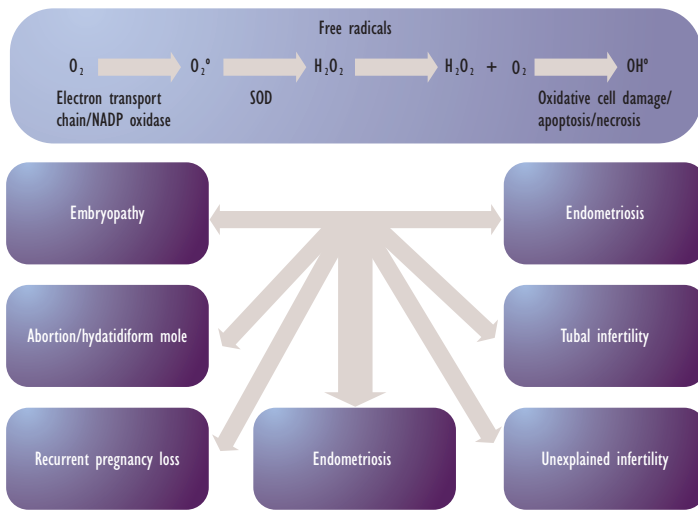
Elevated insulin resistance and hyperhomocysteinemia have been proposed to be caused by OS in patients with polycystic ovarian disease (PCOD).¹⁶ Rosglitazone has been reported to reduce the elevated MDA concentration and increase the total antioxidant status in women with polycystic ovarian syndrome.¹⁷ The decreased antioxidant status and the elevated OS levels may contribute to the increased cardiovascular (CV) morbidity in these patients.¹⁸

Impact of OS on Assisted Reproduction

There may be multiple extraneous sources of ROS generation in the assisted reproduction setting, inclusive of oocytes (four to five per dish), cumulus cell mass (thousands of cells), and the spermatozoa used for insemination¹⁹ (see Figure 2). A perturbed redox state can affect the developing embryo, as a result of suboptimal culture conditions leading to altered gene expression and impaired adenosine triphosphate (ATP) production and resultant impairment in placental and embryo growth.²⁰

Gamete quality and gamete interaction are affected by OS.²¹ The microenvironment of spermatozoa, oocytes, and embryos is modulated by ROS. OS mediates peroxidative damage to the sperm membrane and

Figure 2: The Adverse Effects of Free Radicals in Assisted Reproduction Setting



induces nuclear DNA damage.²² Reactive oxygen species can modulate the fertilizing capabilities of the spermatozoa. There is extensive literature on OS and its role in male infertility and sperm DNA damage and its effects on ART.^{23,24} ROS levels in semen have been reported in a meta-analysis conducted by the authors' group to have a significant effect on the fertilization rates with *in vitro* fertilization (IVF), and the ROS levels can be utilized to counsel patients before IVF.²⁵ The authors' group has investigated the effects of follicular fluid ROS on ART outcomes. ROS in culture media may impact post-fertilization development, i.e. cleavage rate, blastocyst yield, and quality (indicators of ART outcomes). ROS levels in day-one culture media is an important biomarker for embryonic growth.

The *in vitro* culture environment differs from the *in vivo* environment and the OS affects both ovaries as similar levels of the marker were demonstrated in follicles aspirated from the left or the right side.²⁶ Less than 50% of the cleaved embryos progress to blastocyst stage, revealing that blastocyst development *in vitro* lags behind *in vivo* development. Extrinsic factors such as elevated OS levels in *in vitro* culture may contribute to poor embryo development.²⁷ Biomarkers of OS have been identified in patients undergoing IVF/ET. OS markers such as thiobarbituric acid-reactive substances, conjugated dienes and lipid hydroperoxides have been studied in the preovulatory follicular fluid.²⁸ No correlation was seen between these markers and IVF outcome (fertilization rates or biochemical pregnancies).²⁸ A potent antioxidant system may be present in the follicular fluid, as indicated by low levels of all three biomarkers of OS in the follicular fluid. Higher basal total antioxidant capacity (TAC) were demonstrated in follicular fluid of oocytes that subsequently fertilized.²⁹

Higher levels of OS have been demonstrated in women of advanced reproductive age undergoing IVF.³⁰ Lipid peroxidation may be a good indicator of metabolic activity within the follicle. Minimal levels of OS have been shown as necessary for achieving pregnancy with IVF.^{30,31}

Role of Antioxidants in Overcoming OS in Infertility

Antioxidants act as scavengers to neutralize free radicals, and have generated considerable interest in overcoming the adverse and pathological results of OS. OS can cause direct damage to oocytes in developing follicles, oocytes, and spermatozoa in the peritoneal cavity, or the embryo in the fallopian tube,^{19,32} or through an imbalance in redox leading to luteal regression³³ that results in lack of luteal support to pregnancy.¹³ Overcoming the pathological effects of OS may be achieved by reducing the generation of ROS or increasing the amounts of antioxidants available. There are literature reports on the utilization of nutritional supplements and antioxidants such as vitamin C supplementation in patients with infertility. However, there is lack of consensus on the type and dosage of antioxidants to be used. Clinical evidence on the benefits of antioxidant supplementation is equivocal.

Evidence in the literature supports the use of systemic antioxidants for the management of some of the cases of male infertility.³⁴ A randomized, controlled, multi-center study of the effect of vitamin C supplementation (750mg/day) in patients with a luteal phase defect revealed a significantly higher pregnancy rate in the treatment group compared with controls.³⁵ In another study, women with a history of recurrent miscarriages and luteal phase defects had significantly lower concentrations of antioxidants than in healthy women.³⁶

In a double-blind, placebo-controlled pilot study, impact of a nutritional supplement, containing vitamin E, iron, zinc, selenium, and L-arginine was examined.³⁷ The mean mid-luteal progesterone levels increased from 8.2ngm/ml to 12.8ngm/ml, and the patients receiving the supplement experienced a significant increase in ovulation rates and pregnancy rates (33% pregnant; $p < 0.01$) compared with the placebo group.³⁷

A significant negative correlation has been reported between duration of smoking exposure and fertilization rates in IVF procedures. Eliminating the smoking factor would help improve fertility and ART outcomes.³⁸ Prolonged smoking history is associated with elevated levels of OS, and *in vivo* antioxidants can be recommended in infertile women who smoke.³⁹

Strategies to Overcome OS in Assisted Reproduction

The fertilization and embryo development *in vivo* takes place in an environment of low oxygen tension.⁴⁰ During assisted reproductive procedures, it is important to avoid conditions that promote ROS generation and expose gametes and embryos to ROS. During culture, low oxygen tension improves the implantation and pregnancy rate compared with higher oxygen tension.⁴¹ Similarly, higher implantation and clinical pregnancy rates are reported when antioxidant supplemented media was used, rather than standard media without antioxidants. Metal ions can sometimes result in the production of oxidants; they can also increase the production of ROS directly, through the Haber-Weiss reaction. It may be useful to add metal ion chelating agents to the culture media to decrease the production of oxidants.⁴¹

Various antioxidants, including beta-mercaptoethanol, taurine, hypotaurine, vitamin E, and vitamin C when added to *in vitro* culture media, have been reported to result in reduction in blastocyst degeneration, increased blastocyst development rates, and increased hatching of blastocysts and reduction in embryo apoptosis. The addition of ascorbate during cryopreservation reduces the levels of hydrogen peroxide and thus the oxidative distress in mammalian embryos.⁴² As a consequence, the embryo development improved with enhanced blastocyst development rates.

Mechanical removal of ROS in patients with endometriosis undergoing IVF/ET has been studied.⁴³ Cumulus oophorus rinsing is performed to overcome the deleterious effects of ROS in patients with ovarian endometriosis.⁴³ ROS has deleterious effects on both the oocyte and the embryo quality. The deleterious effects of increased TNF- α cytokines and ROS can be circumvented by the rinsing procedure in the peritoneal fluid of patients with endometriosis and unexplained infertility.

Spermatozoa are particularly susceptible to ROS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids and their cytoplasm contains low concentrations of the scavenging enzymes.⁴⁴ Sperm preparation methods have a bearing on the outcomes of the ART. Sperm preparation by centrifugation may be associated with a generation of ROS. It has been reported that seminal plasma is rich in antioxidants and protects the spermatozoa from DNA damage and lipid peroxidation.⁴⁵ Supplementation of the IVF media with N-tert-butyl hydroxylamine (NTBH) and SOD/catalase mimetics was reported to block sperm chromatin disintegration.⁴⁶

The prolongation of the sperm-oocyte co-incubation time of 16–20 hours can lead to increased ROS generation. Two prospective, randomized, controlled studies have advocated shorter sperm-oocyte co-incubation time.^{47,48} Co-incubation times of one to two hours resulted in better quality embryos with significantly improved fertilization and implantation rates. The zona pellucida thickness is significantly less in fertilized oocytes compared with the unfertilized oocytes and correlates positively with embryo quality.⁴⁹ Lower zona pellucida thickness was demonstrated with intracytoplasmic sperm injection (ICSI) and shorter sperm-oocyte exposure times, and this resulted in enhanced fertilization rates. Composition of the media utilized for IVF has significant influence on the oxidant status of the oocytes and pre-implantation embryos.

When OS is diagnosed, treatment plans must focus on identifying and eliminating the source. When a specific cause is identified, medical and surgical management options should be considered to eliminate the source of free radicals. Unlike in the male, specific clinical conditions associated with OS have yet to be identified in women. This complicates the treatment of the primary cause of excessive reactive oxygen species production. Only after treatment of the primary etiology should patients be advised to take antioxidant supplementation.

Conclusion

OS modulates a range of physiological functions and plays a role in pathological processes affecting female reproduction. OS influences the entire reproductive span of a woman's life, and even thereafter, i.e. menopause. The role of OS is becoming increasingly important, as there is newer evidence of its role in conditions such as polycystic ovarian disease, abortions, pre-eclampsia, hydatidiform mole, fetal embryopathies, preterm labor, and intrauterine growth retardation, which lead to an immense burden of maternal and fetal morbidity and mortality. It is important to further elucidate the role of OS in unexplained infertility and recurrent early pregnancy losses, and therefore devise strategies to overcome its adverse effects. There are, for example, on-going trials with antioxidant supplementation for the prevention of pre-eclampsia. They will prove the safety and effectiveness of antioxidants and if they could improve the maternal and fetal outcomes. Successful management of infertility in the ART scenario depends on overcoming OS in the *in vitro* conditions. The results of the studies reviewed here need to be validated by larger randomized, double-blind, case-controlled trials. ■

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