

# The role of free radicals and antioxidants in reproduction

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## Purpose of review

This review summarizes the role of free radicals and oxidative stress in the pathophysiology of human reproduction.

## Recent findings

An extensive review of the literature on the role of oxidative stress in influencing assisted reproduction and its outcome is described in this article. Free radicals or reactive oxygen species mediate their action through many of the proinflammatory cytokines and this mechanism has been proposed as a common underlying factor for endometriosis, ovarian cancer, polycystic ovary disease, and various other pathologies affecting the female reproductive process, as highlighted in this review.

Oxidative stress, sperm DNA damage, and apoptosis have been implicated in male infertility. Elevated reactive oxygen species levels correlate with the poor fertility outcomes seen in the assisted reproductive technology setting.

## Summary

Oxidative stress has been implicated in male and female infertility, including fetal dysmorphogenesis, abortions, and intrauterine growth restriction. Accurate evaluation of seminal oxidative stress by standardized assays may help in the diagnosis and management of male infertility. There is evidence in the literature on the beneficial effects of oral antioxidant supplementation in male infertility. Current ongoing trials will provide answers on the safety and effectiveness of antioxidants in improving maternal and fetal outcomes. Further studies need to be conducted to determine if antioxidant supplementation will prevent fetal developmental defects in high-risk pregnancy with diabetes.

## Keywords

antioxidants, assisted reproduction, female infertility, female reproduction, free radicals, male infertility, oxidative stress

## Abbreviations

<b>ART</b>	assisted reproductive technology
<b>Cu-Zn SOD</b>	copper zinc superoxide dismutase
<b>ICSI</b>	intracytoplasmic sperm injection
<b>IVF</b>	in-vitro fertilization
<b>LPO</b>	lipid peroxidation
<b>MnSOD</b>	manganese superoxide dismutase
<b>PMNL</b>	polymorphonuclear leucocytes
<b>ROS</b>	reactive oxygen species
<b>TAC</b>	total antioxidant capacity
<b>TNF-<math>\alpha</math></b>	tumor necrosis factor alpha

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## Introduction

Aerobic metabolism is associated with the generation of prooxidant molecules called free radicals or reactive oxygen species (ROS) that include the hydroxyl radicals, superoxide anion, hydrogen peroxide, and nitric oxide. There is a complex interaction of the prooxidants and antioxidants, resulting in the maintenance of intracellular homeostasis. Whenever there is an imbalance between the prooxidants and antioxidants, a state of oxidative stress is initiated. Free radicals have a dual role in the reproductive tract. They are also key signal molecules modulating various reproductive functions. Free radicals can influence the oocytes, sperm, and embryos in their microenvironments, for example follicular fluid, hydrosalpingal fluid, and peritoneal fluid. These microenvironments have a direct bearing on quality of oocytes, sperm oocyte interaction, implantation, and early embryo development. Oxidative stress affects both implantation and early embryo development which determines a successful pregnancy. There is a complex interplay of cytokines, hormones, and other stressors that affects cellular generation of free radicals; these molecules act further through the modulation of many transcription factors and gene expression. This review addresses how oxidative stress is involved in the physiological functioning of the female reproductive tract and how it influences the outcomes of assisted reproductive technology (ART).

## Oxidative stress and ovarian function

Aerobic metabolism utilizing oxygen is essential for energy requirements of the gametes, and the free radicals play a significant role in physiological processes within the ovary. Many studies have demonstrated involvement of ROS in the follicular-fluid environment, folliculogenesis, and steroidogenesis [1–3]. The role of

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ROS and antioxidant enzymes, copper zinc superoxide dismutase (Cu–Zn SOD), manganese superoxide dismutase (MnSOD), and glutathione peroxidase, in oocyte maturation was provided by Riley *et al.* [4] using immunohistochemical localization, mRNA expression, and thiobarbituric acid methods that suggested a complex role in ovulation and luteal function in the human ovary [5].

Oxidative stress has been shown to affect the midluteal corpus luteum and steroidogenic capacity both *in vitro* and *in vivo*. In a very interesting study, using corpora lutea collected from pregnant and nonpregnant patients, it was observed that during normal situations Cu–Zn SOD expression parallels the levels of progesterone, with a rise from early luteal to midluteal phase and decrease during regression of the corpus luteum. The mRNA expression, however, of Cu–Zn SOD in the corpus luteum during pregnancy was much higher than those of midcycle corpora lutea. This factor enhanced SOD expression during pregnancy, possibly caused by increased human chorionic gonadotropin (HCG) levels, and may be the cause of apoptosis of the corpora lutea. Similarly, the antioxidant enzymes glutathione peroxidase and MnSOD are considered the markers for cytoplasmic maturation, as these are expressed only in metaphase II oocytes [6]. Decreased developmental potential of oocytes from poorly vascularized follicles has also been attributed to low intrafollicular oxygenation [7,8]. Studies demonstrate intensified lipid peroxidation in the preovulatory Graafian follicle [9] and that glutathione peroxidase may help in maintaining low levels of hydroperoxides inside follicle [10], suggesting an important role of oxidative stress in ovarian function. Oxidative stress and inflammatory process have roles in the pathophysiology of polycystic ovarian disease and drugs such as Rosiglitazone maybe effective by decreasing the levels of oxidative stress [11,12].

### Oxidative stress and the endometrium

Cyclical changes in the endometrium are accompanied by changes in the expression of various antioxidants in the endometrium. Enzymes, such as thioredoxin, have a higher expression in the early secretory phase and this could be important for implantation [13]. It is interesting to note that targeted disruption of the thioredoxin gene in mice was associated with the lethal effects on the early embryo [14]. In addition, increased expression of SOD in the endometrium was observed in the late secretory phase just before menstruation. Tumor necrosis factor alpha (TNF- $\alpha$ )-induced MnSOD expression in human endometrial stromal cells was observed to be via nuclear factor kappa B (NF $\kappa$ B) activation [15]. Estrogen or progesterone withdrawal led to increased expression of cyclooxygenase-2 (COX-2) mRNA and increased

prostaglandin F $_{2\alpha}$  (PGF $_{2\alpha}$ ) synthesis in endometrial cells *in vitro*. These studies suggest a role for ROS-mediated NF $\kappa$ B activation in endometrial physiology as related to implantation and/or menstruation [3].

### Placental oxidative stress and abortions

As implantation is a very well orchestrated process that involves complex interactions between the embryo and the uterine environment, a burst of placental oxidative stress during establishment of maternal circulation may cause early pregnancy loss [16,17]. Spontaneous abortion is accompanied by a significant disruption of the pro-oxidant and antioxidant balance. Oxidative stress may also have a role in patients with recurrent abortions with no known etiology [18]. During pregnancy, there is an increased number of polymorphonuclear leucocytes (PMNL) that may result in increased generation of the superoxide ions [19]. Oxidative stress may modulate expression of cytokine receptors in the placenta, cytotrophoblasts, vascular endothelial cells, and smooth muscle cells [20]. It is not clear, however, if oxidative stress affects the posttranslational modification or the aberrant expression of cytokine receptors that results in a failure to stimulate growth and differentiation.

T helper cells influence production of two kinds of cytokine responses, TH1 or TH2, which are modulated by the antioxidant glutathione [21]. Miscarriages in patients with a history of recurrent abortions were associated with elevated levels of the TH1 response and glutathione, while GSH depletion leads to the inhibition of TH1-type cytokines. The role of oxidative stress and antioxidants is an interesting area for further investigations in women associated with recurrent pregnancy loss.

### Oxidative stress and embryo development

Oxidative stress is involved in defective embryo development and retardation of embryo growth [22,23] that is attributed to induced cell-membrane damage, DNA damage, and apoptosis. Apoptosis results in fragmented embryos, which have limited potential to implant and hence result in poor fertility outcomes [24]. In addition, in-vitro blastocyst formation during ART is suboptimal and antioxidants may improve blastocyst development. Increased oxidative stress in the male germ line has also been associated with poor fertilization rates, impaired embryo development, and increased rates of pregnancy loss [25]. The fertilization and embryo development *in vivo* takes place in an environment of low oxygen tension [26]. During culture, low oxygen tension improves the implantation and pregnancy rate [27]. Similarly, increased implantation and clinical pregnancy rates are reported when antioxidant-supplemented medium was

used for ART rather than standard medium without antioxidants. Metal ions can also induce production of ROS directly through the Haber–Weiss reaction. Addition of metal ion chelating agents to the culture media may decrease the production of oxidants and help in successful embryo development and pregnancy [27]. Addition of ascorbate during cryopreservation has also been reported to reduce the levels of hydrogen peroxide and prevent oxidative distress to mammalian embryos [28]. Antioxidants may play a significant role not only during ART procedures but also in preventing subsequent loss or damage to embryo.

### **Oxidative stress and endometriosis associated infertility**

Increased generation of ROS by peritoneal fluid macrophages, with increased lipid peroxidation in patients with endometriosis, has been demonstrated, whereas other researchers have reported contrary findings [29]. Diminished peritoneal fluid antioxidants [30,31], elevated oxidized lipoproteins [30], lysophosphatidyl choline [31], and other markers of lipid peroxidation provide further evidence of oxidative stress in the peritoneal microenvironment of patients with endometriosis [32–34]. Increased generation of autoantibodies to oxidatively modified lipoproteins that are antigenic has been reported in patients with endometriosis [30, 35]. The investigations of various biomarkers have then revealed presence of oxidative stress locally and systemically in patients with endometriosis.

Activated macrophages, present in increased numbers in the peritoneal fluid of patients with endometriosis, are known to release TNF- $\alpha$ , a pleiotropic cytokine that induces toxic effects on gametes mediated by free radicals [36] and that plays an important role in the pathophysiology of endometriosis-associated infertility. Peritoneal fluid aspirated from the patients with endometriosis inhibited the cleavage of two-cell embryos [37]. Infliximab, an inhibitor of TNF- $\alpha$ , may be potentially investigated for treatment of endometriosis. Further larger studies with sufficient statistical power need to be conducted to assess the role of oxidative stress with a global assessment of the oxidative stress biomarkers and antioxidants in endometriosis.

### **Redox regulation of ovarian senescence**

It is proposed that ovarian senescence is caused by increased oxidative stress in the follicular fluid. A reduction in the expression of glutathione and catalase activity, accompanied by increased expression of the SOD activity, was demonstrated in older women compared with young controls undergoing in-vitro fertilization (IVF) [38]. In a recent study with aging rats, Yeh *et al.* [39] demonstrated PGF2 $\alpha$ -induced luteolysis leading

to reduced expression of glutathione reductase. Oxidative stress can result in disturbances of meiotic spindles of murine oocytes during aging, which can cause degeneration or apoptosis [40]. Poor quality embryos, with the majority being aneuploid, result from ageing gametes.

### **Oxidative stress and assisted reproduction**

Low levels of ROS play a beneficial role in IVF. Lipid peroxidation (LPO) and total antioxidant capacity (TAC) levels in follicular fluid were found to correlate positively with the pregnancy rates in patients undergoing IVF [41]. Oyawoye *et al.* [42] also observed that baseline TAC levels, in the follicular fluid of oocytes that were fertilized subsequently, were significantly higher.

The follicular fluid microenvironment and oxidative stress status provides a window to oocyte quality, fertility outcomes, and success of intracytoplasmic sperm injection (ICSI) [43]. Apoptosis and DNA damage have been reported to be associated with fertilization failure [44]. A higher percentage of human oocytes remaining unfertilized after ICSI demonstrate DNA fragmentation. Levels of selenium-dependent glutathione peroxidase and glutathione *S*-transferases were significantly decreased in follicles yielding oocytes that failed to fertilize during IVF. Minimal levels of ROS are essential and are indicative of metabolic activity within the follicle. High ROS levels in the culture media, however, on the morning after oocyte retrieval correlated with lower blastocyst development, poor fertilization and cleavage rates, and higher embryonic fragmentation following ICSI [45]. Levels of 8-hydroxy-2-deoxyguanosine, an indicator of cellular DNA damage when evaluated in granulosa cells from aspirated oocytes, correlated negatively with fertilization rates and the number of good quality embryos in patients undergoing IVF. Highest index was demonstrated in patients with endometriosis compared with patients who underwent IVF for various other reasons such as tubal factor or male infertility [46].

Oxidative stress affects both ovaries, as similar levels of the marker were demonstrated in follicles aspirated from the left or the right side [47]. Indirect evidence of oxidative stress has been associated with antiphospholipid generation during IVF. Presence of antiphospholipid antibodies in the plasma, and its association with increased oxidative stress in the plasma, was reported in infertility patients undergoing IVF [48–50]. In a pilot study isoprostane 8,12-iso-iPGF2 $\alpha$  was detected in sera as a marker of oxidative stress in patients undergoing IVF [47]. These studies provide evidence of systemic oxidative stress in infertile patients undergoing IVF.

As ROS have deleterious effects on both the oocyte and the embryo quality in patients with endometriosis, various strategies are designed to minimize the exposure of gametes to environments that lead to elevated free-radical generation. Mechanical removal of ROS, and the rinsing of cumulus oophorus during ART, could overcome the deleterious effects of cytokines and high ROS levels in patients diagnosed with endometriosis [51]. Hence, there is scientific evidence in the literature that minimal levels of ROS may be necessary for ART, but excess of ROS can lead to poor outcomes with ART (Fig. 1).

### Role of free radicals in male infertility

Increased generation of ROS in semen affects sperm function, especially fusion events associated with fertilization, and leads to infertility [52–55]. ROS are known to be generated from spermatozoa and leucocytes and the resultant peroxidative damage causes impaired sperm function. Elevated ROS levels correlate negatively with sperm concentration and sperm motility [56]. An accurate two-variable model, utilizing the standard semen analysis and sperm deformity index, has been recommended for identifying infertile males with high levels of ROS [57]. 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 [58]. Sperm-preparation methods have a bearing on the out-

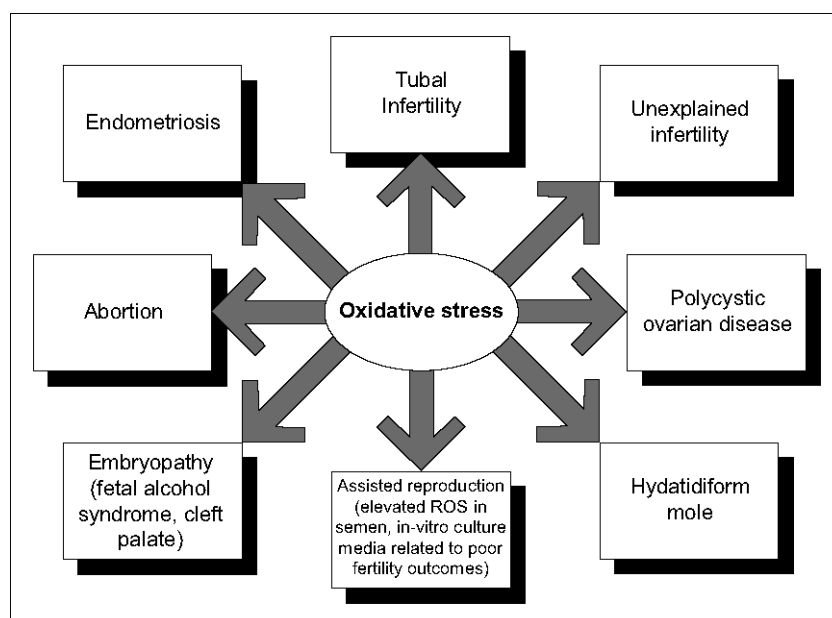
comes of the ART. Sperm preparation by centrifugation may be associated with generation of ROS. It has been reported that seminal plasma is rich in antioxidants and protects the spermatozoa from DNA damage and lipid peroxidation [59]. Supplementation of the IVF media with *N*-tert-butyl hydroxylamine (NTBH) and SOD/catalase mimetics was reported to block the breakdown of sperm chromatin [60]. Recently, a meta-analysis revealed that ROS in semen had a statistically significant impact on the fertilization rates with IVF [61], suggesting that ROS levels in the semen may be important predictors of IVF success and can be helpful in counseling patients on IVF outcomes.

There are reports of increased generation of ROS species with prolonged sperm–oocyte coincubation time of 16–20 h, advocating shorter sperm–oocyte coincubation time [62,63]. In addition, composition of media utilized for IVF has significant influence on the oxidant status of the oocytes and preimplantation embryos. There is current evidence that supplementation of media with antioxidants, such as disulphide reducing agents or divalent chelators of cations, may be beneficial to embryos and improve pregnancy rates [22].

### Role of antioxidant supplementation

Many clinical and research centers are investigating the usefulness of antioxidant supplementation and their role in prevention of pre-eclampsia. As oxidative stress results in luteolysis, antioxidant supplementation, for

**Figure 1** The role of oxidative stress in female reproduction





example vitamin C and vitamin E, has been shown to have beneficial effects in preventing luteal phase deficiency and resultant increased pregnancy rate [64,65] and others have reported no value [66]. Meta-analysis investigating the intervention of vitamin-C supplementation in pregnancy was inconclusive [67]. Another meta-analysis of women taking any of the vitamin supplements started prior to 20 weeks' gestation revealed no reduction in total fetal losses, or in early and late miscarriage, having used the fixed-effects model [68]. Improved pregnancy rates were also reported with combination therapy with the antioxidants pentoxifylline and vitamin-E supplementation for 6 months in patients with thin endometria who were undergoing IVF with oocyte donation [69].

As oxidative stress can induce sperm dysfunction, many sperm preparation techniques, such as density gradient centrifugation and glass wool separation, reduce ROS formation by removing leucocytes, cellular debris, and immotile spermatozoa. Many antioxidants (vitamins C and E, glutathione and  $\beta$ -carotene, pentoxifylline, etc.), supplemented during sperm preparation and ART, improve sperm motility and acrosome reaction [70,71]. Supplementation with vitamin E has also been reported to prevent the deleterious effects of ethanol toxicity on cerebral development in the animal model [72]. A recent meta-analysis reported that vitamin-E supplementation, in higher doses for more than 1 year, resulted in increased mortality [73]. This meta-analysis has created significant debate and rethinking among researchers and clinicians regarding vitamin-E supplementation during pregnancy. Nevertheless, there are essential differences among the population groups and the dosage and duration of supplementation for prevention of preeclampsia. Although many advances are being made in the field of antioxidants therapy, the data are still debatable and need further controlled evaluations in larger populations.

#### Antioxidants and assisted reproductive technology

ROS may originate from the male or female gamete or the embryo or indirectly from the surroundings, which includes the cumulus cells, leucocytes, and culture media. Accurate assessment of the free radicals and oxidative stress levels is essential and will help clinicians screen and identify patients with oxidative stress. Increased levels of day-1 ROS are markers of poor embryo quality, especially ICSI cycles [45]. Various antioxidants, including  $\beta$ -mercaptoethanol [74], protein [75], vitamin E [76], vitamin C [77,78], cysteamine [79, 80], cysteine [81], taurine and hypotaurine [82], and thiols [83], added to the culture medium can improve the developmental ability of the embryos by reducing the effects of ROS. Sperm-manipulation media are sup-

plemented with human serum albumin, polyvinylpyrrolidone, and HEPES, which are DNA protectors [84]. Scavenging of the ROS by various antioxidants has been proposed to lead to a better environment for the preimplanted embryos. Reduction in blastocyst degeneration, increased blastocyst development rates, increased hatching of blastocysts and reduction in embryo apoptosis, and other degenerative prooxidant influence has been reported. It is interesting to observe that blastocyst development *in vitro* always lags behind blastocyst development *in vivo*, as there is a variation in the ability of IVF media and its components to scavenge ROS and prevent DNA damage. Addition of the enzymatic antioxidant, for example SOD, to the culture medium prevented the deleterious effects of oxidative stress on sperm viability and on the embryo development both *in vivo* and *in vitro* [85]. This effect was demonstrated by increased development of two-cell stage embryos to the expanded blastocyst stage in the SOD-supplemented medium. These data suggest that the antioxidants exert beneficial effects on embryo development, possibly by a reduction in the incidence of apoptosis. Taurine, an essential amino acid, also improves spermatozoa motility, capacitation, fertilization, and supports early embryonic development [86]. Antioxidant supplementation has also been reported to have beneficial effects on sperm morphology and preimplantation embryo development and leads to reduction of developmental defects.

#### Future trends

Although many animal studies suggest improved fetal outcomes with antioxidant supplementation [87], it is not clear if antioxidant treatment prevents embryo dysmorphogenesis in women with high-risk pregnancies complicated by diabetes or ethanol intake [72]. Maternal oxidative stress biomarkers have been proposed to provide a risk profile for pregnancies that may be complicated with nonsyndromic congenital heart disease. Further studies need to be conducted to elicit the exact pathways by which oxidative stress causes damage to embryos and to help design interventions for prevention of birth defects. Current ongoing trials, on antioxidant supplementation for prevention of preeclampsia, will also provide answers to the usefulness, safety, and effectiveness of such oral antioxidants.

#### Conclusion

Free radicals and oxidative stress have important roles in modulating many physiological functions in reproduction, as well as in conditions such as infertility, abortion, hydatidiform mole, fetal embryopathies and pregnancy complications such IUGR and preeclampsia. The evaluation of in-vivo oxidative stress is difficult. The minimum well tolerated concentration, and the

physiological levels of ROS in the reproductive tract, need to be defined. Longitudinal studies to assess various biomarkers during pregnancy may help identify their association with congenital fetal malformations or pregnancy complications such as intrauterine growth retardation and preeclampsia. There is emphasis on overcoming oxidative stress during ART.

There is an ongoing debate on the role of antioxidant supplementation in both male and female infertility. Reports of antioxidant therapy in female infertility are few and antioxidant therapies have not resulted in modification of outcomes of many of the diseases investigated. These fields are interesting areas to pursue, especially using preventative approach mainly caused by the high cost of such infertility treatments.

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## References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 352).

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