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The Impact of Oxidative Stress on Female Reproduction and ART: An Evidence-Based Review

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INTRODUCTION

Aerobic metabolism is associated with the generation of pro-oxidant 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 pro-oxidants and antioxidants, resulting in the maintenance of the intracellular homeostasis. Whenever there is an imbalance between the pro-oxidants and antioxidants, a state of oxidative stress (OS) is initiated.

OVERVIEW OF OS AND ROS

Under normal conditions, paired electrons create stable bonds in biomolecules. However, if the bond is weak, it might break, leading to the formation of free radicals. Free radicals are defined as any species with one or more unpaired electrons in the outer orbit that include ROS such as superoxide, hydrogen peroxide, hydroxyl, and singlet oxygen radicals. They are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons, initiating a cascade of reactions of more free radicals leading to uncontrolled chain reactions (1). Free radicals such as the superoxide radical are formed when high-energy electrons leak from the electron transport chain. The dismutation of superoxide results in the formation of hydrogen peroxide. The hydroxyl ion is a major type of ROS that is highly reactive, having the ability to modify purine and pyrimidines and cause damaging DNA strand breaks (2,3).

ROS are formed endogenously as a natural byproduct of aerobic metabolism and through the activity of various metabolic pathways and enzymes of oocytes and embryos. ROS may originate directly from the embryos or their surroundings. Exogenous factors such as oxygen consumption, metallic cations, visible light, amine oxidase, and spermatozoa can inflate the amount of ROS produced by embryos (3,4). Phagocytes, leukocytes, parenchymal steroidogenic cells, and endothelial cells are potential sources of ROS. Enzymes known to generate ROS include plasma membrane NADPH oxidase in phagocytes; oxidases of mitochondrial, microsomal, and peroxisomal origin; and cytosolic xanthine oxidase in the endothelial cells (5).

While controlled production of ROS is necessary for certain physiological functions, higher levels of ROS may overwhelm antioxidant capacity and cause OS to occur (6). Oxygen-free radicals may be produced normally, as a part of cellular metabolism, or as a part of the body's defense mechanisms. There is a complex interplay of cytokines, hormones, and other stressors that affects cellular generation of free radicals. Free radicals then further act through the modulation of gene expression and transcription factors. ROS have important roles in mediating tissue remodeling, hormone signaling, oocyte maturation, folliculogenesis, tubal function, ovarian steroidogenesis, cyclical endometrial changes, and germ cell function (2,3,7). However, during times of environmental stress, ROS levels can increase dramatically, leading to significant damage to cell structures. There is an assortment of antioxidants that hinder ROS production, scavenge ROS, and repair the cell damage they inflict (8,9). Nonenzymatic antioxidants consist of vitamin C, taurine, hypotaurine, cysteamine, and glutathione. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and glutaredoxin (6). Intracellular homeostasis is maintained as a result of the complex interaction between pro-oxidants and antioxidants.

OS is caused by the relentless formation of free radicals within an environment lacking proper antioxidant balance, resulting in pathological changes in cells. OS is thought to have cytotoxic effects by instigating the peroxidation of membrane phospholipids and altering most types of cellular molecules such as lipids, proteins, and nucleic acids. Subsequently, these changes could lead to an increase in membrane permeability, loss of membrane integrity, enzyme inactivation, structural damage to DNA, mitochondrial alterations, adenosine triphosphate depletion, and apoptosis (4,6,10). Free radicals can influence the oocyte, sperm, and embryos in their follicular fluid, tubal fluid, and peritoneal fluid microenvironments, thus influencing reproductive outcome (3).

PHYSIOLOGICAL ROLE OF ROS IN FEMALE REPRODUCTION

Various biomarkers of OS have been studied in the female reproductive tract. ROS and the transcripts of the various antioxidant enzymes have been localized and different studies have confirmed their presence in the female reproductive tract. ROS may act as important mediators in hormone signaling, oocyte maturation, ovarian steroidogenesis, ovulation, luteolysis, luteal

maintenance in pregnancy, implantation, compaction, blastocyst development, germ cell function, and corpus luteum formation (2,6).

ROS and Folliculogenesis

ROS are thought to play a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis, and luteolysis. Folliculogenesis refers to the maturation of the ovarian follicle, a densely packed shell of somatic cells that contains an immature oocyte. The developmental process entails the progression of a number of small primordial follicles into large preovulatory follicles. Follicular fluid ROS levels may represent the physiological ranges of ROS required for the normal development of the oocyte and the subsequent embryo (5). Controlled OS is thought to play a pivotal role in ovulation. Inflammatory-like modifications first occur in the theca interna and granulose layers of follicles in response to hCG during luteinization. The final stages of oocyte maturation before follicle rupture are orchestrated with the production of various cytokines, kinins, prostaglandins, proteolytic enzymes, nitric oxide, and steroids. These events have been demonstrated to influence blood flow in the ovaries during the periovulatory period (11).

The follicular fluid environment surrounding the oocytes may play a critical role in fertilization and embryo development, influencing IVF outcome parameters such as fertilization, embryo cleavage, and pregnancy rates (6). In addition to granulosa cells, growth factors, and steroid hormones, the follicular fluid environment contains leukocytes, macrophages, and cytokines, which can all produce ROS (5). Ovarian folliculogenesis also involves local autocrine and paracrine factors, such as the nitric oxide (NO) radical. Follicular NO is thought to be produced by either endothelial NO synthase or inducible NO synthase. NO exerts its effects through the activation of various iron-containing enzymes (12). Some studies have shown a relationship between NO concentrations and follicular growth and programmed follicular cell death, implicating the involvement of the free radical in both of these processes (13,14). Low concentrations of NO may prevent apoptosis, whereas at higher concentrations the effects of NO may be pathological, promoting cell death by peroxynitrite generation. Cells involved in steroidogenesis such as theca cells, granulose lutein cells, and hilus cells show stronger oxidative enzyme activity, suggesting an association between OS and ovarian steroidogenesis.

The expression of various markers of OS has been demonstrated in normally cycling ovaries (15,16). The concentrations of various OS markers have been demonstrated to be lower in the follicular fluid than in the serum, suggesting that follicular fluid contains high concentrations of antioxidant systems, which help protect an oocyte from oxidative damage (17). Primordial, primary, preantral, nondominant antral follicles in follicular phase, dominant follicles, and atretic follicles have been studied for superoxide dismutase (SOD) expression as a representative of enzymatic antioxidant (18) SOD is a metal-containing antioxidant enzyme that catalyzes the decomposition of superoxide into hydrogen peroxide and oxygen, protecting the cells from harmful free radicals of oxygen. SOD was found to be present in the ovary, particularly in the theca interna cells in the antral follicles (18). Therefore, theca interna cells may act as important protectors of the oocyte from OS during oocyte maturation. The preovulatory follicle has a potent antioxidant defense, which can be exhausted by intense peroxidation (19). Transferrin, a blood plasma glycoprotein that binds iron, is known to suppress ROS generation and has been proven an important factor for the successful development of follicles (20). The antioxidant factor, ascorbic acid can be depleted both by oxidant scavenging and impaired cellular recycling of vitamin C. Ascorbic acid deficiency characteristically results in ovarian atrophy, extensive follicular atresia, and the premature resumption of meiosis, illustrating the importance of its protective role against OS. Other antioxidant enzymes, such as catalase and other nonenzymatic antioxidants such as vitamin E, the peroxidase cofactor–reduced glutathione, and the carotenoid lutein have been suggested to protect the oocyte and the embryo from OS by detoxifying and neutralizing ROS production (5).

ROS and the Endometrial Cycle

OS is involved in the modulation of cyclical changes in the endometrium. Fluctuations in the expression of SOD in the endometrium have been investigated. Altered SOD and ROS levels have been demonstrated in the endometrium during the late-secretory phase, just before menstruation (21). An elevated lipid peroxide concentration and decreased SOD concentrations have been reported in human endometrium in the late-secretory phase, and these changes may be responsible for the breakdown of the endometrium, implicating the involvement of OS in the process of menstruation (21). The expression of endothelial NO synthase (NOS) and inducible NOS have been demonstrated in the human endometrium and the endometrial vessels (12,22). Endothelial NOS is distributed in glandular surface epithelial cells in the human endometrium (23). NO is thought to regulate the microvasculature of the endometrium. Expression of endothelial NOS mRNA has been detected in the midsecretory phase and late-secretory phase, indicating its involvement in the decidualization of the endometrium and menstruation. Endothelial NOS is also thought to bring about changes that prepare the endometrium for implantation. Recent studies exploring the underlying mechanisms of endometrial shedding have established that estrogen and progesterone withdrawal in endometrial cells cultured in vitro leads to a decrease in SOD activity, thereby increasing ROS concentrations. In turn, ROS may activate nuclear factor kappa B, which stimulates increased cyclooxygenase-2 mRNA expression and prostaglandin F2α synthesis, facilitating the physiological changes required for endometrial shedding and/or implantation to occur (21).

VEGF and Ang-2 are key regulators of endometrial angiogenesis. VEGF and Ang-2 are induced by hypoxia and ROS (24) and have been observed to be upregulated in the endometria of patients taking long-term progestin-only contraceptives. The changes in VEGF and Ang-2 expression are thought to play an integral role in producing the abnormally distended, fragile vessels that are the cause of abnormal uterine bleeding associated with long-term progestin-only contraceptives use. The later induces the abnormal angiogenesis by decreasing endometrial blood flow, inducing hypoxia (25). In vitro, hypoxia was demonstrated to increase OS by inducing the expression of nitrotyrosine, a marker of peroxinitrite anion generation in cultured endometrial microvascular endothelial cells. OS is thus implicated in the genesis of endometrial

pathophysiology seen in long-term progestin-only contraceptives users (25).

ROLE OF OS IN FEMALE INFERTILITY

OS induces infertility in women through a variety of mechanisms. We have discussed how ovarian follicles experiencing OS can lead to direct damage to oocytes. Oocytes and spermatozoa can also experience direct damage, which can lead to impaired fertilization due to an environment of OS in the peritoneal cavity. Even when fertilization occurs, apoptosis leading to embryo fragmentation, implantation failure, abortion, or congenital abnormalities in offspring can occur. OS in the fallopian tubes can cause direct adverse effects on the embryo. Defects in the endometrium, which normally supports the embryo and its development, can arise when there is an ROS-antioxidant imbalance in the female reproductive tract (26). ROS-antioxidant imbalance is also implicated in luteal regression and insufficient luteal hormonal support for the continuation of a pregnancy (8). OS has been implicated in many other causes of infertility, such as endometriosis, hydrosalpinx, polycystic ovarian disease, unexplained infertility, and recurrent pregnancy loss (27).

OS and Endometriosis

The association between endometriosis and infertility remains a highly controversial topic of debate. Severe cases of infertility associated with endometriosis are thought to possibly result from mechanical blockage of the sperm-egg union by endometriomata, adhesions, and pelvic anatomy malformations. However, the pathogenesis of infertility experienced by patients with mild to moderate endometriosis and with no anatomical distortions is poorly understood.

Women with endometriosis have been reported to have an increased volume of peritoneal fluid, containing increased concentrations of peritoneal macrophages, cytokines, and prostaglandins. Activated peritoneal macrophages have been implicated in the pathology of endometriosis as they may be responsible for increased production of ROS (28). It has been suggested that ROS may increase growth and adhesion of endometrial cells in the peritoneal cavity, promoting endometriosis adhesions and infertility (29).

There are several hypotheses as to why OS may occur in relation to endometriosis. There is considerable evidence that suggests that menstrual reflux transplants cell debris into the peritoneal cavity and is associated with the development of endometriosis. Erythrocytes release hemoglobin and hem, which act as proinflammatory factors. Hem contains the redox-generating iron molecule (30). The presence of iron, (31), macrophages (32), and/or environmental contaminants such as polychlorinated biphenyls (33) in the peritoneal fluid may disturb the balance between ROS and antioxidants, resulting in endometriosis and tissue growth. Circulating levels of OS from other sources may also contribute to the pathogenesis of disease. A definitive conclusion about the association between OS and endometriosis is difficult to reach as most of the research studies investigating this relationship differ greatly in many regards including the selection of the control population, eligibility criteria, markers of OS and antioxidant status, and the biological medium in which OS was measured (1).

An increase in ROS production by peritoneal fluid macrophages, with increased lipid peroxidation, has been demonstrated in endometriosis patients (34), whereas other researchers have reported contrary findings (10). Epitopes produced as a result of lipid peroxidation have been demonstrated in macrophage-enriched areas of both the endometrium and endometriosis implants. Jackson et al. reported a weak association between the thiobarbituric acid reactive substances, a measure of overall OS and endometriosis, after adjusting for confounding factors such as age, BMI, gravidity, serum vitamin E, and serum lipid levels (35). Women with idiopathic infertility and endometriosis have been seen to have higher peritoneal fluid concentrations of ROS than tubal ligation control patients (10). However, this effect has not been observed to correlate with the severity of endometriosis and was not observed to be significant, suggesting that in patients with the disease, peritoneal fluid ROS may not directly cause infertility. OS may contribute to angiogenesis in ectopic endometrial implants and aids the progression of endometriosis by increasing VEGF production (24). This effect is partly mediated by glycodelin, a glycoprotein whose expression is increased by OS. Glycodelin may act as an autocrine factor within ectopic endometrial tissue by augmenting VEGF expression (24).

Greater amounts of NO and NOS have been detectable in the endometrium of women with endometriosis (1). Increased expression of inducible NOS and increased levels of endothelial NOS in the glandular endometrium have been reported in patients with endometriosis. Variations in the expression of the endothelial NOS gene may be involved in endometrial angiogenesis, thus modulating the process of endometriosis. These changes in NOS expression could also alter endometrial receptivity and impair embryo implantation. Some studies have found the peritoneal fluid from patients with endometriosis to contain increased concentrations of NO. Elevated concentrations of NO, such as those produced by activated macrophages, can hinder infertility in a myriad of ways, including changing the composition of the peritoneal fluid environment that hosts the processes of ovulation, gamete transport, sperm-oocyte interaction, fertilization, and early embryonic development (1).

Endometriosis patients have exhibited increased lipid-protein complex modification in the endometrium. Lipid peroxide concentrations have been demonstrated to be the highest among patients with endometriosis. The peritoneal fluid of women with endometriosis has been observed to have insufficient antioxidant defense, having a lower total antioxidant capacity (TAC) and significantly reduced levels of the individual antioxidant enzymes such as SOD (36,37). The concentrations of SOD have been demonstrated at statistically significant lower concentrations in infertile women with endometriosis compared with fertile controls.

Various studies have failed to demonstrate a difference in ROS, NO, lipid peroxide, and antioxidant levels in the peritoneal fluid of women with endometriosis compared to fertile women (38,10). This might be explained by the fact that only persistent markers of OS, such as enzymes or stable byproducts of oxidative reactions, may still be detected at the time endometriosis is diagnosed. Another possible reason might be that OS occurs only locally and therefore would not result in an increase in total peritoneal fluid ROS concentrations.

OS may contribute to the pathogenesis of endometriosis via molecular genetic pathways. Investigation of endometriotic tissue has yielded results showing gene deletion of mitochondrial

DNA resulting in its rearrangement. Differences in gene expression levels in ectopic and eutopic endometria have been elucidated, including 904 differentially expressed genes and the differential expression of the glutathione-S-transferase gene family, which are implicated in the metabolism of the potent antioxidant glutathione. The cellular responses to OS, which include cell proliferation and angiogenesis, may also be determined by differential gene expression (39).

An imbalance between ROS and antioxidant levels may play an important role in the pathogenesis of endometriosis-associated infertility. Increased concentrations of ROS in the oviductal fluid could have adverse effects on oocyte and spermatozoa viability and the process of fertilization and embryo transport in the oviduct. Also, the associated presence of activated neutrophils and macrophages and proinflammatory factors in the oviductal fluid could significantly amplify ROS production by foci of endometriosis (29). A significant increase in ROS production could result in oxidative damage to the sperm plasma and acrosomal membranes, leading to a loss of motility and the ability of spermatozoa to bind and penetrate the oocyte, respectively. OS resulting in DNA damage may lead to failed fertilization, reduced embryo quality, pregnancy failure, and spontaneous abortion.

Therapies for autoimmune diseases, which share many similarities with endometriosis, may be useful in treating endometriosis. Pathological levels of tumor necrosis factor alpha (TNF-α) may be present in the female reproductive tract in women with endometriosis. In the female endometrium, TNF-α plays a role in the normal physiology of endometrial proliferation and shedding and also in the pathogenesis of endometriosis (40). Abnormally high levels of TNF-α have been demonstrated in the peritoneal fluid of women with endometriosis, and an increase in its levels appears to be positively correlated with the stage of endometriosis (40,41).

It has been shown in an in vitro experiment that spermatozoa quality declined following incubation with TNF-α in a dose-dependent and time-dependent manner (42). This may offer some explanation for the endometriosis-associated infertility. In the same experiment, sperm motility and membrane and chromatin integrities were higher in the samples incubated with TNF-α plus infliximab (a monoclonal antibody that binds both soluble and membrane forms of TNF-α, neutralizing its toxic effects) than in the samples treated with TNF-α only. It has been suggested that infliximab could potentially be used to help treat female infertility caused by endometriosis in those with elevated levels of TNF-α in their peritoneal fluid.

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 (43).

OS and Hydrosalpinx

Although IVF is considered to be the best fertility treatment for hydrosalpinx, some investigations have shown the presence of a hydrosalpinx to decrease the success rates of IVF. Fluid within the hydrosalpinx appears to reduce embryo implantation rates and increase the risk of miscarriage, motivating some physicians to advise removing the tube or separating it from the uterus prior to undergoing IVF. The adverse effect of hydrosalpinges has been shown to be reversible by salpingectomy prior to IVF (44).

The exact mechanism by which hydrosalpingeal fluid (HSF) induces its embryotoxic effect is unknown, but it is hypothesized that an OS-mediated mechanism may be involved in this phenomenon. Lab studies have demonstrated the presence of ROS, antioxidants, and lipid peroxidation products in hydrosalpingeal fluid. At low levels, ROS in the tubal fluid has been shown to be positively correlated with blastocyst development. Low levels of ROS, beneath a threshold for being deleterious to embryos, may represent normal ROS generation by a functional endosalpinx, whereas extensive endosalpingeal damage may yield nondetectable levels of ROS in the HSF. Therefore, detection of ROS at low concentrations might serve as a marker of normal tubal secretory function. Levels of IL-6 in the HSF were found to be positively correlated with blastocyst development rates, suggesting its contribution in preventing some tubal fluid samples from becoming embrytoxic (Bedaiwy et al., 2005). IL-1b, which is known to inhibit ovarian follicular cell apoptosis, was also found in all tubal fluid samples tested and may be a marker of normal tubal secretory function (45).

Fluid leaking from Fallopian tubes enlarged with hydrosalpinx has been shown to exert a concentration-dependent embryotoxic effect (46). The presence of toxic substances, at high concentrations, in HSF is thought to mediate its negative effects. HSF has also been shown to reduce endometrial integrins, which may facilitate implantation. HSF flow into the endometrial cavity may lead to mechanical flushing of the embryos from the uterus. Excision of hydrosalpinges is suggested to restore integrins to normal and improve implantation rates. The tubal epithelium may secrete cytokines, leukotrienes, or prostaglandins into the sequestered fluid that could directly alter endometrial function. Cytokines, associated with inflammatory processes and involved in embryotoxic effects, have been implicated in the poor outcome of IVF-ET in women with distally occluded fallopian tubes. Bedaiwy et al. (2005) studied the biochemical nature of HSF and found TNF-α in 100 percent of all HSF samples. TNF-α is a cytotoxic, angiogenic cytokine, produced by macrophages and many other types of cells.

OS and Unexplained Infertility

It is hypothesized that elevated levels of ROS disturb the pro-oxidant/antioxidant balance in peritoneal fluid and may be the cause of infertility in women who do not have any other obvious cause. Elevated levels can damage the ovum after its release from the ovary, the zygote/embryo, and the spermatozoa, which are very sensitive to OS (8). Studies comparing ROS levels in peritoneal fluid between women undergoing laparoscopy for infertility evaluation and fertile women undergoing tubal ligation have shown levels of peritoneal fluid ROS to be significantly higher in patients with unexplained infertility compared with the fertile women (10). Elevated ROS levels in patients with unexplained infertility imply reduced levels of antioxidants such as vitamin E and glutathione, resulting in a reduced ability to scavenge ROS and neutralize its toxic effects (10). This was demonstrated in a study where concentrations of antioxidants in patients with unexplained infertility have been shown to be significantly lower than in fertile patients, suggesting a potential use for antioxidant

supplementation to treat the high levels of ROS in patients with idiopathic infertility.

AGE-RELATED FERTILITY DECLINE, MENOPAUSE, AND ROS

The fertility potential of the average woman begins to decline appreciably at the age of thirty-five years and begins to decline dramatically beyond the age of forty years. ROS may play a role in age-related decrease in estrogen production. SOD and glutathione peroxidase expression decreases in the ovary from the premenopausal to menopausal period (47). SOD and glutathione peroxidase levels are significantly and positively correlated with aromatase enzyme activity.

Higher levels of OS have been demonstrated in women of advanced reproductive age undergoing IVF (48). Ovarian senescence is thought to result from increased OS in the follicular fluid. Free radical–induced damage may be implicated, at least partly, for the age-related decline in quantity and quality of follicle reserves (49). This process may involve oxidative damage to mitochondrial DNA, proteins, and lipids. ROS are known to significantly perturb the intracellular calcium (Ca) homeostasis in the oocytes and cause aging of the oocytes (50). IVF patients of advanced reproductive age may exhibit a reduced expression of genes mainly involved in the neutralization of ROS (48) such as decrease in the expression of SOD1, SOD2, and catalase mRNA content, representing the first evidence that reproductive aging can downregulate the gene expression of granulosa cells (51). This downregulation of genes involved in the front-line defense against ROS was associated with the accumulation of oxidative damage mainly affecting mitochondria. Age-related changes that have adverse consequences in granulosa cells may be one of the mechanisms by which advanced reproductive aging causes a reduction in the developmental competence of oocytes. These changes may lead to effects often seen in pregnancies in older women, which include damaged cytoskeleton fibers, causing degeneration or apoptosis. In addition, impaired fertilization and poor-quality embryos, most of which are aneuploid, result from the aging process.

OS AND MALE GAMETES

The paternal genome is of paramount importance in normal embryo and fetal development. ROS-induced sperm damage during sperm transport through the seminiferous tubules and epididymis is one of the most important mechanisms leading to sperm DNA damage (52–57). These result in single- and double-stranded DNA fragmentation (primary damage) and the generation of secondary DNA damage of the 8-OH-2′-deoxyguanosine type. Fertilization of the oocyte by a spermatozoon with unrepaired primary or secondary DNA damage may result in implantation failure, embryo development arrest, pregnancy loss, or birth defects (58–61). In addition, recent studies suggest that sperm DNA fragmentation may be associated with an increase in sperm aneuploidy (61,62). Sperm aneuploidy is mainly the result of meiotic alterations during spermatogenesis (63). ROS- and/or caspase or endonuclease-induced DNA fragmentation may be increased in aneuploid sperm during passage through the epididymis (57). Therefore, couples diagnosed with recurrent pregnancy loss may benefit from testing of sperm DNA fragmentation.

OS AND ITS IMPACT ON ART

OS has an important role in ovulation process. We discussed above how follicular fluid microenvironment has a crucial role in determining the quality of the oocyte. The oocyte quality in turn impacts the fertilization rate and the embryo quality. OS markers have been localized in the follicular fluid in patients undergoing IVF/embryo transfer (5,64–66).

Low intrafollicular oxygenation has been associated with decreased oocyte developmental potential as reflected by increasing frequency of oocyte cytoplasmic defects, impaired cleavage, and abnormal chromosomal segregation in oocytes from poorly vascularized follicles (67). ROS may be responsible for causing increased embryo fragmentation, resulting from increasing apoptosis (68) (refer Figure 64.1). Thus, increasing ROS levels are not conducive to embryo growth and result in impaired development. Current studies are focusing on the ability of growth factors to protect embryos cultured in vitro from the detrimental effects of ROS such as apoptosis. These growth factors are normally found in the fallopian tubes and endometrium. The factors being investigated are insulin growth factor, and epidermal growth factor in mouse embryos, which in many respects are similar to human embryos (69).

The effects of follicular OS on oocyte maturation, fertilization, and pregnancy have also been studied (66). Follicular fluid ROS and lipid peroxidation levels may be markers for success with IVF. Patients who became pregnant following IVF or ICSI had higher lipid peroxidation levels and TAC in follicular fluid. However, both markers were unable to predict embryo quality. Pregnancy rates and levels of lipid peroxidation and TAC demonstrated a positive correlation. Levels of follicular fluid ROS were reported to be significantly lower in patients who did not become pregnant compared with those who became pregnant (5). Thus, intrafollicular ROS levels may be viewed as a potential marker for predicting success with IVF. OS in follicular fluid from women undergoing IVF was inversely correlated with the women's age (48). Using a thermochemiluminescence assay, the slope was found to positively correlate with maximal serum estradiol levels, number of mature oocytes, and number of cleaved embryos and inversely with the number of gonadotrophin ampoules used. The pregnancy rate achieved was 28 percent, and all pregnancies occurred when the thermochemiluminescence amplitude was small. This is in agreement with another study that reported minimal levels of OS were necessary for achieving pregnancy (66). A recent large study in 156 couples undergoing ART demonstrated high follicular fluid levels of homocysteine and their inverse association with embryo quality in women with endometriosis (70). Elevated homocysteine levels are caused by heightened OS and lead to poor oocyte and embryo quality in women with endometriosis. However, the view that low levels of ROS in follicular fluid have beneficial effects on IVF outcomes (4) is not universally held. Recent studies have reported that high levels of ROS in follicular fluid lead to decreased fertilization potential of the oocytes in ART cycles (71).

Other OS markers such as thiobarbituric acid–reactive substances, conjugated dienes, and lipid hydroperoxides have been studied in the preovulatory follicular fluid (17). No correlation was seen between these markers and IVF outcome (fertilization rates or biochemical pregnancies) (17).

8-Hydroxy-2-deoxyguanosine is a reliable indicator of DNA damage caused by OS. This compound is an indicator of OS in

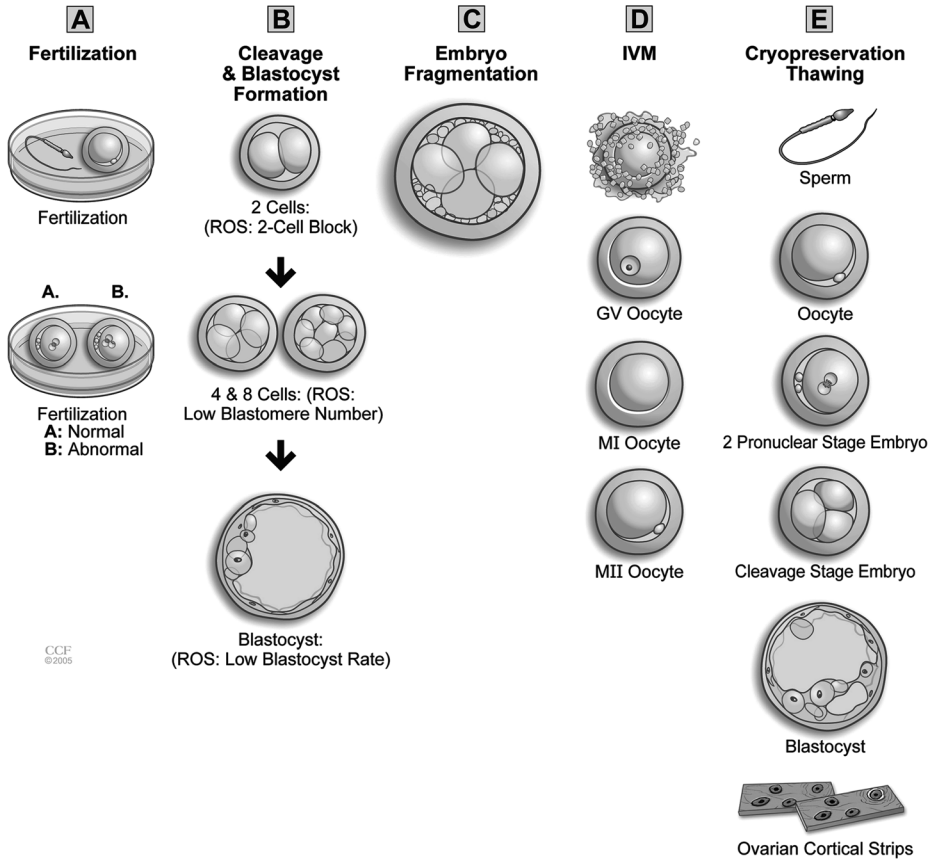

Figure 64.1. Impact of OS in different ART settings.

various other disease processes such as renal carcinogenesis and diabetes mellitus. Higher levels of 8-hydroxy 2-deoxyguanosine were associated with lower fertilization rates and poor embryo quality (72). High levels of 8-hydroxy 2-deoxyguanosine are also found in granulosa cells of patients with endometriosis, and this may impair the quality of oocytes.

Bedaiwy et al. in a study from our group reported that slow early embryo development (seven cells on day 3), high fragmentation (10 percent), and reduced formation of morphologically normal blastocysts may be associated with increased levels of ROS in the culture media on day 1. Moreover, high day 1 ROS levels in culture media had no relationship with the fertilization rate (FR) in conventional IVF cycles but were significantly related to higher FRs and blastocyst development rates with ICSI cycles (46).

Literature reports have shown that women who became pregnant after IVF therapy had a tendency toward higher levels of TAC in their follicular fluid compared to those who did not achieve pregnancy (66). The mean TAC in fluid from follicles that yielded oocytes that were successfully fertilized was significantly greater than the mean TAC from follicular fluid associated with oocytes that were not. Similarly, mean glutathione peroxidase levels were increased, in follicles yielding oocytes that were subsequently fertilized (73). Conversely, the mean TAC of fluid from follicles whose oocyte gave rise to an embryo that survived till time of transfer was reported to be significantly lower than the mean TAC in follicular fluid associated with oocytes that gave rise to nonviable embryos (64). TAC levels in day 1 culture media appear to be an additional biochemical marker reflecting the OS status during early embryonic growth. Day 1 TAC levels significantly correlated with the clinical pregnancy rates in ICSI cycles (74). On a different front, high TAC level has been reported as a marker for poor response to ovulation induction in women with polycystic ovarian syndrome (75).

Levels of selenium in follicular fluid of women with unexplained infertility were found to be lower than those in women with tubal factor or male factor infertility (73). Higher levels of SOD activity were present in fluid from follicles whose oocytes did not fertilize compared with those that did (76). These discrepancies may be due to the fact that the studies measured different parameters.

Smoking has been associated with prolonged and dose-dependent adverse effects on ovarian function (77). According to a meta-analysis, the overall value of the odds ratio for the risk of infertility associated with smoking was 1.60 [95 percent confidence interval (CI) 1.34–1.91]. ARTs, including IVF, are further shedding light on the effects smoking has on follicular health. Intrafollicular exposure to cotinine increases lipid peroxidation in the follicle.

Further large studies need to determine the correlation between ROS activity levels and TAC levels in the follicular fluid on ovulation process, oocyte quality, developmental competence, and fertilization potential of oocytes, as ROS may be having differing effects at different stages of ART.

REDOX AND EMBRYO DEVELOPMENT

Physiological levels of redox may be important for embryogenesis (refer Figure 64.2). Overproduction of ROS is detrimental

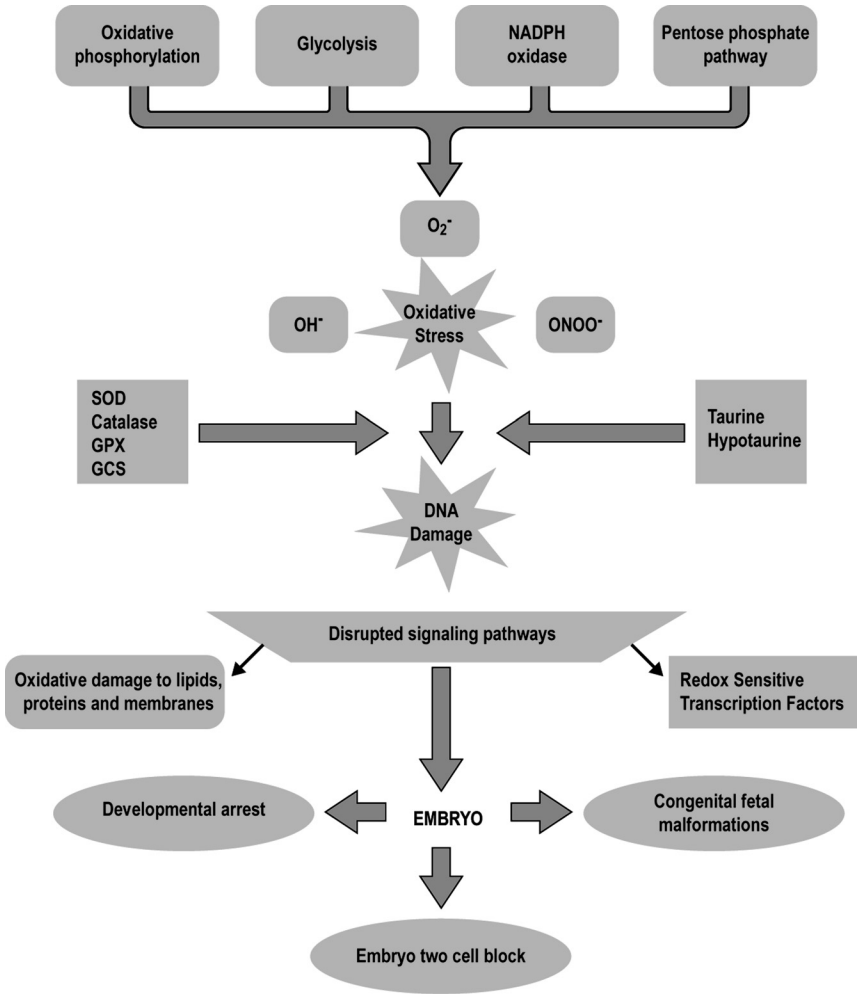

Figure 64.2. Redox and embryo development.

for the embryo, resulting from impaired intracellular milieu and disturbed metabolism (78,79). OS can be generated in the sperms and leucocytes and on sperm-mediated oocyte activation and on the activation of the embryonic genome. Oxidative phosphorylation, NADPH oxidase, and xanthine oxidase are predominant sources of ROS generation in oocytes and embryos. Oxidative phosphorylation is a process necessary for the generation of ATP in order to meet embryo energy requirements, and it results in ROS production. Electrons leak from the electron transport chain at the inner mitochondrial membranes. These electrons are transferred to the oxygen molecule, resulting in an unpaired electron in the orbit. This leads to the generation of the superoxide molecule. The other points of generation of ROS are the cytoplasmic NADPH oxidase, cytochrome p450 enzymes, and the xanthine oxidoreductase enzymes. Excessive OS can have deleterious effects on the cellular milieu and can result in impaired cellular growth in the embryo or apoptosis resulting in embryo fragmentation. Thus, OS-mediated damage of macromolecules plays a role in fetal embryopathies. Thioredoxins are a widely distributed group of small proteins with strong reducing activities, and their expression was found to be essential for early differentiation and morphogenesis of the mouse embryo (80).

Deficient folate levels in the mother result in elevated homocysteine levels. The homocysteine-induced OS has been proposed as a potential factor for causing apoptosis and disrupting palate development and causing cleft palate (81). OS-mediated damage of the macromolecules has been proposed as a mechanism of thalidomide-induced embryopathy (82,83). Hyperglycemia/diabetes-induced downregulation of cycloxygenase-2 gene expression in the embryo results in low PGE2 levels and diabetic embryopathy (84).

Preimplantation embryos are not a static entity as demonstrated by sequential culture. Embryos pass through many hurdles during their developmental process and have ever-changing needs. Preimplantation embryonic development is associated with a change in preference of energy metabolism pathways. Embryos possess inherent energy requirements that are met by ATP generation from oxidative phosphorylation and glycolysis. Blastocyst development is accompanied by a shift in pathway of ATP generation from oxidative phosphorylation to an increasing dependence on ATP generation from glycolysis. Increased glucose uptake in the postcompaction stage meets the increased energy demands of an embryo. Blastocyst development from the two-cell-stage embryo is modulated by the ratio of pyruvate to lactate in the culture medium as this in turn

affects the intracellular pyruvate to lactate ratio. Excessive generation of ROS occurs at certain critical points accompanied by increased energy demands such as embryonic genome activation, embryonic compaction, and hatching (85). Minimal levels of ROS may play a role during the critical points of embryogenesis. Excessive levels of ROS have adverse impacts on embryo quality and competence (6,78,86). The literature reports that a reduction in OS levels leads to better ART outcomes (87,88).

In the ART setting, a majority of retrieved mature oocytes fertilize, but of these only up to 70 percent undergo the first three cleavage divisions during the first three days in culture (89). Less than 50 percent of the cleaved embryos undergo cavitation and proceed to blastocyst formation by day 5 in culture (90,91). Similarly, only approximately 30 percent of day 3 embryos will progress to develop into morphologically normal blastocysts. OS has been implicated as a causative factor associated with the poor fertility outcomes in ART.

During the first trimester, an embryo grows best under low oxygen concentrations as documented in maternofetal oxygen diffusion studies (92). In human embryos, elevated blastulation rates have been reported by decreasing the oxygen tension (5 percent O_2) and maintaining low illumination levels throughout the embryo manipulation period (93). High O_2 concentrations during in vitro cultures lead to an increase in hydrogen peroxide (H_2O_2) levels, DNA fragmentation, and reduction in embryo development competency. ROS such as H_2O_2 are responsible for programmed cell death, also known as apoptosis, and may cause the failure of blastocyst development and preimplantation embryo death. An animal study has emphasized the protective role of the enzyme G6PD (glucose 6-phosphate dehydrogenase) against OS. The protection of the embryos against OS prevented the embryopathies (94).

Early embryo development in mammals, from fertilization through differentiation of principal organ systems, occurs in a low-oxygen environment (83). A marginal improvement in preimplantation embryonic viability has been reported under low oxygen concentrations in patients undergoing IVF and ICSI (95). Lower concentrations of oxygen in in vitro culture of porcine embryos decreased the H_2O_2 content and resulted in reduced DNA fragmentation, which thereby improved developmental ability (96). The higher oxygen concentrations of 20 percent have been associated with lower developmental competence. Accelerated development was seen under low (5 percent) oxygen concentrations.

OS AND GAMETE CRYOPRESERVATION

ROS are generated from the semen cells during the cooling process (97). Cryopreservation enhances lipid peroxidation, as ROS-induced membrane lipid damage, DNA damage, and apoptosis have been demonstrated in frozen spermatozoa (97). Reduction of antioxidant defenses also adversely affects cryopreserved spermatozoa. Supplementation of the thawing media with antioxidants such as glutathione helps improve spermatozoa function and in vitro fertilizing capabilities (98). Cryopreserved oocytes because of oxidative toxicity were reported to show DNA damage on comet assay (99).

Ovarian transplantation and oocyte cryopreservation continue to have poor outcomes, which have been proposed to be caused by ischemia and resultant OS. Ischemia time strongly correlates with the ovarian tissue damage, and treatment with the antioxidant vitamin C reduces the stromal tissue apoptosis and damage (100).

STRATEGIES TO OVERCOME OS IN ASSISTED REPRODUCTION

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 (refer Figure 64.1). In human IVF/ICSI procedures, the clinical pregnancy rates have remained unchanged at 30–40 percent (101). It is hypothesized that the altered redox state in in vitro conditions may play a role in poor ART outcomes, and controlling OS may improve ART outcomes (68,78). Fertilization and embryo development in vivo occur in an environment of low oxygen tension (83). It has been noted 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 and apoptosis (102).

During ART procedures, it is important to emulate in vivo conditions by avoiding conditions that promote ROS generation. Achieving that has been shown to lead to a reduction in blastocyst degeneration, increased blastocyst development rates, increased hatching of blastocysts, and reduction in embryo apoptosis, and other degenerative pro-oxidant influence has been reported (78). The available strategies include the following.

1. Ensuring in vitro culture under low–oxygen tension conditions: During culture, low–oxygen tension conditions improve the implantation and pregnancy rate better than high oxygen tension (103).
2. Metal ion culture media supplementation: It has been shown that metal ions may enhance the production of oxidants. As a result, it was suggested that it may be useful to add metal ion–chelating agents to culture media to decrease the production of oxidants (103).
3. Enzymatic and nonenzymatic antioxidant culture media supplementation: Higher implantation and clinical pregnancy rates are reported when antioxidant-supplemented media is used rather than standard media without antioxidants. Various nonenzymatic antioxidants including beta-mercaptoethanol (104), protein (102), vitamin E (96), vitamin C (105,106), cysteamine (107,108), cysteine (109), taurine and hypotaurine (110), and thiols (111) added to the culture media with the purpose of improving the developmental ability of the embryos by reducing the effects of ROS.

Also, the addition of the enzymatic antioxidant, for example, SOD to the culture media prevented the deleterious effects of OS on sperm viability and on the embryo development both in vivo and in vitro (112). This was demonstrated by increased development of the two-cell-stage embryos to the expanded blastocyst stage in the SOD-supplemented media. Mechanical removal of ROS in IVF/ET has been studied as a method to improve IVF outcome (113). The rinsing of cumulus oophorus has been shown to overcome the deleterious effects of ROS in patients with ovarian endometriosis (113).

4. Control of sperm ROS production and sperm chromatin damage: 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 (114). The seminal plasma is rich in antioxidants and protects the spermatozoa from DNA damage and lipid peroxidation (115). Sperm preparation techniques such as density gradient centrifugation and glass wool separation reduce the ROS formation by removing the leucocytes, cellular debris, and immotile spermatozoa. It has been shown that sperm preparation methods affect ART outcomes. Sperm preparation by centrifugation may be associated with generation of ROS. Taurine, an essential amino acid, is an antioxidant that has been shown to improve spermatozoa motility, capacitation, and fertilization and support early embryonic development (116). Also many antioxidant (vitamins C and E, glutathione and beta-carotene, pentoxifylline, etc.) supplementation of sperm preparation media have been shown to improve sperm motility and acrosome reaction (117,118). Supplementation of IVF media with *N*-tert-butyl hydroxylamine and SOD/catalase mimetics was reported to block the breakdown of sperm chromatin (119). Standard sperm preparation media are supplemented with human serum albumin, polyvinylpyrrolidine, and HEPES, which are DNA protectors (120). Adding ascorbate during cryopreservation reduces the levels of hydrogen peroxide and thus the OS in mammalian embryos (89). As a consequence, embryo development improved with enhanced blastocyst development rates.

5. Reducing sperm-oocyte coincubation time: Reports suggest that a prolonged sperm-oocyte coincubation time (sixteen to twenty hours) increases the generation of ROS. Two prospective randomized controlled studies have advocated using a shorter sperm-oocyte coincubation time (121,122). Coincubation times of one to two hours resulted in better quality embryos and significantly improved fertilization and implantation rates (123).

TROPHOBLASTIC OS AND PREGNANCY: ROLE IN ABORTIONS, HYDATIDIFORM MOLE, AND PREECLAMPSIA

Although oxygen is essential for sustaining life in cells, it undergoes extensive metabolism that can result in the production of toxic derivatives. This metabolism is mainly confined to the electron transport chain in the mitochondria that ultimately results in the generation of ATP, which supports cell metabolism. The end products of oxygen metabolism may include molecules in an activated electronic state that have unpaired electrons and are highly reactive with molecules found in biological systems. Collectively, these activated molecular species derived from oxygen metabolism are designated as ROS (124). ROS extensively damage cellular organelles including the mitochondria, nuclear and mitochondrial DNA, and cell membrane, ultimately leading to cellular demise (125–127).

Normal human placentation is determined for the most part by the proper invasion of the uterine spiral arteries by a genomically normal trophoblast. This invasion governs the changes in the anatomy of the placental vasculature to ensure optimum perfusion by the maternal vessels. Definite metabolic changes occur in embryos during the transition from first to second trimester. It is evident that during the period of embryonic organogenesis, the prevailing oxygen tension is low and metabolism is largely anaerobic (128). Thus, the production of ROS is reduced perhaps to prevent DNA damage induced by oxidants. This is also supported by animal research that shows increased blastocyst rate at low oxygen tension (129). At the end of the first trimester, there occurs a definite rise in oxygen tension in the intervillous space from less than 20 mmHg to more than 50 mmHg (130,131), leading to a burst in OS. Studies show that lower oxygen tension in the first trimester stimulates the invasive capacity of the trophoblast (132). This is probably due to increased activity of integrins that help trophoblast cells to proliferate. Persistent low oxygen tension also diminishes placental proliferation and invasion, and hence increased oxygen tension enables persistence of cytotrophoblast proliferation (133). It is suggested that impaired placental development or degeneration of syncytiotrophoblast in early pregnancy may be an effect of placental OS that may lead to complications such as recurrent abortions, preeclampsia, and congenital anomalies in diabetes (83). Several biomarkers have been associated with preeclampsia and increased OS, and some of the primary culprits are NOS-1, an isoform of NADPH oxidase, and endothelin 1 (134). It is possible that some of these factors may play an inhibitory role in cell proliferation and maturation and trigger OS in the human placenta by altering the balance between oxidant (increased MDA levels) and antioxidants (decreased GSH, GSSG, and AA). This can result in cell apoptosis leading to derangements in placental invasion and early abortion. In one study, the placental circulation was investigated using immunohistochemical analysis for heat shock protein (HSP 70i), a marker for cellular stress such as nitrotyrosine residues, and hydroxynonenal, as markers of protein and lipid oxidative damage, respectively (135). In this case-control study in normal pregnancies, intervillous blood flow increased with gestational age, being detected in nine of twenty-five cases at eight to nine weeks but in eighteen of twenty at twelve to thirteen weeks.

OS AND ITS RELATIONSHIP TO IVM OF OOCYTES

Follicle development and maturation of the oocyte is a dynamic process with high levels of metabolic activity. In vitro maturation of oocytes constitutes the in vitro advancement from diplotene stage of prophase I to metaphase II oocyte, along with cytoplasmic maturation, which is essential for the fertilization and early development of the embryo. The free radicals generated during this phase are numerous. Whenever the balance between the pro- and antioxidants in the cell is disturbed, OS results. Preserving fertility and treatment of infertility has merged in the recent years, thus introducing the concepts of in vitro maturation of immature oocytes that otherwise would have been lost in the physiological process. There are many scientific reports in the literature that analyze the potential correlation between the various markers of OS and the antioxidant protection with oocyte quality, developmental competence, fertilization capability, and blastocyst development. Various challenges hinder the selection of in vitro maturation of oocyte as an established mode of assisted reproduction in spite of the advantages of absent hyperstimulation syndrome and low cost. It is evident that the quality of in vitro mature oocytes are suboptimal since embryos derived from them have increased cleavage blocks. A well-documented fact that may be responsible for varied effects on cells is the generation of OS within cell culture media. It is evident that increased OS

generated in the in vitro media may have varied effects on ART outcomes (78). The generation of ROS being an invariable phenomenon in external culture may also influence follicular and oocyte development in vitro. The oxidative insult on developing oocytes might be a responsible factor for low outcomes in IVM or may be responsible for aneuploidy in the fertilized oocytes (136). This phenomenon has led researchers to find methods to prevent such cell damage due to OS, but the final consensus for supplementing media with antioxidants in order to enhance oocyte development and quality is yet to be achieved though empirical usage seems to be feasible.

ROLE OF THERAPEUTIC ANTIOXIDANT SUPPLEMENTATION

Antioxidant supplementation of sperm preparation and gamete culture media was discussed in detail above. In this section, the preconceptional therapeutic use of antioxidants is discussed. It has been shown that OS results in luteolysis and that oral antioxidant supplementation, for example, vitamin C and vitamin E, have beneficial effects in preventing luteal phase deficiency and resulting in higher pregnancy rate (137,138). Other studies failed to demonstrate this favorable effect of antioxidant supplementation (139). A meta-analysis investigating the effect of vitamin C supplementation on pregnancy outcome was inconclusive (140). Another meta-analysis that used the fixed effects' model for women taking any of the vitamin supplements starting prior to twenty weeks gestation revealed no reduction in total fetal losses or in early and late miscarriage (141). Improved pregnancy rates were also reported with combination oral therapy with the antioxidants pentoxifylline and vitamin E supplementation for six months in patients with thin endometrium, undergoing IVF with oocyte donation because of history of radiotherapy (142).

Since OS can induce sperm dysfunction, many recent literature reports have emphasized the importance of the beneficial antioxidant effects of folate and zinc in male subfertility. Nutritional factors such as folate, zinc, and thiols may lead to fertility enhancement through their antiapoptotic effect and by prevention of DNA damage (143). Although many advances are being made in the field of antioxidants therapy, the data are still debatable and need further controlled evaluations in larger population.

CONCLUSIONS

ROS plays an essential role in the pathogenesis of many reproductive processes. In male factor infertility, OS attacks the fluidity of the sperm plasma membrane and the integrity of DNA in the sperm nucleus. ROS-induced DNA damage may accelerate the process of germ cell apoptosis, leading to the decline in sperm counts associated with male infertility. OS modulates a range of physiological functions and plays a role in pathological processes affecting female reproductive life span and even thereafter, that is, menopause. ROS-mediated female fertility disorders share many pathogenic similarities with the ones on the male side. The role of OS is becoming increasingly important as there is newer evidence of its role in conditions such as polycystic ovarian disease, abortions, preeclampsia, hydatidiform mole, fetal embryopathies, preterm labor, and intrauterine growth retardation. 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, ongoing trials with antioxidant supplementation, which will provide evidence on the safety and effectiveness of antioxidants and if they could improve the maternal and fetal outcomes. High follicular fluid ROS levels are associated with negative IVF outcomes, particularly in smokers. Successful management of infertility in the ART scenario depends on overcoming OS in the in vitro conditions.

KEY POINTS

- OS has been implicated in different reproductive scenarios such as endometriosis, folliculogenesis, oocyte maturation, and sperm DNA damage and is detrimental to both natural and assisted fertility.
- Many extrinsic and intrinsic conditions exist in ART setting that can be modified to reduce the toxic effects of ROS.
- ART laboratory personnel should avoid procedures that are known to be deleterious, especially when safer procedures preventing OS can be used.
- Although nutritional factors folate, zinc, and thiols may lead to fertility enhancement, the data are debatable and need evaluation in controlled studies on large population.

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