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Comprehensive Invited Review

Redox Considerations in Female Reproductive Function and Assisted Reproduction: From Molecular Mechanisms to Health Implications

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ABSTRACT

Physiological levels of reactive oxygen species (ROS) play an important regulatory role through various signaling transduction pathways in folliculogenesis, oocyte maturation, endometrial cycle, luteolysis, implantation, embryogenesis, and pregnancy. Persistent and elevated generation of ROS leads to a disturbance of redox potential that in turn causes oxidative stress (OS). Our literature review captures the role of ROS in modulating a range of physiological functions and pathological processes affecting the female reproductive life span and even thereafter (*i.e.*, menopause). The role of OS in female reproduction is becoming increasingly important, as recent evidence suggest that it plays a part in conditions such as polycystic ovarian disease, endometriosis, spontaneous abortions, preeclampsia, hydatidiform mole, embryopathies, preterm labor, and intrauterine growth retardation. OS has been implicated in different reproductive scenarios and is detrimental to both natural and assisted fertility. Many extrinsic and intrinsic conditions exist in assisted reproduction settings that can be tailored to reduce the toxic effects of ROS. Laboratory personnel should avoid procedures that are known to be deleterious, especially when safer procedures that can prevent OS are available. Although antioxidants such as folate, zinc, and thiols may help enhance fertility, the available data are contentious and must be evaluated in controlled studies with larger populations. *Antioxid. Redox Signal.* 10, 1375–1403.

I. INTRODUCTION

FREE RADICAL SPECIES are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrates, or any nearby molecule, causing a cascade of chain reactions resulting in cellular damage and disease. There are two major types of free radical species: reactive oxygen species (ROS) and reactive nitrogen species (RNS).The three major types of ROS are superoxide ($O_2^{-\cdot}$), formed when electrons leak from the electron transport chain; hydrogen peroxide (H_2O_2), resulting from the dismutation of superoxide or directly from the action of oxidase enzymes, and hydroxyl ($HO^{\cdot}$), a highly reactive species that can modify purines and pyrimidines and cause strand breaks that result in DNA damage. ROS, which have been implicated in >100 diseases, have both physiological and pathological roles in the female reproductive tract.

Free radicals and electrons are important messengers that operate in cell-signaling pathways in the redox chain. The effects of free radicals are complex and multifaceted. Reproductive cells and tissues remain stable when free radical production and the scavenging antioxidants remain in balance. The sequential mechanism of $O_2^{-\cdot}$ and H_2O_2 generation, mitochondrial damage, caspase activation, and apoptotic induction can affect the oocytes as well as the embryo. ROS can modulate cellular functions, and OS can impair the intracellular milieu, resulting in diseased cells or endangered cell survival.

ROS can affect a variety of physiological functions in the reproductive tract, and excessive levels can result in various pathologies affecting female reproduction. Oxidant status can influence early embryo development by modifying key transcription factors and hence modifying gene expression.

This review will focus on redox homeostasis and OS generation in female reproduction and elucidate the role of ROS in physiological processes such as folliculogenesis, oocyte maturation, ovulation, corpus luteum formation, endometrial cycle, luteolysis, implantation, embryogenesis, and pregnancy. The importance of the critical balance that exists between proxidants and antioxidants in the female reproductive tract is discussed, and the impact of oxidative stress, DNA damage, and apoptosis on gamete and embryonic development is described. The review provides evidence from the literature on oocyte and embryonic metabolism and its close linkage to their redox potential. Our review also highlights that OS in the male reproductive tract has become a matter of concern, due to the potentially toxic effects of uncontrolled ROS levels on sperm quality and function, and amelioration of seminal OS is important for natural and assisted fertility. The review encapsulates the role of OS which is becoming increasingly important as new evidence suggests that it plays a role in pathological conditions such as polycystic ovarian disease, endometriosis, spontaneous abortions, preeclampsia, hydatidiform mole, fetal teratogenicity, preterm labor, and intrauterine growth retardation (IUGR). It also highlights how OS modulates natural and assisted fertility and the importance of strategies to intercept OS to overcome its adverse effects.

II. REDOX HOMEOSTASIS AND GENERATION OF OXIDATIVE STRESS

Redox status is determined by a balance between pro-oxidant generation and neutralization by antioxidants. Maintaining redox homeostasis is important to the generation of high quality gametes and embryos. Altering the redox status may impact signaling pathways, transcription factors, and epigenetic mechanisms and cause a reduction in oocyte and embryo quality. Thus, an understanding of the reactions that maintain normal redox homeostasis will provide insight into the mechanisms behind the generation of OS response in the female reproductive function and during assisted reproduction techniques (ART).

Redox reactions, or oxidation–reduction reactions, involve the transfer of electrons, resulting in an oxidized or reduced state. Compounds that have a negative reduction potential (calculated from electron flow) are considered strong reducing agents. A negative reduction potential correlates with a lower affinity for electrons; thus, these compounds become oxidized.

Conversely, compounds with a positive reduction potential are stronger oxidizing agents. They have a higher affinity for electrons and undergo reduction. An example is molecular oxygen, a known oxidizing agent with an extreme affinity for electrons. Oxygen in its radical form may react easily with fatty acids, altering the biological structure of membranes (28). Therefore, the amount of catalytic oxygen must be monitored when the radical form of oxygen is present.

ROS are normally formed during the reduction of oxygen by free electrons as a by-product of various metabolic pathways and contribute to the cell's redox status (see Fig. 2) (9). Ninety-eight percent of inspired oxygen is reduced during lipolysis, with the production of chemical energy and several other biologic processes in the human body. Incomplete reduction of the remaining 2% leads to the formation of the three major types of ROS. Inhibiting oxidative phosphorylation decreases the concentration of ROS, which suggests that this metabolic cycle leads to the generation of ROS. Furthermore, since the inhibition of NADPH oxidase (which produces the superoxide radical and H_2O_2) and xanthine oxidase has the same effect, the same conclusions can also be applied (85). ROS includes radical species with an unpaired electron, such as superoxide, hydroperoxyl, hydroxyl, peroxyl, and alkoxyl radicals (see Fig. 2). The nonradical derivates of the radical species are H_2O_2, HOCL, fatty acid derivatives, hydroperoxides, reactive aldehydes, and singlet oxygen (88).

ROS also may be derived from exogenous sources. For example, in a hyperoxic environment, the enzymatic activity for the generation of the superoxide radical increases. Certain metallic cations such as copper (Cu) and iron (Fe) also may contribute significantly to the generation of ROS (89). However, metallic ion chelators such as ethylenediamine tetra-acetic acid (EDTA) and transferrin work to bind metals, thereby inhibiting their ability to react and produce ROS. Visible light also can be a factor in ROS production, possibly as a result of the oxidation of bases in DNA or DNA strand breaks (85).

Reactive nitrogen species (RNS) include nitric oxide (NO) and nitrogen dioxide, as well as nonreactive species such as peroxynitrite, nitrosamines, and others. NO, an important RNS that is present in the body, is specifically synthesized by nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline (see Fig. 3). Although this molecule has been reported to act therapeutically as a vasodilating agent, excess NO is toxic, and maintaining a normal concentration of NO is vital (4). NO has an unpaired electron, making it a highly reactive free radical that can damage proteins, carbohydrates, nucleotides, and lipids. Together with other inflammatory mediators, it results in cell and tissue damage, low grade, sterile inflammation, and adhesions (59). NOS have been shown to generate H_2O_2, superoxide, and NO. Superoxide generation is increased in L-arginine-depleted cells. Under these conditions, superoxide reacts with NO, resulting in increased generation of peroxynitrite and cell toxicity. Moreover, the specific NOS blocker *N*-nitro-L-arginine methyl ester blocks the generation of superoxide and peroxynitrite. Also, peroxynitrite and its metabolite are capable of inducing cytotoxic effects by causing lipid peroxidation and nitrozation of several tyrosine molecules that regulate enzyme function and signal transduction and Na^+ channel inactivation. Together, these findings suggest that the action of NO in a cell depends on its concentration; the cellular redox state; and the abundance of metals, protein, thiols, and low-molecular weight thiols, as well as other nucleophilic targets (185).

The effects of NO are proposed to be mediated through cyclic guanosine monophosphate (cGMP) as a second messenger or by generation of ROS resulting from interaction of NO with superoxide radicals (86). NO effects are concentration dependent. At low concentrations ($<1\ \mu M$), the transduction effect of NO is mediated by activation of soluble guanyl cyclase and mediated through cGMP (90, 185).

III. REDOX PATHWAYS IN THE CONTROL OF PHYSIOLOGICAL EVENTS IN FEMALE REPRODUCTION

According to Inoue *et al*. (108), mitochondrial generation of ROS occurs mainly at complex I (where NADH dehydrogenase acts) and complex III, a component of the Q-b (where conversion of ubiquinol to ubisemquinone to ubiquinone takes place) (Fig.1). At complex IV in the electron transport chain (ETC), molecular oxygen normally is converted to water. However, as the electrons are being passed down the ETC to produce adenosine 5′-triphosphate (ATP), molecular oxygen may gain an electron. As molecular oxygen combines with this lone electron, it is transformed into the fast-reacting superoxide molecule, which may react with fatty acids (Fig. 1) (38, 85, 108). This may induce a morphological change in the cell that can result in questionable cell viability or lead to apoptosis (28).

The contribution of ETC to ROS generation is balanced by antioxidant systems. Certain drugs such as rotenone, thenoyltrifluoroacetone, and antimycin A have been shown to inhibit the actions of complex I, II, and III, respectively. Cytochrome c is the fourth complex in the ETC sequence, and inhibitive actions of NO, carbon monoxide, or cellulose nitrate (CN) at this complex may cause an overflow of free electrons. These electrons are dangerous because they can potentially lead to a reaction with molecular oxygen that converts it to the superoxide radical (Fig. 2). However, the superoxide radical is converted into H_2O_2 by mitochondrial SOD_2 (superoxide dismutase 2) and further modified by GSH peroxidase to produce

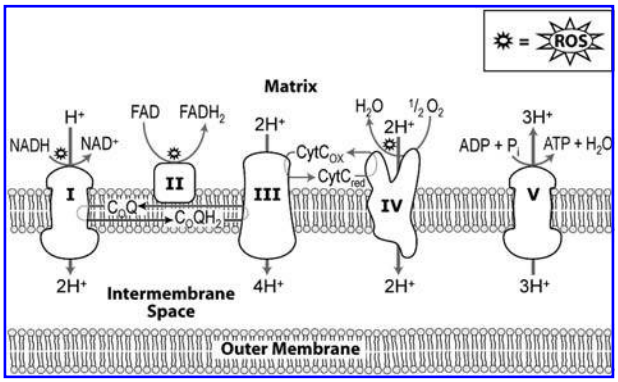

FIG. 1. ROS production from the electron transport chain.

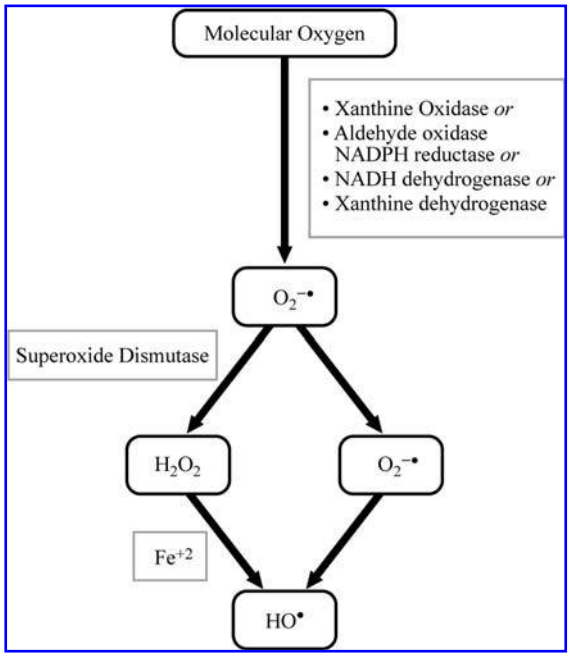

FIG. 2. Generation of superoxide radical.

water. The presence of these antioxidants is vital to maintain the homeostatic operations of the ETC. If a decrease in these antioxidants occurs, overproduction of ROS and cell damage will result (108).

Inoue *et al*. also showed that superoxide radicals may be generated in the mitochondria and the peroxisomes. Dismutation reactions by Cu,Zn-SOD and manganese (Mn-SOD) in both cellular organelles can neutralize excess ROS. ROS has been shown to decrease GSH levels, resulting in apoptosis (28, 108).

The metabolism of oocytes and embryos is tightly regulated and is an important determinant of their redox potential. Glycolysis, Kreb's cycle, and the pentose phosphate pathways are the oocyte's main metabolic pathways. Glucose is metabolized via the glycolytic pathway in cumulus cells and through the pentose phosphate pathway in oocytes. A surge in oocyte metabolism at the recommencement of meiosis may cause an oxidative burst. Dumollard *et al*. (63) studied the role of glucose in mouse oocytes, an ideal model due to its remarkable similarities to human gametes. It was hypothesized that glucose served no role in mouse oocytes since glycolysis was assumed to be inhibited. This group also discovered that reduced nicotinamide adenine dinucleotide (NADPH) in the mouse oocyte is not supplied by the pentose phosphate pathway; therefore glucose metabolism would not affect the intracellular redox potential.

Although Dumollard *et al*. discovered that glucose metabolism was not a crucial factor in any redox pathways in the mouse oocytes; they did find that pyruvate and lactate are essential in this model. Lactate dehydrogenase was found to directly reduce the redox potential of the cell. Collectively, it was found that lactate dehydrogenase synthesizes pyruvate and lactate in the cytosol to set the cytosolic redox potential (63).

Dumollard and colleagues also demonstrated that NADPH is regenerated through the conversion of isocitrate to α-ketoglutarate via NADP dependent-isocitrate dehydrogenase (NADP-ICDH). Therefore, maintenance of these two pathways is vital to prevent OS (62).

Oxidative insults to the oocytes are more likely under *in vitro* conditions due to exposure to higher oxygen concentrations than occur *in vivo*. Conflicting evidence exists as to whether hypoxia results in an increase or decrease in ROS. Reports in the literature demonstrate that hypoxia modulates the redox status differentially under *in vivo* and *in vitro* conditions. Mitochondrial generation of ROS by cardiomyocytes increases in response to hypoxia (128), and prolonged exposure to hypoxia changes the redox status of the cytochrome c oxidase enzyme (43). This is particularly important with respect to reproductive processes, as oxidative stress due to hypoxia is considered a contributing factor in preeclampsia (18), whereas OS due to hyperoxia is more pertinent in assisted reproductive technologies (ART) and *in vitro* conditions for gametes and embryos (121, 176).

Complex interaction between media supplements and oocyte metabolism sets the oxidation reduction potential *in vitro*. Simulating the concentration of follicular fluid metabolites such as glucose and lactate in the *in vitro* maturation media has been investigated in caprine oocytes (98). The glucose and pyruvate concentrations did not have any adverse effects on the developmental competence of oocytes. In porcine and mammalian oocytes, contradictory findings have demonstrated that glucose metabolism is critical to nuclear and cytoplasmic maturation through the glycolytic and PPP pathways (97, 251). Energy sources were reported to be critical in resumption of meiosis and also in meiotic maturation of oocytes. Karja *et al*. have reported that oxygen concentration modulates the metabolite requirements of porcine embryos developed after the *in vitro* culture of the oocyte (119). The higher concentration of supplemented glucose led to increased generation of ROS in Day 1 embryos and reduced embryonic developmental competence (119). Oxidization–reduction potential of the oocyte is thus regulated by multiple factors and is species specific. To summarize, energy sources must be present in the right concentrations, ratios, and combinations in relation to each other and the other components in the medium and are important determinants of the developmental competence of mammalian oocytes.

IV. ROS-INDUCED PROGRAMMED CELL DEATH IN GAMETES AND EMBRYOS

Apoptosis, programmed cell death (PCD), is a major component of normal development, maintenance of tissue homeostasis, and removal of damaged cells. Generation of ROS, depolarization of mitochondrial membrane potential, reduction in antioxidant levels, activation of proapoptotic proteins, and blockade of the ETC leads to apoptosis. This in turn can lead to oocyte demise, embryonic fragmentation, and embryonic wastage. ROS and antioxidants appear to have a physiological role in reproductive processes, including folliculogenesis, oocyte maturation, luteal regression, endometrial shedding, and fertilization (180, 189, 221). Macrophages, neutrophils, and granulosa cells in Graffian follicles are a source of ROS, which are balanced by antioxidants. During folliculogenesis, important antioxidants such as catalase, superoxide dismutase (SOD),

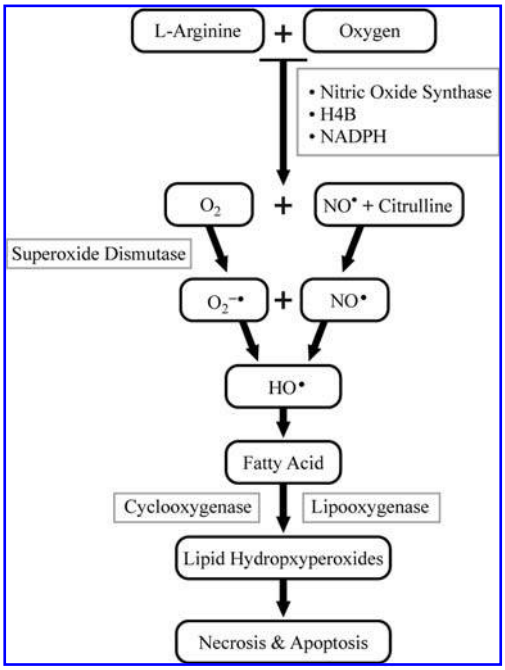

FIG. 3. Mechanism of RNS formation.

GSH transferase, paraoxanase, heat shock protein 27, and protein isomerase protect the oocytes against toxic injury due to oxidative stress (23). An imbalance between pro-oxidants and antioxidants has been postulated in female infertility based on its potential effects on ovulation, fertilization, embryo development, and implantation (9).

Mitochondria are the most abundant organelles in both oocytes and pre-implantation embryos (234). However, their underdeveloped morphology means that mitochondrial generation of ATP is low at these developmental stages. Events such as meiotic maturation, compaction, and blastulation may lead to increased energy demands that may be met with elevated ATP generation in the mitochondria (62). ROS can affect mitochondrial functions in oocytes and embryos (127). Mitochondrial DNA lacks histones, making it prone to oxidative injury. Moreover, mitochondria are one of the main sites for ROS production, which means they are the first cell organelle to be affected. When mitochondria are damaged, ROS can leak into the cytoplasm in increasing amounts (41). Mitochondria are central to metabolic activities, so any disturbance in these activities can lead to profound problems of ATP generation, which is essential for gamete functions. Mitochondrial dysfunction may lead to cell cycle arrest or cell death, which is set off by oxidative stress (136, 137).

Apoptosis is an ongoing process in ovarian development and folliculogenesis, and results in follicle atresia, follicle degeneration, and fertility decline. ROS are generated in cells actively engaged in steroidogenesis (91, 247) which is linked with the mitochondrial ETS. ROS generation is countered by an expression of various antioxidants such as SOD, GPX, and GSH. Suppression of GSH synthesis leads to increased rates of antral follicle atresia in rats (138). It has been hypothesized that the gonadotropins inhibit follicle apoptosis by enhancing the follicle GSH levels (231). Maintenance of adequate levels of reduced GSH to circumvent ROS and apoptosis is important for folliculogenesis and oocyte maturation (Fig. 4).

The mechanisms behind the induction of apoptosis and the factors involved in cellular protection have been demonstrated in many reports in the literature. Shibayam–Imazu *et al*. (203) studied whether vitamin K_2-treated human ovarian cancer TYK-nu cells might produce ROS during the induction of apoptosis and whether ROS might be associated with the dissipation of mitochondrial transmembrane potential. They demonstrated that the increased intracellular levels of superoxide observed in these cancer-treated cells can predict future apoptosis by vitamin K_2 (Fig. 5). Moreover, since cycloheximide inhibited apoptosis, it was suggested that protein synthesis was mandatory to produce the superoxide molecule. When an inhibitor of GSH synthesis, buthionine sulfoximine (BSO), was added to vitamin K_2-treated cells, the number of apoptotic cells was 1.3-fold greater than cells treated with vitamin K_2 alone. This study further confirmed the important role of GSH in preventing programmed cell death. Finally, it deduced the possible depolarization in the mitochondrial membrane subsequent to the production of superoxide in TYK-nu cell. This depolarization may lead to activation of procaspase 3, promoting conversion to caspase and ultimately resulting in apoptosis (203).

Apoptosis also may be induced through a cascade of another set of events caused by OS. In the lipid bilayer, voltage-dependent anion channels coexist with adenine nucleotide translocase, which has sulfhydryl groups attached (Fig. 6). These ligands can induce the release of additional free electrons, imposing OS on the cell. These free electrons would then oxidize the sulfhydryl groups on the ligand complex, causing a membrane permeability transition (Fig. 6). This will further release SOD and cytochrome c, events leading to apoptosis (108).

Tarin *et al*. had proposed that oocyte aging is accompanied by free radical-induced mitochondrial injury, leading to an age-related higher incidence of aneuploidy (225). Redox-induced apoptosis of human oocytes in resting follicles *in vitro* was demonstrated to be dose- and time-dependent by Zhang *et al*. (249). Ovulated oocytes also can undergo demise *in vitro* as a result of apoptosis (169). Maintenance of redox pathways is important for the protection of gametes against OS. On a molecular level, ROS can alter many redox pathways and may eventually lead to apoptosis in oocytes and embryo.

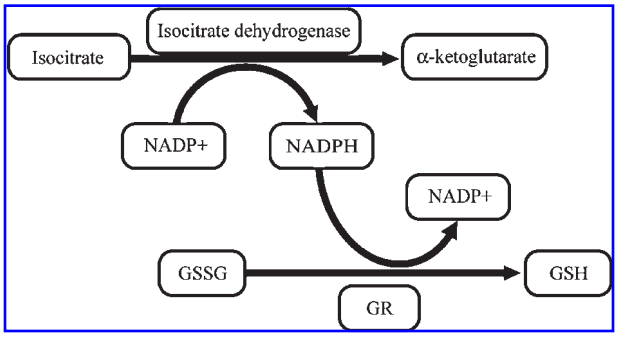

FIG. 4. GSH and redox regulation in the oocytes.

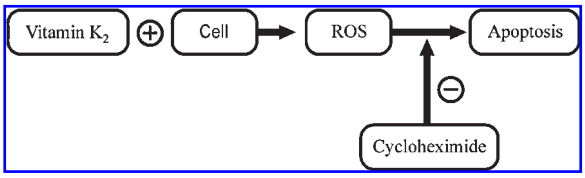

FIG. 5. ROS and induction of cellular apoptosis.

Proteins and mRNAs, both of which have DNA repair capabilities, are stored in the oocyte during the developmental process. The repair genes are expressed early in mammalian embryonic development. However, these repair mechanisms may not be very effective in *in vitro* pre-implantation embryonic development. The gene expression profiles need to be examined (243).

V. EXOGENOUS AND ENDOGENOUS ANTIOXIDANTS: ROLE IN REDOX REGULATION

Antioxidants are substances that significantly delay or inhibit the oxidation of a substrate when present in low concentration relative to that of the substrate (85). These scavengers oppose the actions of ROS, serving a protective role. Antioxidants are necessary for homeostatic purposes; an imbalance in the equilibrium of antioxidants and pro-oxidants can result in OS, a phenomenon that is becoming recognized as a key element in the pathogenesis of several diseases.

Antioxidants are present in both enzymatic and nonenzymatic forms (Table 1). Enzymatic or natural antioxidants include SOD, catalase, GSH peroxidase, and GSH reductase (4). SOD is one of the most important enzymatic antioxidants because it is the first antioxidant that attempts to neutralize ROS. Under normal conditions, the neutralization reaction involves the reduction of the superoxide molecule to H_2O_2 and water (171). H_2O_2 also may be neutralized by either GSH peroxidase or catalase (236).

Antioxidants such as SOD, Cu,Zn-SOD, Mn-SOD, GSH peroxidase, and γ-glutamyl synthetase have been investigated by immunohistochemical localization and mRNA expression. The expression of various antioxidant biomarkers has been demonstrated in normal cycling human ovaries. All follicular stages have been examined for SOD expression, including primordial, primary, preantral, and nondominant antral follicles in the follicular phase, dominant follicles, and atretic follicles. ROS may have a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis, and luteolysis. A delicate balance exits between ROS and antioxidant enzymes in the ovarian tissues where antioxidant enzymes neutralize ROS production and protect the oocyte and embryo.

Immunohistochemistry confirms the presence of SOD in the ovary with an intense staining in the theca interna cells of the antral follicles (218). Antibody to Ad4-binding protein (Ad4BP) was utilized to localize Ad4BP in the nuclei of theca and granulosa cells. Ad4BP is a steroidogenic transcription factor that induces transcription of the steroidogenic P450 enzyme (218) and controls steroidogenesis in the ovaries. The correlation between Ad4BP and SOD expression suggests an association between OS and ovarian steroidogenesis.

The senescent process may involve oxidative damage to mitochondrial DNA, proteins, and lipids. A decrease in intracellular ATP and the GSH (GSH)/GSH disulphide (GSSG) ratio and an increase in cytosolic calcium ions have been reported. These changes may harm cytoskeleton fibers and impair fertilization and embryo development, all of which occur more frequently in pregnancies in older women (135, 224).

Tatone *et al*. (227) studied whether levels of these important antioxidants, specifically catalase and SOD, decrease as age increases. Variants of SOD1 and SOD2, as well as catalase mRNA expressions, were analyzed in the granulosa cells from periovulatory follicles. The results showed that levels of these antioxidants in the granulosa cells were lower in older women. When protein levels of the antioxidants were examined by Western blot analysis, the result was consistent, showing a statistically significant decrease in the levels of SOD1, SOD2, and catalase protein density with increasing age. This study was the first to present evidence that reproductive aging can also affect gene expression in granulosa cells. Therefore, as women age, they should be advised to take additional supplements of antioxidants to compensate for the decreasing levels of SOD and catalase (227).

Nonenzymatic antioxidants such as vitamin C, vitamin E, selenium, zinc, taurine, hypotaurine, GSH, beta carotene, and carotene usually are obtained from dietary sources (199). GSH is considered the main nonenzymatic antioxidant and is seen in very high concentrations in oocytes and embryos. This increased concentration allows for large stores of GSH in oocytes for decondensation of the sperm nucleus, as well as embryo protection until the blastocyst stage. GSH is considered a necessary antioxidant because depletion of GSH results in DNA damage and increased H_2O_2 concentrations (85).

The GSH system is one of the key antioxidant enzymes present in different systems, including the reproductive tract. Glutathione peroxidase (GPx) occurs in five different forms (GPx1 and GPx4 acting in most tissues and GPx5 present in the epididymis), all of which neutralize hydrogen peroxides and/or alkyl hydroperoxides (171). However, each GPx does not reduce all forms of hydrogen peroxide. For example, GPx1 and GPx2 reduce only soluble hydroperoxides such as H_2O_2; some reduce hydroperoxides such as hydroxyperoxy fatty acids,

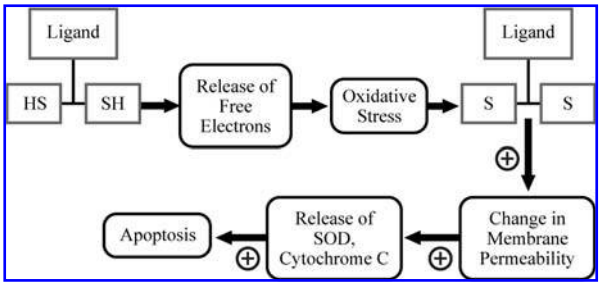

FIG. 6. OS: induction of apoptosis through altered membrane permeability.

TABLE 1. ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS

Antioxidant	Localization in the female reproductive tract	Mechanistic actions
Enzymatic antioxidants		
Superoxide dismutase: Cytoplasmic (Cu, Zn-SOD) Mitochondrial (Mn-SOD)	Endometrial glandular cells	Neutralizes intra- and extracellular superoxide anions
Catalase	Tubal fluid	Neutralizes intra- and extracellular hydrogen peroxide
Glutathione peroxidase	Glandular epithelium in endometrium	Reduction of hydrogen peroxide and lipid peroxides and other peroxides to water or alcohol. Eliminates hydroxyl radical.
Non-enzymatic antioxidants		
Vitamin E	Follicular fluid	Major chain breaking antioxidant in membranes, directly neutralizes superoxide anion, hydrogen peroxide, and hydroxyl radical.
Vitamin C	Ovary	Chain breaking antioxidant, competitively protects the Lipoproteins from peroxyl radicals and recycles vitamin E. Diverse antioxidant function. Reduces presence of Sulfhydryl.
Glutathione	Tubal fluid, oocyte/embryo	Metabolizes hydrogen peroxide and hydroxyl radical
Cysteamine	Follicular fluid	Precursor of hypotaurine
Ascorbate	Follicular fluid	Blocks DNA damage
Taurine	Follicular fluid, Tubal fluid	Neutralizes hydroxyl radicals, precursor of taurine.
Transferrin	Tubal fluid	Metal chelation
Thioredoxin	Oocyte/embryo	Protects embryo against oxidative stress and embryopathic effects.

cumene hydroperoxide or t-butyl hydroperoxide; GPx4, and to some extent GPx3, also reduce hydroperoxides of more complex lipids such as phosphatidylcholine hydroperoxide. GPx4 also is capable of reducing hydroperoxide groups of thymine, lipoproteins, and cholesterol esters, and is unique in acting on hydroperoxides integrated in membranes.

GPx is a member of the GSH system, along with GSH, GSH-S-transferase, and GSH reductase (Fig. 4). As H_2O_2 is reduced to water and oxygen, GSH (GSH) is oxidized to GSSG by GPx. The reverse reaction involves the enzyme GSH reductase, which uses NADPH to donate a proton to GSSG, recycling GSH (Fig. 4) (171). Cysteine and cysteamine (CSH) increase the GSH content of the oocyte and enhance the outcomes with *in vitro* maturation (IVM).

CSH not only acts as a scavenger but also helps maintain GSH levels in the oocyte. Guerin *et al*. (85) have hypothesized a mechanism for maintaining necessary GSH levels. One pathway begins with CSH traveling from the serum into the follicular fluid; as it transforms into cystamine, CSH catalyzes the conversion of glutamylcysteine synthetase to GSH, another important antioxidant. A second pathway that converts CSH to cystamine initiates the conversion of cystine to cystiene, which can produce GSH through the enzyme glutamylcysteine synthetase. Therefore, CSH is a necessary antioxidant to ensure high levels of GSH and also converts to hypotaurine, another antioxidant (85).

Taurine and hypotaurine are scavenger molecules that help to maintain the redox equilibrium levels in gametes. Levels of

several amino acid, including taurine, change dynamically with folliculogenesis, estrus cyclicity, and follicle dominance (159) and have been proposed to act as antioxidants and promote oocyte capacitation. Taurine makes its contribution to the antioxidant system by neutralizing cytotoxic aldehydes that result from lipid peroxidation, while hypotaurine neutralizes hydroxyl radicals and lipid peroxidation products.

The thioredoxin system is a group of many antioxidative proteins such as thioredoxin and peroxiredoxins (PRDX) involved in maintenance of redox homeostasis. PRXDs are expressed in bovine oocytes and embryos and may have a role in oocyte maturation *in vitro* and embryo development (132). Thioredoxin has been proposed to be involved in redox regulation during embryogenesis as well (123, 124). Supplementing culture media with thioredoxin is known to overcome mouse two cell block (150, 151).

Vitamin C helps maintain cellular redox status by preventing oxidative DNA damage and leading to increased levels of hypotaurine. Although ascorbate has antioxidant properties if transition metals are present, it can switch from acting as an antioxidant to producing pro-oxidant effects. Vitamin E is another important antioxidant that prevents the production of superoxide and anion by inhibiting NADPH oxidase, the enzyme that produces ROS (85).

Antioxidants are vital to the body's defense against excess ROS levels, with each antioxidant making a unique contribution to preventing diseases attributed to the harmful effects of OS. Therefore, an ideal concentration of antioxidants must be maintained to ensure protection against ROS-induced damage.

VI. DIFFERENTIAL REDOX REQUIREMENTS IN MODULATION OF PRE-TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL CELL SIGNALING CASCADES IN FEMALE REPRODUCTION

Once produced, ROS can react further with other molecules to modify many cellular components. Baker and Aitken (28) cemented the idea that ROS are hazardous to the cell. At complex IV in the ETC, molecular oxygen normally is converted to water. However, as the electrons are being passed down the ETC to produce ATP, molecular oxygen may gain an electron and transform into the fast-reacting superoxide molecule, which may react with fatty acids. This may induce a morphological change in the cell that can affect the cell's viability or lead to apoptosis (28).

Oxidative stress has been determined to be responsible for >100 diseases (88). This problem can be further understood by examining events at the molecular level. Excessive and persistent production of ROS can induce negative effects in many signaling pathways, changing their normal outcomes.

Although many pathways have an altered result due to the effects of ROS, the ROS does not always directly affect the pathway's target. It may interact with the intermediary reaction steps by acting as a second messenger to induce an abnormal final outcome (109). For example, ROS tends to cause increased tyrosine phosphorylation, enhancing the effects of tyrosine kinases while inhibiting the effects of tyrosine phosphatases. Specifically, on a protein level, all tyrosine phosphatases share a cysteine residue at the active site. ROS may inhibit the enzyme in one of two ways. One possibility is through the use of 1 m*M* H_2O_2; the H_2O_2 attacks the cysteine residue, converting cysteine to a sulfenic acid by replacing the sulfhydryl group with a hydroxyl group, thus inactivating the enzyme. The second possibility is through the actions of GSSG, which can be converted to its reduced form, GSH, resulting in an alteration of the catalytic site (61).

A superfluous amount of ROS also can induce a negative systematic effect through insulin-mediated pathways (58). Normally, signaling by insulin requires autophosphorylation of insulin kinase, an enzyme containing a tyrosine residue in the active site. Moreover, the insulin response is amplified by a pro-oxidative shift in intracellular GSH redox state, which was proven by the discovery of antioxidants inhibiting kinase activities. Since insulin may produce H_2O_2, this ROS can induce a positive feedback system on insulin, generating even more ROS and inhibiting more phosphatase enzymes.

Mitogen-activated protein kinases (MAPK) signaling pathways are among the most important pathways present in the human body and normally are tightly controlled. MAPK pathways are major regulators of gene transcription in response to oxidative stress. MAPK signaling cascades are regulated by phosphorylation and dephosphorylation on serine and/or threonine residues and respond to activation of receptor tyrosine kinases, protein tyrosine kinases, receptors of cytokines and growth factor, and heterotrimeric G protein-coupled receptors (61). However, the presence of an abundance of ROS can alter the normal effects of these cascade signaling pathways. For example, it has been shown that JNK and p38 can be activated due to ROS. JNK normally is inhibited by the enzyme GSH-S-transferase as it prevents phosphorylation. However, after the addition of H_2O_2, this complex can be dissociated and phosphorylation can occur. A similar process occurs with apoptosis signaling-regulation kinase (ASK1) and Trx. Trx normally inhibits ASK1, also preventing phosphorylation from occurring; however, once ROS species are present, the kinase can be activated through dissociation of the ASK-1-Trx complex (61).

The levels of intracellular calcium also must be tightly regulated since calcium plays a role in many reproductive physiological processes. Nevertheless, the presence of ROS species can further increase calcium levels, activating a number of pathways involving calcium, such as caldmodulin-dependent pathways. Similarly, the transcription factor AP-1, composed of c-Fos and c-Jun proteins, is responsible for many different processes such as activation of the interleukin-2 gene. Oxidative stress factors such as ultraviolet radiation and H_2O_2 will enhance the expression of the AP-1 transcription factor and subsequently, its protein component. Nuclear factor kappa beta, which plays a role in apoptosis by controlling responses in relation to inflammatory or immune means and controlling genes that regulate the cell cycle and cell cycle inhibitor p21, likewise can be activated through ROS generation (61, 93). Hypoxia-inducible factors (HIF), transcription factors modulated by oxygen concentration, are important in embryonic growth regulation. HIF-1 is particularly sensitive to low oxygen con-

centration and can activate genes such as erythropoietin that are important for embryonic growth and development (56, 94).

In a study conducted by Burdon *et al*., the role of cyclooxygenase (COX) enzymes and their relation to OS and apoptosis were examined in murine placental samples (36). They discovered that OS does, in fact, increase during gestation and that there are significant changes in the concentrations of COX-1 and COX-2 enzymes throughout gestation. Peak levels of COX-1 and COX-2 enzymes coincided with the greatest numbers of cells undergoing apoptosis. A possible mechanism is that OS levels increase with increasing gestational age, leading to elevated concentration of COX-1 and COX-2 enzymes, possibly through activation of p38 MAPK and nuclear factor kappa beta pathways (36).

OS disrupts cellular function at the level of signaling pathways, altering their outcomes and leading to further dysfunction. Maintaining the normal redox homeostatic levels of antioxidants and pro-oxidants is extremely important to ensure cell viability and proper cellular function in the reproductive tract.

VII. REGULATORY ROLE OF ROS IN THE ENDOMETRIAL CYCLE

ROS play a modulatory role in the physiologic functions of the female reproductive system. The endometrial cycle consists of proliferative, secretory, and menstrual phases, all of which are controlled by hormonal changes. OS is implicated in the regulation of these cyclic changes in the endometrium (Fig. 7). Variations in endometrial SOD levels have been reported. Modified measures of SOD and ROS within the endometrium have been exhibited in the late secretory phase of the cycle, just prior to the onset of menstruation (215). Late secretory phase human endometrium has been demonstrated to have elevated lipid peroxide concentrations and decreased SOD concentrations (Fig. 7). These distortions may be responsible for the breakdown of the endometrium, and the evidence of the involvement of OS in menstruation (Fig. 7) (215). A study by Serviddio *et al*. evaluated the effects of changing hormone levels during the menstrual cycle on the redox balance and lipid peroxidation in nor-

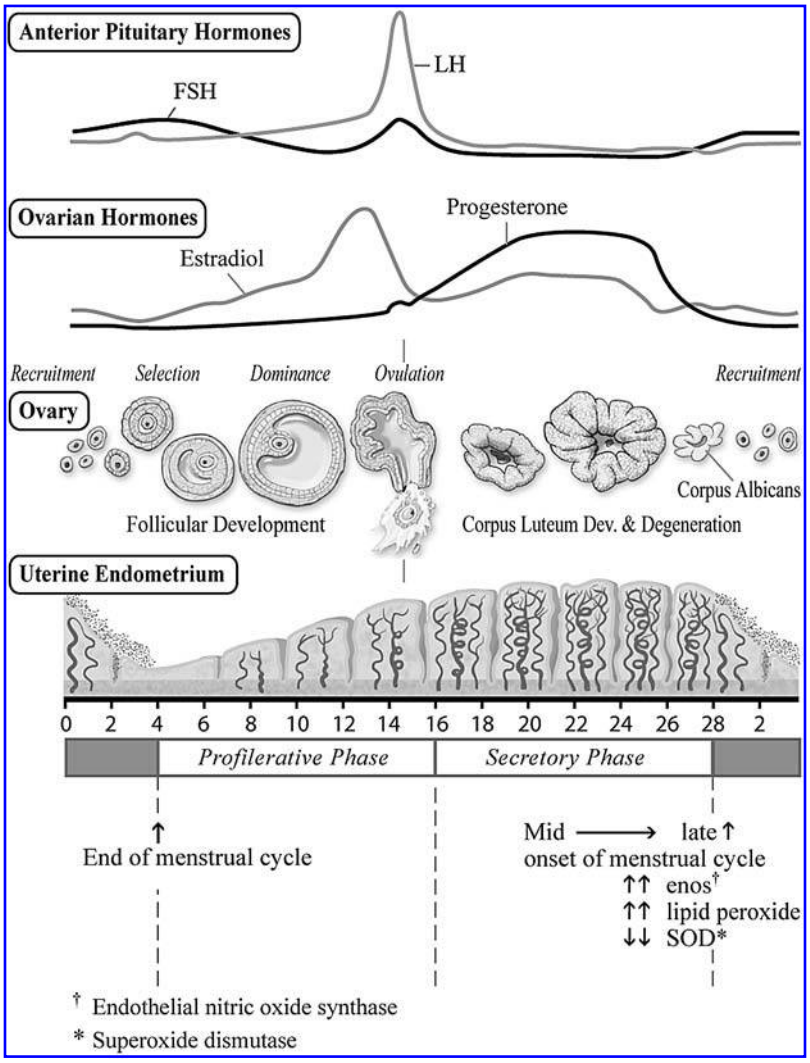

FIG. 7. Modulation of redox balance throughout the endometrial cycle.

mal human endometrial cells. The researchers observed that the hormonal pattern was involved in maintaining optimal redox balance in the endometrium through regulating the level of GSH and its metabolism (197).

NO regulates endometrial microvasculature. Inducible NOS and endothelial NOS expression have been demonstrated in human endometrium and endometrial vessels (161, 185). Endothelial NOS is disseminated in glandular surface epithelial cells in the human endometrium (232). Expression of endothelial NOS mRNA has been demonstrated in the mid-secretory phase and late secretory phase of the endometrial cycle, suggesting that endothelial NOS is involved in the decidualization of the endometrium and menstruation (Fig. 7). Endothelial NOS is thought to produce physical changes that prepare the endometrium for implantation.

Literature reports of studies exploring the underlying mechanisms of endometrial shedding have revealed that estrogen and progesterone withdrawal in endometrial cells cultured *in vitro* decreases in SOD activity, leading to increased, unhindered levels of ROS. ROS may initiate a cascade of events by activating nuclear factor kappa B, which excites increased COX-2 mRNA expression and prostaglandin F2α synthesis. These physiological changes facilitate the endometrial processes of shedding and/or implantation (215), and reduced expression of SOD leads to failed pregnancy (216).

The formation of endometrial vasculature has been shown to be largely regulated by VEGF and Ang-2. These key regulatory factors are induced by hypoxia and ROS and are upregulated in the endometria of patients on long-term, progestin-only contraception. Long-term progestin-only contraceptives are thought to decrease endometrial blood flow, ultimately inducing hypoxia and causing abnormal angiogenesis (99). *In vitro* studies have shown that hypoxia stimulates the expression of nitrotyrosine in cultured endometrial microvascular endothelial cells, signifying peroxynitrite anion generation. Therefore, oxidative stress may account for endometrial pathophysiology in patients using long-term progestin-only contraceptives (99).

VIII. ROLE OF OXIDATIVE STRESS IN PREGNANCY AND ASSOCIATED COMPLICATIONS

Pregnancy is associated with an inflammatory response characterized by leukocyte activation (101, 190). An increased generation of superoxide free radicals by polymorphonuclear leukocytes has been demonstrated in women in the first trimester of pregnancy (72, 101). During the second trimester, increased oxidant formation and even the existence of OS is regarded as normal (72). Elevated levels of lipid peroxides, vitamin E, and free radicals generated by placental mitochondria have been reported in pregnancy (57, 133, 148). A prospective study in which blood samples were routinely drawn from women with uncomplicated pregnancies reported that lipid peroxide levels remained the same, while vitamin E levels increased with the gestational age (240).

Normal human placentation is determined mainly by the appropriate invasion of the uterine spiral arteries by a genomically normal trophoblast. This infiltration regulates anatomical changes in the placental vasculature to ensure maximal fetal perfusion by the maternal vessels. During the changeover from first to second trimester, embryos undergo marked metabolic changes. The period of embryonic organogenesis is seen to occur under an environment of prevailing low oxygen tension with metabolism that is chiefly anaerobic (114). Consequently, it is feasible that ROS production is reduced to prevent oxidant-induced DNA damage.

One of the fundamental points with respect to the role of oxygen signaling and ROS in preeclampsia is that it is believed that the pathophysiology of this condition is established in the first trimester of pregnancy. For the majority of this time, the placenta exists in physiological hypoxia (1–2%) due to the presence of trophoblast plugs in the maternal spiral arteries that prevent the flow of maternal blood into the intervillous space (106, 114, 183). These plugs are dislodged at ~10–12 weeks of gestation, resulting in an increase in oxygen tension in the intervillous space. A distinct rise in intervillous space oxygen tension from $<$20 to $>$50 mm Hg occurs at the close of the first trimester (115, 183). The timing of this onset of blood flow is important as the syncytiotrophoblast of the placenta lacks protection because it does not express catalase enzymes or Mn-SOD enzymes until this time (241, 242). The premature onset of blood flow in the intervillous space causes placental OS and has been associated with miscarriage (112). Lower oxygen tension in early pregnancy prevents trophoblast differentiation into an invasive type, and conversely it facilitates persistent cytotrophoblast proliferation, fibronectin synthesis, and alpha(5) integrin expression (39). Placental OS may cause impaired placental development or lead to syncytiotrophoblast degeneration in early pregnancy, which may culminate in complications such as recurrent abortions, preeclampsia, and congenital anomalies in diabetes (37).

A. Recurrent pregnancy loss

Recurrent pregnancy loss (RPL), characterized by three or more consecutive pregnancy losses before 20 weeks of gestation, affects 0.5–3% of women of reproductive age (2). The etiology of RPL remains unclear and is a scientific challenge. Causative factors may be varied and multiple and include genetic abnormalities, uterine anomalies, autoimmune diseases such as systemic lupus erythematosus, antiphospholipid syndrome, blood clotting disorders such as hyperhomocysteinemia or other thrombophilias, infectious diseases, endocrinopathies, polycystic ovary syndrome, sperm DNA fragmentation, and sperm meiotic alterations (47, 212).

Fifty to 60% of RPL cases are of unknown etiology and are thought to have pathophysiology related to endothelial damage, impaired placental vascularization, and immune impairment. Abnormal placentation results in syncytiotrophoblast dysfunction and placental OS, which appears to result in early abortion, implicating OS as a possible factor in the etiology of idiopathic RPL (37). Syncytiotrophoblast are known to be very sensitive to oxidant-induced damage in the first trimester. Increased plasma levels of lipid peroxides and GSH and lower levels of vitamin E and beta-carotene have been shown in pa-

tients with recurrent abortion (207). A large case-control study reported that elevated risk of recurrent pregnancy loss was associated with the null genotype polymorphism of the GSH S-transferase enzymes in patients with recurrent pregnancy loss. Interestingly, pregnant women with a history of RPL have been observed to have significantly elevated plasma GSH levels, reflecting a response to increased amounts of OS (143).

B. The effect of paternal factors on pregnancy outcomes

Paternal factors greatly influence embryonic and fetal development. Sperm DNA may be damaged by exposure to ROS during sperm transport through the seminiferous tubules and epididymis (21, 22, 50, 157, 210). This may cause primary damage such as single- and double-stranded DNA fragmentation and may also lead to the generation of 8-OH-2′-deoxyguanosine-type secondary damage. Spermatozoal DNA is an important site of action by which ROS can affect the ability of a man to father a biological child. The percentage of sperm with damaged DNA is negatively correlated with the fertilization rate (214). Embryo development may be affected by damaged spermatozoa DNA, although DNA damage can be repaired by oocytes to some extent. Oocyte fertilization by spermatozoa with unrepaired primary or secondary DNA damage may lead to implantation failure, embryo development arrest, pregnancy loss, or birth defects (40, 76, 192). Recent studies have suggested that sperm DNA fragmentation may be related to an increase in sperm aneuploidy (186), which is mainly a result of meiotic alterations during spermatogenesis (21, 186). During passage through the epididymis, aneuploid sperm may experience increased ROS- and/or caspase-induced or endonuclease-induced DNA fragmentation (134). Couples struggling with RPL may benefit from sperm DNA fragmentation testing of semen (71).

Hyperhomocysteinemia is a disorder of homocysteine metabolism associated with preeclampsia, as well as fetal neural tube defects, recurrent pregnancy loss, and placental abruption. Homocysteine is a thiol-containing amino acid with pro-oxidant properties, implicated in the sulfurylation and methylation metabolic pathways. Plasma homocysteine levels usually fall during normal pregnancy (152). At elevated levels, homocysteine induces OS that is hypothesized to cause apoptosis and disruption of palate development and lead to early pregnancy loss (122).

OS due to increased lipid peroxidation is related to the pathophysiology of recurrent pregnancy loss due to antiphospholipid (aPL) antibody syndrome (74). The aPL antibody syndrome, an autoimmune cause of recurrent pregnancy loss, is delineated by high titers of aPL or lupus anticoagulant associated with pregnancy loss or history of a thrombotic event and/or autoimmune thrombocytopenia. A pregnancy loss rate of 50%, 84% of which were fetal deaths, was demonstrated in a series of women positive for aPL antibodies (34). Although the mechanism of aPL antibody formation is not fully understood, oxidative stress is suspected to play a role. Increased low density lipoprotein (LDL) oxidation is known to convert the antigenic character of some oxidized phospholipids, making them subjects of a directed attack by aPL antibodies (102).

C. Preeclampsia

Preeclampsia is a complex, multisystem disorder affecting 5–8% of all pregnancies (95). It is characterized as a state of OS and manifests clinically as hypertension, proteinuria, and the highest maternal and fetal morbidity and mortality of all pregnancy complications in humans. Increased generation of free radicals and decreased levels of antioxidants can result in a state of OS that is involved in the etiopathogenesis of preeclampsia. Investigations have shown that at 10–12 weeks, the placenta undergoes changes that result in an oxidative burst (115). There are many literature reports demonstrating that the pregnancy at high altitudes results in prolonged hypoxia leading to oxidative stress (248). OS may be one of the contributory factors at term pregnancy, but not the sole factor in etiopathogenesis of preeclampsia.

Preeclampsia has been thought to be a two-stage disorder. In the first stage, extravillous trophoblast cells that have grown out from the placenta and formed plugs in the maternal spiral arteries are dislodged and followed by the onset of maternal blood flow into the intervillous space, and this results in the oxidative burst to which the placenta is exposed. The second stage involves a maternal response, such as ROS generation, to the placental factor. Mitochondrial activity increases, which leads to increased ROS production (148, 202). The pathogenesis of preeclampsia is established in the first trimester (where hypoxia is normal). The inadequate remodeling of the spiral arteries during the first trimester results in a decreased perfusion of the placenta in the third trimester leading to oxidative stress which may play an important role in the pathogenesis of preeclampsia. Abnormal placentation leads to placental ischemia, resulting in the generation of placental oxidative stress (181).

Increased levels of lipid peroxidation have been described in preeclampsia (35). Localized in the placental syncytial microvillus membrane, NADPH oxidase may play a role in placental lipid peroxidation by producing increased amounts of the superoxide radical (140, 178). Significantly increased levels of lipid peroxidation, accompanied by a trend toward elevated protein carbonyl concentrations, have been established in the placenta of women with preeclampsia (25, 237, 253). Lipid peroxidation results in primary lipid peroxidation products such as lipid hydroperoxides, and secondary products such as malondialdehyde (MDA) and lipid peroxides. Also, the capacity of antioxidants to scavenge ROS was reported to be upregulated in the placenta of some preeclamptic patients. Interestingly, depleted and insufficient antioxidant reserves may tilt the balance in favor of pro-oxidation. Mean placental levels of the enzymatic antioxidants GSH and SOD were significantly reduced in women with preeclampsia (139). In a case control study, Aydin *et al*. found that patients with preeclampsia exhibited a significant increase in plasma MDA levels and a significant decrease in SOD activity (27). Plasma MDA, SOD activity, and diastolic blood pressure were well correlated, indicating a relationship between the severity of the disease process and the level of lipid peroxidation. Leukocytes may mediate tissue injury in preeclampsia via endothelial cell activation, leading to increased serum adhesion molecules such as vascular cell adhesion molecule-1 and sE-selectin. While there have been conflicting reports on the plasma levels of sE-selectin in preeclamp-

sia, Aydin *et al*. found significantly higher sE-selectin levels in patients with preeclampsia, which were also found to correlate with diastolic blood pressure (27). Another indicator of endothelial cell injury that has been shown to increase in preeclampsia is fibronectin (213). Fibronectins are a family of ubiquitous, high molecular weight extracellular matrix glycoproteins produced by the endothelium and other cells. They are thought to play a part in many different fundamental tissue interactions, including cell adhesion and migration. Aydin *et al*. (27) found that fibronectin levels increased significantly in preeclampsia and correlated with diastolic blood pressure.

Injured endothelial cells in preeclampsia also produce endothelin, a potent endogenous vasoconstrictor peptide that is thought to participate in the regulation of vascular tone. Plasma endothelin-1 (ET-1) concentrations have been observed to increase in preeclamptic patients (58), suggesting the possible contribution of ET-1 to the hypertension that characterizes preeclampsia. Unopposed effects of ET-1 also could favor smooth muscle contraction and impaired uteroplacental blood flow (27). NO levels also have been explored in preeclampsia with mixed results. NO, known to inhibit platelet aggregation and adhesion to vascular endothelial surfaces, has been shown to be reduced (196), unchanged (206), or elevated (208) in preeclamptic patients. In a case control study, preeclamptic patients' serum NO levels were reported to be significantly decreased and negatively correlated with diastolic blood pressure (27). Preeclampsia, therefore, may be associated with endothelial dysfunction and reduced NO-mediated vascular relaxation, that lead to a poorly perfused fetoplacental unit, the source of ROS and lipid peroxides.

NOS-1, an isoform of NADPH oxidase and endothelin-1, has been identified as a significant biomarker associated with preeclampsia and OS (148). These factors may play an inhibitory role in cell proliferation and maturation, triggering placental OS by altering the redox balance. As a result, cellular apoptosis leading to derangements in placental invasion causing early spontaneous abortion can occur. Placental circulation was analyzed using immunohistochemical detection of heat shock protein (HSP 70i), an indicator of cellular stress. Markers such as nitrotyrosine residues and hydroxynenal were also assessed as indicators of protein and lipid oxidative damage, respectively (113). This case control study showed that in normal pregnancies, the onset of intervillous blood flow increased with gestational age, detected in 9 out of 25 cases at 8–9 weeks, but in 18 of 20 cases at 12–13 weeks. In abnormal pregnancies, flow was detected in nearly all cases (22 of 25) at 8–9 weeks.

D. Hydatidiform mole

It has been hypothesized that the pathogenesis of preeclampsia and hydatidiform mole involve OS-induced damage, as inflammatory states with elevated levels of cytokines such as TNF-α and interleukin-6 have been demonstrated (175, 198). Hydatidiform mole is a placental defect that can involve OS-induced damage and is characterized by grape-like degeneration of the placenta and genotypic abnormalities known to promote early miscarriage. Patients with complete hydatidiform mole have demonstrated decreased antioxidant response in comparison to control subjects (4). The pathological role of DNA damage is related to a lower total antioxidant capacity in patients with complete hydatidiform mole (92).

In general, an abundance of investigations have illustrated that markers of OS often are increased and endogenous antioxidant levels tend reduced in women experiencing OS, which mediates the pathological mechanisms implicated in adverse pregnancy outcomes (103, 104).

IX. ROLE OF OXIDATIVE STRESS IN MALE AND FEMALE INFERTILITY

A. Male infertility

Defective sperm function is the most prevalent cause of male infertility, a difficulty faced by at least 24% of couples attending infertility clinics (105). Despite an abundance of published research on the subject, the origin of sperm dysfunction is not well understood. Controlled ROS generation mediates the normal physiological function of spermatozoa, whereas uncontrolled production may precipitate pathological conditions (11, 193).

In the male reproductive tract, a balance normally exists between ROS production and antioxidant scavenging activities. As a result, only minimal amounts of ROS remain that are needed during the fertilization process. For sperm to undergo an acrosome reaction and fuse with the oocyte, hyperactivation of the spermatozoa must occur by way of capacitation. *In vitro* experiments have shown that ROS is significantly involved in the regulation of these normal sperm functions (83). Aitken *et al*. found that levels of ROS can augment the ability of human spermatozoa to bind with zona pellucida, whereas the addition of vitamin E was reported to reverse this effect (14). Other investigations have shown that incubation of spermatozoa with low concentrations of H_2O_2 may stimulate sperm capacitation, hyperactivation, and the capacity of the spermatozoa to undergo the acrosome reaction and oocyte fusion (15, 52, 84, 125). Conversely, antioxidant enzymes such as catalase and SOD hinder capacitation (52). ROS may contribute to the acrosome reaction through its effect on phosholipase A2 and also may facilitate binding of the spermatozoa to the zona pellucida by inhibiting tyrosine phosphatase activity (10). This results in enhanced tyrosine phosphorylation, which is essential for interaction and binding between spermatozoal membrane molecules and ZP 3 proteins on the zona pellucida. ROS also may act in the male reproductive system as second messenger molecules that transmit signals by increasing the influx of calcium ions, resulting in a series of chain reactions that increase ATP production.

In recent years, OS in the male reproductive tract has become a matter of concern due to the potentially toxic effects of uncontrolled ROS levels on sperm quality and function. Studies have detected high ROS levels in semen samples of 25–40% of infertile men (55, 163). Furthermore, antioxidant capacity has been found to be higher in the seminal plasma from fertile men compared with that of infertile men (131). However, it is generally accepted that pathological levels of ROS detected in semen of infertile men are more likely a consequence of increased ROS production rather than reduced antioxidant levels

in seminal plasma (252). Excessive ROS production may overwhelm the antioxidant defense strategies of spermatozoa and seminal plasma, resulting in OS (54, 200, 204, 205). ROS is thought to impair the fertilizing potential of men by affecting various mechanisms of oocyte fertilization by spermatozoa.

Essentially, each human ejaculate is replete with potential sources of ROS that have the potential to cause injury by way of lipid peroxidation, DNA damage, and/or apoptosis. The extent of oxidative-induced injury to sperm cells depends on the nature and amount of ROS, duration of exposure, and extracellular factors such as temperature; oxygen tension, and the composition of the surrounding environment, including ions, proteins, and ROS scavengers (193). Polyunsaturated fatty acids are found in abundance in the spermatozoa membrane and are susceptible to oxidative attack due to the presence of double bonds (8). In spermatozoa, lipid peroxidation occurs in a self-propagating manner that can be halted only by antioxidant action. Fatty acid peroxidation leads to the loss of membrane fluidity and a reduction in the activity of membrane enzymes and ion channels, resulting in the inhibition of normal cellular mechanisms required for fertilization. Resulting membrane fluidity can render spermatozoa unable to initiate the biochemical reactions required for acrosome reaction, zona pellucida binding, and oocyte penetration (16, 83). Also, peroxidative damage to the sperm membrane may impair motility (Fig. 8). ROS also may inflict peroxidative injury to DNA bases and phosphodiester backbones (Fig. 8).

Spermatozoa of infertile men are commonly observed to have DNA fragmentation, a direct effect of high ROS levels (126, 217). Experimentally, artificial exposure to ROS has been shown to induce various types of DNA abnormalities, which include base modification, production of base-free sites, deletions, frame shifts, DNA cross-links, and chromosomal rearrangements (7, 64). High levels of OS in the seminal fluid have been related to multiple single- and double-strand breaks in sperm DNA (233). ROS-induced DNA damage may accelerate the apoptotic process, leading to a decreased sperm count and, therefore, a reduced chance of initiating a pregnancy. Apoptosis removes abnormal human germ cells and prevents overproduction. In mature spermatozoa, it has been positively associated with ROS levels. Caspase levels also have been significantly related to levels of OS.

Major sources of excessive ROS production in the male reproductive tract include immotile or morphologically abnormal spermatozoa, leukocytes, and morphologically normal but functionally abnormal spermatozoa (13, 110, 172). Intracellular mechanisms of spermatozoal ROS generation occur at the level of plasma membrane (NADPH-oxidase system) (Fig. 8) (12) and mitochondria (NADPH-dependent oxido-reductase system) (77). Defective spermatogenesis may lead to an excess of residual cytoplasm in the spermatozoal midpiece. Patients who have a high percentage of spermatozoa with excess residual cytoplasm in the midpiece exhibit higher levels of ROS production. Oxidative damage of morphologically normal, mature spermatozoa may occur by exposure to ROS-producing immature sperm during their co-migration from seminiferous tubules to the epididymis. Leukocytes contribute to OS in the male reproductive system by generating extracellular ROS in prostatic and seminal vesicle secretions (155, 201). Infection or inflammation of the genital tract stimulates the activation of leukocytes, increasing their capacity to produce ROS to100 times that of the amount of ROS generated by nonactivated leukocytes (172). ROS produced by leukocytes may cause sperm damage in cases where seminal leukocyte concentrations are abnormally high (53) or when seminal plasma is removed during sperm preparation for assisted reproduction (110). Seminal leukocytes also may stimulate ROS production by human spermatozoa. This effect may be mediated by direct cell–cell contact or by soluble products released by leukocytes (155).

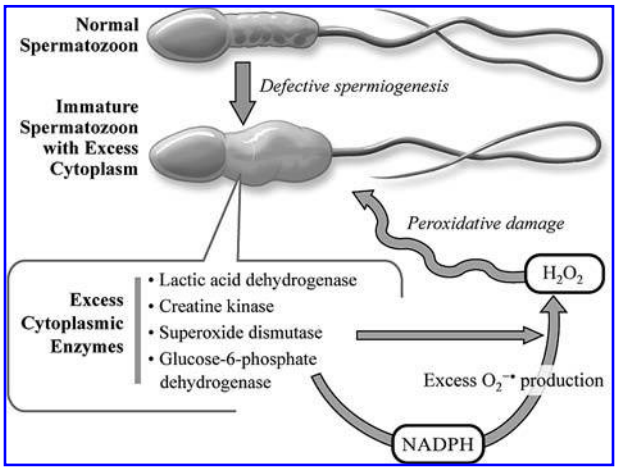

FIG. 8. Mechanism of ROS-induced damage by morphologically abnormal spermatozoa.

Spermatozoa lack the necessary cytoplasmic antioxidant enzymes needed to repair oxidative damage and have greater amounts of polyunsaturated fatty acids present in their membranes, making them particularly vulnerable to excessive ROS levels (8). This deficiency is compensated for by tight sperm DNA packaging and the seminal plasma. The latter provides the enzymatic antioxidants SOD, the GSH peroxidase/GSH reductase system, and catalase and the nonenzymatic antioxidants vitamin C, vitamin E, taurine, and hypotaurine (193). Wherever ROS levels become pathologically increased, these antioxidants confer protection to spermatozoa by preventing, intercepting, and repairing ROS damage.

B. Female infertility

The prevalence of female infertility ranges from 7% to 28%, depending on age. In general, an estimated 84% of couples conceive after 1 year of intercourse, and 92% of couples conceive after 2 years. Approximately 40–50% of infertility is due to female causes, and some 1.3 million American couples receive medical advice or treatment for infertility every year.

OS impacts fertilization and can further induce apoptosis, resulting in embryo fragmentation, implantation failure, or abortion. In the fallopian tubes, OS may induce damaging effects in an embryo. The endometrium, which facilitates embryo implantation and development, can become defective when the female reproductive tract experiences an ROS–antioxidant imbalance (107). OS may hinder the support required for the continuation of a pregnancy by causing luteal regression and insufficient luteal hormone levels (3). Several other known

causes of infertility, such as endometriosis, hydrosalpinx, polycystic ovarian disease, unexplained infertility, and RPL may be attributed to OS in the environment (5)

X. OXIDATIVE STRESS AND FEMALE GYNECOLOGIC CONDITIONS

A. Role of oxidative free radicals in pathogenesis of endometriosis

OS is thought to have an integral role in the pathogenesis of endometriosis, a complex disease characterized by the extrauterine presence of endometrial glands and stroma. Jackson *et al*. determined a weak association between the thiobarbituric acid reactive substances, a measure of overall OS, and endometriosis adjusted for confounding factors such as age, BMI, gravidity, serum vitamin E levels, and serum lipid levels (111). To date, a definitive conclusion on the relationship between OS and endometriosis has yet to be reached. The range of research investigating this association differs significantly in many ways, including the selection of control population, eligibility criteria, OS and antioxidant status markers used, and the biological medium in which OS was assessed (86).

Several hypotheses strive to explain the association of OS with endometriosis. Retrograde menstruation is implicated in the development of endometriosis as it transplants cell debris into the peritoneal cavity. Erythrocytes yield the pro-inflammatory factors hemoglobin and haem containing the redox-generating iron molecule (179). Disruption of the balance between ROS and antioxidants in the peritoneal fluid, precipitating endometriosis and tissue growth, may result from the presence of iron (24), macrophages (146), and environmental contaminants such as polychlorinated biphenyls (60).

In some instances, the peritoneal fluid from patients with endometriosis contains increased concentrations of NO. Abnormally elevated NO concentrations, as generated by activated macrophages, can counteract fertility in variable ways, including altering the composition of the peritoneal fluid environment that accommodates the processes of ovulation, gamete transport, sperm oocyte interaction, fertilization, and early embryonic development (86).

Peritoneal fluid from patients with endometriosis has been shown to exhibit inadequate antioxidant defense, with lower total antioxidant capacity (TAC), and significantly reduced levels of individual antioxidant enzymes such as SOD (173, 219). Infertile women with endometriosis statistically have significantly lower concentrations of SOD compared with fertile controls.

Despite the various associations found between OS in peritoneal fluid and endometriosis, a considerable number of studies have failed to demonstrate a difference in ROS, NO, lipid peroxide, and antioxidant levels in peritoneal fluid of women with endometriosis, compared to fertile controls (100, 239). This may be accounted for by the fact that only persistent markers of OS such as enzymes or stable byproducts of oxidative reactions may still be observed by the time endometriosis is realized. Another possible explanation might be that oxidative stress is experienced locally, therefore failing to result in an increased total ROS levels in peritoneal fluid.

Circulating levels of OS from other sources, such as the endometrium and ectopic endometrial implants, may also contribute to the pathogenesis of endometriosis. The endometria of patients with endometriosis have demonstrated increased lipid–protein complex modification, resulting in high lipid peroxide concentrations (173, 219). Epitopes produced as a result of lipid peroxidation have been demonstrated in macrophage-enriched areas of both the endometrium and endometriosis implants (145). A study showed that high levels of various antioxidants inhibit the proliferation of endometrial stromal cells, and moderate levels of OS promote endometrial stromal cell proliferation. It also was found that the highest tested level of OS inhibits proliferation. This can be attributed to the biphasic dose-response to OS in which only moderate doses of ROS instigate growth/proliferation, whereas higher doses cannot, due to direct cytotoxic effects and higher rates of apoptosis (75).

The endometrium of women with endometriosis has been described to have elevated levels of NO and NOS (86) , and increased expression of NOS and higher endothelial NOS levels in the glandular endometrium of patients with endometriosis have been reported. Altered NOS expression may affect endometrial receptivity and hinder embryo implantation. Deviations in endothelial NOS gene expression may also induce endometrial angiogenesis, thereby facilitating the development of endometriosis (86). OS contributes to angiogenesis in ectopic endometrial implants by increasing vascular endothelial growth factors (VEGF) production (164). This effect is partially mediated by glycodelin, a glycoprotein with increased expression due to oxidative stress. Glycodelin acts as an autocrine factor that augments VEGF expression within ectopic endometrial tissue (164).

The pathogenesis of endometriosis may be attributed to OS by way of molecular genetic pathways. Gene deletion of mitochondria resulting in its rearrangement has been observed in endometriotic tissue. Ectopic and eutopic endometria have exhibited differential gene expression, including 904 differentially expressed genes and differential expression of the GSH-S-transferase gene family that are involved in GSH antioxidant metabolism. Cell proliferation and angiogenesis are cellular responses to OS that also may be determined by differential gene expression (246).

An imbalance between ROS and antioxidants resulting in OS may be implicated in the pathogenesis of infertility associated with endometriosis. Elevated ROS levels in oviductal fluid might have adverse effects, impairing oocyte and spermatozoa viability and fertilization and embryo transport within the oviduct. Oxidative stress is known to occur accompanied by the presence of activated neutrophils and macrophages, pro-inflammatory factors that could significantly amplify ROS production in the oviductal fluid by foci of endometriosis (20). Substantial increases in ROS production might result in oxidative damage to sperm plasma and acrosomal membranes, impairing motility and hindering the ability of spermatozoa to bind to and penetrate an oocyte. DNA damage secondary to OS may bring about failed fertilization, reduced embryo quality, failure of pregnancy, and spontaneous abortion.

Tumor necrosis factor-a (TNF-α) is a major pro-inflammatory cytokine known to impair GSH production by several mechanisms, creating an environment conducive to the development of OS. OS itself induces TNF-α production. This pathogenic cycle of GSH disturbances and enhanced TNF-α production may be active in the female reproductive tract in endometriosis. TNF-α is involved in the normal physiology of

female endometrium, playing a role in endometrial proliferation and shedding. However, abnormally high levels of TNF-α are pathogenic and have been demonstrated in the peritoneal fluid of women with endometriosis, with elevated levels appearing to be positively correlated with disease progression (31, 33). An *in vitro* study investigating endometriosis-associated infertility has shown that spermatozoa quality decreases following incubation with TNF-α in a dose- and time-dependent manner (191).

B. Oxidative stress and fertility outcomes with hydrosalpinx

Hydrosalpinx, a blocked, dilated and fluid-filled fallopian tube usually caused by previous tubal infection, has been shown to adversely affect fertility and decreases the success rates of *in vitro* fertilization (IVF). This is problematic, as IVF is considered the best fertility treatment for women with hydrosalpinx. Hydrosalpingeal fluid is thought to reduce embryo implantation rates and increase the risk of miscarriage. Proposed solutions have included removing the affected fallopian tube or separating it from the uterus prior to beginning IVF treatment. Salpingectomy prior to IVF has been shown to reverse the unfavorable effects of hydrosalpinges (211).

OS is thought to play a role in the mechanism by which hydrosalpingeal fluid (HSF) induces its embryotoxic effect (44). Laboratory investigations have demonstrated the presence of ROS, antioxidants, and lipid-peroxidation products in hydrosalpingeal fluid. Physiologic levels of ROS, below the threshold for being deleterious to embryos, may denote normal generation of ROS by a functional, healthy endosalpinx, whereas considerable endosalpingeal damage may result in nondetectable levels of ROS in the HSF. Low levels of ROS in tubal fluid have been demonstrated to positively correlate with blastocyst development. Therefore, normal tubal secretory function may be marked by the detection of ROS at low concentrations. Interleukins such as IL-1β and IL-6 are involved in acute phase reactions and have also been considered as markers of normal tubal secretory function. IL-6 levels in HSF have been shown to be positively correlated with blastocyst development rates and may prevent tubal fluid from becoming embryotoxic. IL-1β acts to inhibit ovarian follicular cell apoptosis and has been detected in normal tubal fluid (211).

Fallopian tubes enlarged with hydrosalpinx can leak fluid, which has demonstrated concentration-dependent embryotoxic effects. These adverse effects may be mediated by high concentrations of toxic substances in HSF. HSF may hinder implantation be reducing endometrial integrins. Integrins can be restored to normal with the excision of hydrosalpinges, improving implantation rates. The flow of HSF into the endometrial cavity may mechanically flush embryos from the uterus. Cytokines, leukotrienes, and prostaglandins may be secreted by tubal epithelium into sequestered fluid and can directly modify endometrial function. In women with distally occluded fallopian tubes, pro-inflammatory cytokines with embryotoxic effects are thought to produce poor IVF/embryo transfer (ET) outcomes. In a study by Barmat *et al*., the cytotoxic, angiogenic cytokine TNF-α was detected in all HSF samples, providing further evidence for OS involvement in the pathology of hydrosalpinx (29).

C. Unexplained infertility

Elevated ROS levels, which disrupt the peritoneal fluid balance of oxidants and antioxidants, are thought to establish infertility in women where other etiological factors are not identified. Increased ROS levels may damage the ovum released from the ovary, the zygote/embryo, and spermatozoa, which are highly sensitive and susceptible to the deleterious effects of OS (4). Investigations comparing peritoneal fluid ROS levels between women undergoing laparoscopic infertility evaluation and fertile women undergoing tubal ligation have demonstrated peritoneal fluid ROS levels to be significantly higher in women with idiopathic infertility compared to fertile women (239). Higher ROS levels in patients with unexplained infertility suggest reduced levels of antioxidants such as vitamin E and GSH that would ultimately reduce ROS-scavenging ability and prevent the neutralization of toxic ROS effects (239). This assertion is supported by a study in which concentrations of antioxidants in idiopathic infertility patients were found to be significantly reduced compared to those in fertile patients.

D. Oxidative damage to ovaries and induction of menopause

At the age of 35, the average woman's fertility potential begins to decline considerably. By the age of 40, this decline occurs more dramatically. Pre- and postovulatory aging of the oocytes have been associated with congenital anomalies, behavioral alterations, learning disabilities in later life, and constitutional diseases such as diabetes mellitus and schizophrenia (225). OS occurs at menopause because of the loss of estrogens, which have antioxidant effects on LDL. Estrogens confer cardioprotection by lowering protein oxidation and antioxidant properties.

SOD and GSH peroxidase expression have been shown to decrease in the premenopausal to menopausal ovary (156). SOD and GSH peroxidase levels exhibit a significant and positive relationship with aromatase enzyme activity (244). Higher amounts of OS have been evidenced in women undergoing IVF who are of advanced reproductive age (244). Ovarian senescence is caused by increased OS in the follicular fluid (Fig. 9). OS-induced injury may account for the age-related decline in follicle number and quality and damage of oocytes (224). This process may entail ROS adversely affecting mitochondrial DNA, proteins, and lipids. Decreased intracellular ATP levels, decreased GSH/GSH disulphide ratios, and increased cytosolic calcium have been observed in aged oocytes. ROS are known to be responsible for disrupting intracellular calcium homeostasis in oocytes, causing them to age (220).

Menopause is associated with alterations in antioxidant gene expression. Diminished expression of genes involved in the neutralization of ROS has been shown in IVF patients of advanced reproductive age (244). Decreased expression of SOD1, SOD_2, and catalase mRNA provides evidence of the downregulation of granulosa cell gene expression due to reproductive aging (227). This downregulation of genes coding for protective antioxidants is associated with an accumulation of OS, resulting primarily in damage to the mitochondria. The association between advanced reproductive age and reduced developmental competence in oocytes may be mediated by age-related changes that cause damaging effects in granulosa cells.

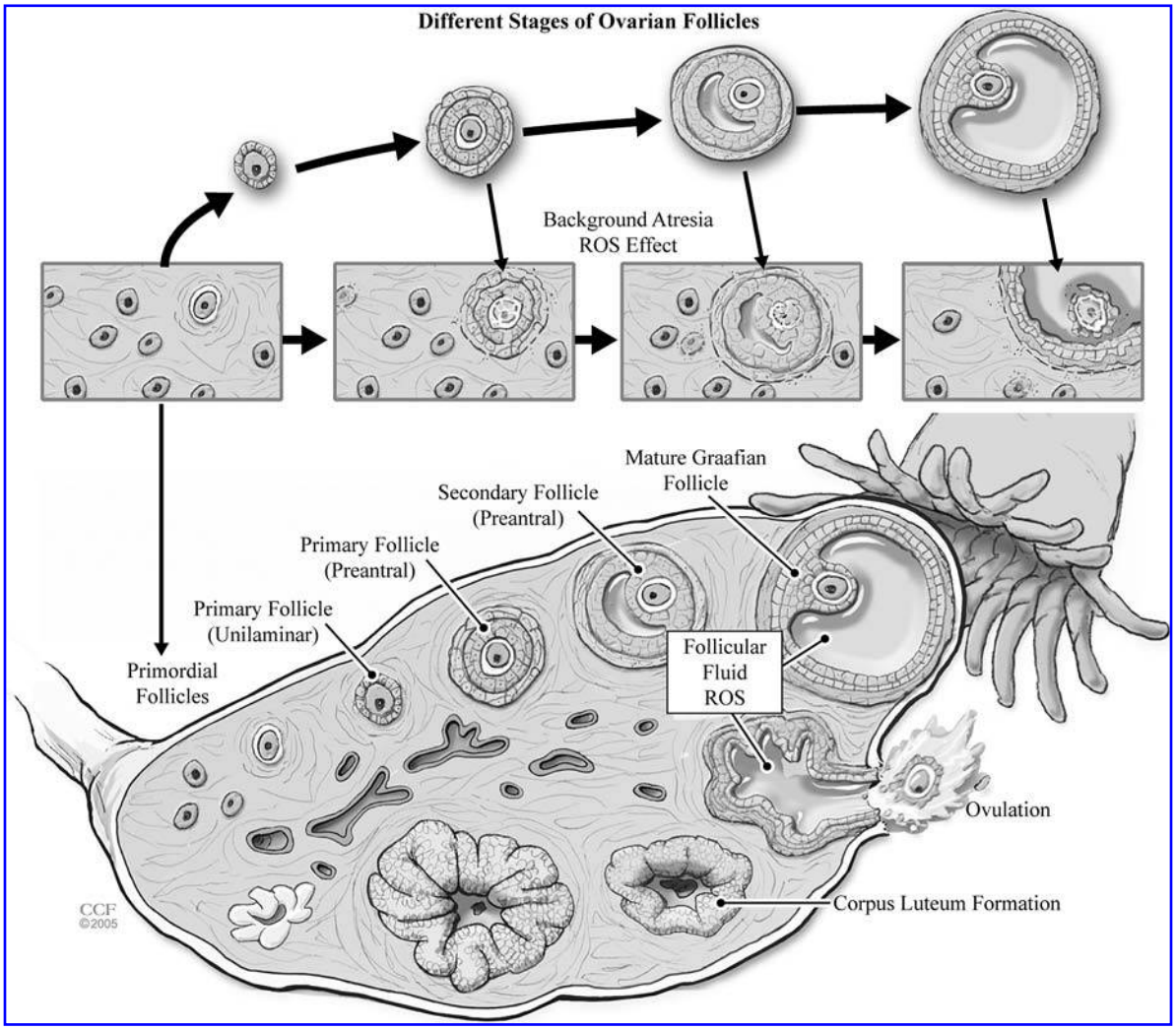

FIG. 9. ROS in folliculogenesis and assisted reproduction.

These changes may promote phenomena often observed in the pregnancies of older women such as damaged cytoskeleton and impaired fertilization and embryo development.

XI. IMPLICATIONS OF OXIDATIVE STRESS IN ASSISTED REPRODUCTION

OS influences oocyte and embryo quality and thus, assisted reproductive techniques (ART) outcomes. ROS appear to play a significant role in the modulation of gamete interaction and successful fertilization. ROS in culture media may impact post-fertilization development (i.e., cleavage rate, blastocyst yield, and quality—indicators of ART outcomes). Generation of ROS is inherent in spermatozoa and contaminating leukocytes. OS mediates peroxidative damage to the sperm membrane or of the oocyte and induces nuclear DNA damage. ROS can modulate the fertilizing capabilities of the spermatozoa.

Spermatozoa selected for ART usually originate from an environment experiencing OS, and a significant proportion may have damaged DNA (233). Sperm preparation techniques may influence the amount of ROS produced and the degree of OS experienced by spermatozoa. For example, studies indicate that repeated cycles of centrifugation significantly increase ROS production by spermatozoa (5). The duration of centrifugation is thought to be more critical for provoking ROS formation by spermatozoa than the force of centrifugation (201). Exposure to high ROS levels may cause DNA fragmentation in spermatozoa. When assisted reproductive techniques such as intrauterine insemination (IUI) or IVF are used, collateral peroxidative damage to the sperm plasma membrane ensures that fertilization cannot occur. However, in cases where intracytoplasmic sperm injection (ICSI) is the technique of choice, this natural selection barrier is bypassed and spermatozoa with DNA damage may be directly injected into the oocyte (11, 233).

Although the use of frozen-thawed semen has practical advantages in ART, cryopreservation has an adverse impact on sperm parameters in most mammals. A study investigated the hypothesis that DNA damage observed in cryopreserved sperm is attributable or related to OS. After a series of experiments, it was found that cryopreservation and ROS such as H_2O_2 pro-

moted DNA instability in ram sperm. The effect of cryopreservation on DNA damage appeared more rapidly compared with the effect of H_2O_2 on fresh sperm. Ram sperm motility was the parameter most sensitive to the effects of oxidative stress. The researchers ultimately concluded that DNA instability seen in ram sperm due to cryopreservation was in part due to the generation of lipid peroxide, although it is not thought to be the primary inducer of DNA damage (170).

A. Role of oxidative stress in in vitro *fertilization/intracytoplasmic sperm injection*

In addition to the observed effects of ART on male gametes, oxidative stress markers have been indicated in the follicular fluid of women undergoing IVF/ET. The follicular fluid microenvironment plays an integral part in shaping the quality of the oocyte. Low intrafollicular oxygenation is thought to decrease oocyte developmental potential by increasing the likelihood of oocyte cytoplasmic defects, impaired cleavage, and abnormal chromosomal segregation in oocytes of poorly vascularized follicles (235). Oocyte quality is known to exert a direct influence on fertilization rates, embryo quality, and viability (26, 162, 165, 167).

ROS production is implicated in the activation of apoptosis cell signaling, causing increased embryo fragmentation, arrest, or demise. Recent studies have explored the potential benefit of using supplements containing growth factors to protect *in vitro* cultured embryos from ROS-mediated apoptosis. Growth factors under investigation in mouse embryos include insulin growth factor (IGF)-1 and epidermal growth factor (EGF), which are endogenous in human endometrium and fallopian tubes (130).

OS in follicular fluid is postulated to influence oocyte maturation, fertilization, and pregnancy (Fig. 9). Follicular fluid ROS and lipid peroxidation levels may be viewed as markers for success with IVF. Patients who have become pregnant after undergoing a successful ART procedure have higher levels of lipid peroxidation and TAC in their follicular fluid compared to the follicular fluid of women who did not become pregnant after undergoing ART (165). The mean TAC in follicular fluid yielding oocytes that were successfully fertilized was shown to be significantly greater than the mean TAC levels from follicular fluid associated with oocytes that did not become fertilized. Also, follicles yielding oocytes that were subsequently fertilized have been shown to exhibit increased mean levels of GSH peroxidase (168). Conversely, mean TAC levels of fluid from follicles from which oocytes developed into embryos that persisted until transfer were reported to be significantly lower than mean TAC levels of follicular fluid associated with oocytes giving rise to nonviable embryos (162). TAC levels in Day 1 culture media are thought to serve as an additional biochemical marker indicating oxidative stress status during the early stages of embryonic growth. Day 1 TAC levels correlated significantly with clinical pregnancy rates in ICSI cycles. High TAC levels have also been considered as markers for poor response to ovulation induction in women with polycystic ovarian syndrome (73).

Pregnancy rates have been shown to be positively correlated with lipid peroxidation and TAC levels. Furthermore, significantly lower levels of follicular fluid ROS were reported in patients who did not achieve pregnancy with IVF/ICSI, when compared to those who became pregnant (26), suggesting that minimal levels of OS may be necessary for achieving pregnancy (165). Interestingly, the degree of OS in the follicular fluid of women undergoing IVF has been inversely correlated with patient age (244).

The belief that low levels of follicular fluid ROS have beneficial effects on ART outcomes has been challenged. Recent investigations have shown that high levels of ROS in follicular fluid are related to decreased fertilization potential of oocytes in ART cycles (51). A recent study analyzed the follicular fluid of patients undergoing ART, showing elevated levels of homocysteine caused by heightened oxidative stress, leading to poor oocyte and embryo quality in women with endometriosis (65). Decreased oocyte quality in patients with endometriosis also might be indicated by high levels of 8-hydroxy2-deoxyguanosine detected in their granulosa cells. 8-Hydroxy2-deoxyguanosine is a known and reliable indicator of oxidative stress-induced DNA damage. Elevated levels of 8-hydroxy2-deoxyguanosine also have been associated with low rates of fertilization and deteriorated embryo quality (195). Other markers of OS such as thiobarbituric acid-reactive substances, conjugated dienes, and lipid hydroperoxides in preovulatory follicular fluid have been measured and are reported to be unrelated to IVF outcome (117).

In a study from our group, Bedaiwy *et al*. (32) reported that slow early embryo development (7 cells on Day 3), high fragmentation (10%), and lessened formation of morphologically normal blastocysts may be associated with higher levels of ROS in the culture media on Day 1. Furthermore, although high Day 1 ROS levels in the culture media did not relate to fertilization rates (FR) in conventional IVF cycles, they were significantly related to higher embryonic fragmentation, lower FR, and blastocyst development rates in ICSI cycles (32).

It is known that reduced atmospheric oxygen concentration during ART procedures is beneficial to embryo development. One study aimed to establish the optimal oxygen concentration for oocyte maturation. It was observed that markers of high quality *in vitro* maturation systems such as oxygen uptake and mitochondrial membrane potential were higher for *in vivo*-matured oocytes and oocytes matured under 5% O2, compared with oocytes matured under 20% O2. Embryo cell numbers were shown to be 20–45% significantly higher when cumulus–oocyte complexes were matured at 5% O2. The overall findings suggest that oocytes matured in physiological concentrations of oxygen have better development and metabolic activity, which more closely resembles in vivo maturation (176).

Additional investigations studying a large sample size are needed to determine and understand the relationship between ROS activity levels and TAC levels in the male and female reproductive tracts. As ROS may exert differing effects at various stages of ART, more attention should be devoted towards uncovering how ROS and TAC modulate the ovulation process, oocyte quality, developmental capacity, and fertilization potential.

B. Role of ROS in in vitro *maturation of oocytes (IVM)*

Oocytes are a sparse resource and get depleted with increasing maternal age. A fixed pool of follicles exists in the ovary

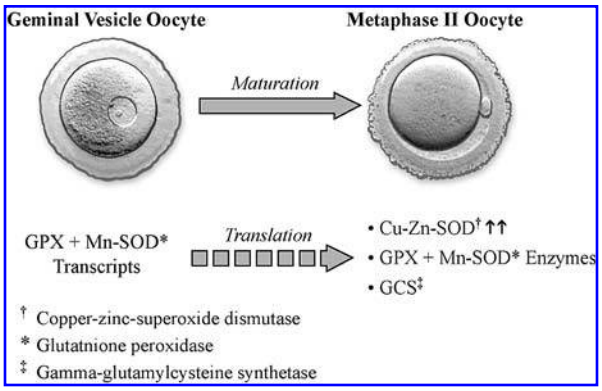

FIG. 10. Changing antioxidant profiles with oocyte maturation.

that undergoes folliculogenesis, oocyte selection, and development in each cycle throughout the reproductive period. *In vitro* maturation (IVM) of the oocyte is a novel technology explored by many researchers over the past two decades. IVM can be a core technology that can help in conserving this scarce resource and help generate large numbers of mature oocytes. There is also a body of evidence supporting the fusion of techniques and technologies, combining retrieval of immature oocytes, followed by IVM or cryopreservation of oocytes, followed by IVM.

Many of the literature reports highlight the differences between oocyte maturation *in vivo* and *in vitro* (120, 144). The majority of these reports conclude that successful IVM requires a coordinated nuclear and cytoplasmic maturation. *In vivo*, oocyte maturation is a complex process influenced by the interplay of the gonadotropin, the oocyte–cumulus oocyte cell interactions, and the enzymatic activities taking place at the molecular level (Fig. 10). Differential expression of enzymes such as Cu,Zn-SOD, Mn-SOD, and GSH peroxidase has been reported with oocyte maturation (67). It is important to replicate the *in vivo* conditions in the *in vitro* circumstances. Fertility outcomes with IVM are still not favorable. Retrieval of immature oocytes, and their culture and maturation *in vitro* not only eliminates the complications of stimulated cycles but also provides a good number of oocytes available for ART. Although the *in vitro* media is subject to modifications, the optimum media that will yield the best results in terms of *in vitro* maturation, fertilization, and pregnancy outcomes is yet to be designed.

In vitro culture media is always exposed to OS due to the generation of ROS from cellular metabolism and external factors (70). A plethora of extrinsic and intrinsic factors governs the generation of free radicals in the media, such as oxygen concentration, light, oocyte handling, and overall metabolism of the oocytes themselves (9). The lack of proper defense or consumption of antioxidant defense due to generation of ROS in the media allows increased damage to the oocyte DNA, or accelerated apoptosis in an unabated manner.

IVM can be a powerful tool for fertility preservation as well as a means of avoiding ovarian hyperstimulation in women undergoing ART. IVM of immature oocytes is a safer, cheaper, and simpler procedure for patients compared with conventional ART and promises to be one of the most applied ART techniques in the future.

XII. STRATEGIES TO MODULATE THE INFLUENCE OF ROS

A. Strategies to ameliorate oxidative stress in IVF/ICSI setting

ROS may originate from the male or female gamete, the embryo, or indirectly from the surroundings, including the cumulus cells, leucocytes, and culture media. ROS generated in IVF media can cause detrimental effects on mitochondria, DNA, RNA, sperm–oocyte fusion, sperm decondensation, and male pronucleus formation (9). Accurate assessment of the levels of free radicals and OS is essential and will help clinicians screen and identify patients with OS. Elevated levels of day 1 ROS are a marker of poor embryo quality, especially for ICSI cycles (32). Various antioxidants, including beta-mercaptoethanol, protein, vitamin E, vitamin C, cysteamine, cysteine taurine, hypotaurine, and thiols added to the culture media can improve the developmental ability of the embryo by reducing the effects of ROS. Sperm manipulation media are supplemented with human serum albumin, polyvinylpyrrolidine, and HEPES, which are DNA protectors (69). Scavenging of the ROS by various antioxidants has been proposed to lead to a better environment for pre-implanted embryos. Reduction in blastocyst degeneration, increased blastocyst development rates, increased hatching of blastocysts, and reduction in embryo apoptosis and other degenerative pro-oxidant influences have been reported. It is interesting to observe that blastocyst development *in vitro* always lags behind blastocyst development *in vivo* due to variation in the ability of IVF media and its components to scavenge ROS and prevent DNA damage. Addition of an enzymatic antioxidant (e.g., SOD to the culture media) prevented the deleterious effects of OS on sperm viability and on embryo development both *in vivo* and *in vitro*. This was demonstrated by increased development of the two-cell stage embryos to the expanded blastocyst stage in the SOD-supplemented media (19).

These data suggest that 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, and fertilization, and supports early embryonic development (87). Antioxidant supplementation also has been reported to have beneficial effects on sperm morphology and pre-implantation embryo development and leads to a reduction in the incidence of developmental defects.

Success rates in IVF are influenced by maternal age, number of oocytes retrieved, and the quality of the embryos transferred. Embryo quality is influenced by extrinsic factors like culture media. The role of IVF media in generating ROS and its damaging effects is complex not completely understood. When ROS are generated, they can diffuse into the cells and damage lipids, proteins, nucleic acids, DNA, and RNA. A large number of extrinsic factors that modulate OS can influence successful outcomes of IVF/ET (Fig. 11), including oxygen concentration, ionizing radiation, and levels of the antioxidants EDTA, SOD, and catalase (Fig. 11) (160).

Potential cellular sources of ROS in conventional IVF are different from those with ICSI (32). In ICSI, the oocyte is devoid of any cumulus cells, and therefore only the oocyte, injected spermatozoa, and the injected culture medium are po-

tential sources of ROS. In contrast, in conventional IVF-ET, ROS may originate from multiple oocytes per dish, large cumulus cell mass, or the spermatozoa used for insemination.

Evidence suggests that media supplementation with antioxidants, disulphide reducing agents, or divalent chelators of cations may be beneficial to embryos studied under *in vitro* conditions (85). The mouse two-cell embryo block can be prevented by antioxidant supplementation (80, 149). Co-incubation of mouse embryos that were exposed to exogenously-induced ROS with vitamin C significantly increased blastocyst development rates (238). Insulin-like growth factors I and II and epidermal growth factors have also been reported to have a positive effect on embryo development in mouse embryos that were exposed to exogenous OS (130). The addition of beta-mercaptoethanol, a thiol protector, reduced levels of apoptosis and blastocyst degeneration in bovine blastocysts (121). This also was associated with increased synthesis of GSH induced by beta-mercaptoethanol.

The embryo in the first trimester grows under low oxygen concentration as seen in maternal–fetal oxygen diffusion studies (113). In human embryos, the blastulation rate increased to 58.5% with low oxygen tension (5% O_2) and low illumination maintained throughout the period of embryo manipulation (154). Removal of environmental pollutants via air filtration may protect *in vitro* embryo growth. It has been recommended that low oxygen concentrations be used at all stages, such as insemination, fertilization, and embryo culture (41). Decreased development of inner cell mass and reduced proportion of transferred blastocysts developing into embryo also have been reported (118). Adoption of low oxygen concentration has been proposed as a standard for embryo culture, especially blastocyst production (30).

Optimal concentrations of individual amino acids, antioxidants, vitamins, and energy sources in culture media were determined and applied to improve the embryo quality and achieve higher blastulation hatching rates with human embryos (19). Different co-culture systems utilized for IVF are associated with improved embryo survival and increased pregnancy rates (209). The cells in co-culture produce enzymatic and nonenzymatic antioxidants and confer a protective effect on the embryos (238). Repeated change of media and use of sequential culture systems may help reduce exposure to ROS. The metabolic requirements of the embryos change with the different stages of development (93). Culture media incubated with fragmented embryos showed a progressive decline in antioxidant capacity (166). Hence, preventing exposure to fragmented embryos and defective spermatozoa would help surmount OS.

Supplementation with vitamin C and E also has been investigated. Antioxidant supplementation with albumin, low molecular weight thiol, and proteins (10% serum substitute supplement) has been used for *in vitro* oocyte maturation and embryo culture (1, 70). Supplementing media with vitamin E increased the number of bovine embryos that reached the expanded blastocyst stage (158).

Traces of metallic ions such as iron or copper in the culture medium can have very significant deleterious effects via the Fenton and Haber–Weiss reactions (85). This observation emphasizes the importance of water quality for the culture media. It is important to note that supplementation with metal chelators is beneficial toward embryo growth, especially transferrin, penicillamine, EDTA, and histidine. Currently there are no available tests for embryologists to assess the redox status of culture medium.

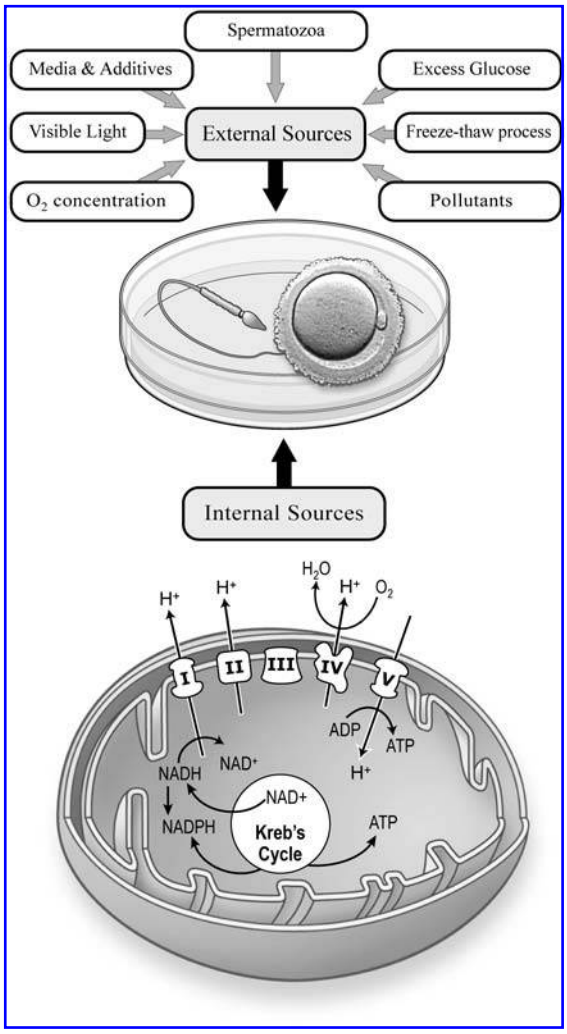

FIG. 11. Sources of OS in an assisted reproductive technique setting.

B. Optimizing in vitro *culture media to overcome oxidative stress in* in vitro *maturation of oocytes*

IVM outcomes continue to be poor, and the optimization of this technology is an area that needs to be tackled by future research studies. To enhance fertility outcomes of IVM, the focus must be on developing an optimal culture system for *in vitro* maturation. Taking into account the possible impact of ROS in the culture medium on oocyte maturation, there has to be a supplemented mechanism to nullify the effects of free radicals. Basic research studies are needed to define the precise equilibrium of the physiologically relevant antioxidants, their potency, and proportions of antioxidants present in the follicular microenvironment that are associated with oocyte optimal developmental competence *in vitro*. *In vivo* oocyte maturation lags behind development *in vivo*, and future translational re-

search needs to be directed towards being able to replicate *in vivo* conditions *in vitro*.

The physiological system has enzymatic (SOD, catalase, GSH peroxidase) and nonenzymatic (ascorbic acid, alpha-tocopherol, thiols) antioxidant defenses that keep ROS activity at a normal balance and minimize the insult. Scavengers reportedly are present in the endometrial and tubal secretions (85, 87). But the *in vitro* system mostly lacks such defenses, and it is this fact that leads researchers to study the effects of supplementing the follicular media with vitamin C. Many studies indicate that the addition of ascorbic acid does prevent oocyte membrane damage and increases basement membrane turnover (42, 222, 226, 228), leading to increased follicle integrity and survival (147). Some researchers proposed that certain concentrations of alpha-tocopherol or ascorbic acid do facilitate meiotic maturation of cumulus free oocytes and can protect cumulus cell DNA damage and apoptosis (222). It has been suggested that vitamin supplementations help oocyte maturation not only by alleviating OS but also by promoting maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK) activity (222).

Ali *et al*. (19) in their experimental studies on bovine oocytes found the antioxidants supplemented during *in vitro* maturation of the oocytes have beneficial effects on the development of the morula and blastocyst stages. In their study, the bovine oocytes were matured, fertilized (under 20% O_2), and embryos cultured (under 7% O_2) *in vitro* with/without supplementation with antioxidant cysteine, *N*-acetyl-L-cysteine (NAC), catalase, and SOD. They found a significant improvement in the proportion of oocytes undergoing morula and blastocyst development with the addition of cysteine to the maturation medium. There was no additive beneficial effect of any other antioxidants used in their study. During IVF addition of antioxidants significantly ($p < 0.05$) reduced the bovine embryo development.

Oocytes also can be protected from oxidant-induced early apoptosis by supplementing media with vitamin E and C (68, 147, 229). Alpha tocopherol scavenges lipid peroxy radicals and acts as a chain-breaking antioxidant. Though there are controversies regarding dietary supplementation of vitamins and their effects on reproductive functions (223), evidence does point towards positive effects in attenuating age-induced abnormal chromosomal alignments and segregation of metaphase-II spindles in mouse oocytes (223). A recent study by our group demonstrated that OS has a detrimental effect on microtubular dynamics and chromosomal alignment in mouse oocytes. A beneficial role of vitamin C in protecting metaphase-II mouse oocyte spindle structure and chromosomal alignment against oxidant H_2O_2-induced damage when added to media simultaneously with the inducer was observed (46). This study suggests that ascorbic acid might prevent OS-induced microtubular break down and thus prevent aneuploidy in mature oocytes in IVM.

Alpha-tocopherol and ascorbic acid both act as crucial antioxidants in the intra- as well as extracellular environment. The role of ascorbic acid was demonstrated in a study on isolated murine oocyte–granulosa cell at certain concentrations of the metabolite glucose. It was found that 0.5 m*M* ascorbic acid significantly reduced the apoptosis of the complexes (68). Vitamin E is an important lipid-soluble vitamin with alpha-tocopherol being its most potent form. It plays a vital role in preventing oxidative damage like lipid peroxidation caused by ROS.

The role of both the antioxidants in IVM and the vitamin E regeneration capability of ascorbic acid was evaluated by Dalvit *et al*. (49). The researchers reported that the concentration of alpha-tocopherol naturally present in the COCs decreased by half during IVM but remained constant in the presence of ascorbic acid. They also found that addition of both the antioxidants significantly ($p < 0.05$) decreased the blastocyst formation rate. Oocytes have protective enzymes like the SOD, GSH peroxidase, and catalase expressed in both the cumulus cells and oocytes (42).

Tao *et al*. (222) demonstrated that L-ascorbic acid and alpha-tocopherol supplementation caused more denuded oocytes to attain meiotic maturation and prevented cumulus cell DNA fragmentation. However, this effect was not seen in oocytes with cumulus cells, and both the antioxidants compromised the viability of the denuded oocytes and the COCs (222). Ascorbic acid 2-O-α-glucoside (AA-2G), a stable derivative of ascorbate, enhances the cytoplasmic maturation of porcine oocytes, essential for postfertilization developmental competence by preventing OS and providing a steady and continuous supply of ascorbic acid during IVM (226).

Optimal concentration of oxygen, metabolites such as glucose, cumulus cell density, and supplements is required for reduction of OS generation and enhancing the cytoplasmic maturation, fertilization potential, and developmental competence of *in vitro*-matured oocytes.

The purported implication of antioxidant supplementation is to optimize the IVM technique and improve fertility outcomes with this technique. The rationale behind IVM is to avoid the effects of hormonal hyperstimulation and reduce patient time and drug costs associated with other assisted reproductive technologies.

C. Sperm preparation techniques for assisted reproductive techniques

Reducing seminal OS is important for both natural and assisted fertility. Sperm quality is one of the most important factors affecting the outcome of ART. Human spermatozoa are highly vulnerable to OS as they have a high content of polyunsaturated fatty acids and lack the capacity for DNA repair. The resulting DNA fragmentation may lead to reduced fertility. Investigators have studied the benefits and side effects of antioxidant therapy, a measure that aims to reduce or avoid the occurrence of DNA fragmentation related to free radical exposure. Therapeutic strategies addressing male infertility may vary according to whether the ROS is generated predominantly from spermatozoal or leukocyte sources. As seminal leukocytes are assumed to be potentially harmful, causes of abnormal leukocyte infiltration in semen, such as inflammation, infection, and cigarette smoking should be recognized and addressed. ART sperm preparation techniques such as density gradient and swim-up separate out good quality spermatozoa and are used to minimize the amount of interaction between ROS-generating cells such as spermatozoa and/or seminal leukocytes with normal spermatozoa. However, the repeated cycles of centrifugation needed for this procedure may actually induce spermatozoa to generate ROS. Therefore, sperm preparation techniques

with minimized centrifugation periods are required to reduce the risk of ROS-mediated injury to sperm.

As previously mentioned, during sperm preparation for ART, spermatozoa are washed free from their seminal plasma and left susceptible to OS due to a lack of antioxidant protection. Increased ROS production by spermatozoa, mounted with the removal of seminal antioxidant defense, put spermatozoa at a high risk for experiencing oxidative damage. This phenomenon has prompted researchers to investigate the potential benefits of supplementing sperm preparation and gamete culture media with antioxidants. HEPES [(2-hydroxyethyl) piperazine-1-ethanesulfonic acid] was found to be the most potent protector as determined by plasmid relaxation assay, which measures the plasmid DNA damage (69). IVF media supplemented with human serum albumin (10 mgm/ml), glucose (2.78 *M*mol), 1% polyvinyl alcohol, 5% polyvinylpyrrolidine, sucrose (100 m*M*), 60% percoll, human tubal fluid, catalase (1 and 10 IU), and HEPES (21 m*M*ol) scavenges ROS and confers protection from DNA damage.

D. Role of oral antioxidants

Several nutritional and biochemical factors are said to regulate male reproductive function and in deficiency states may precipitate subfertility. The micronutrient folate has a role in DNA synthesis and mediates spermatogenesis. Zinc also has been named a factor in testicular development, sperm maturation, and testosterone synthesis. Thiols, such as GSH, check the levels of ROS generated by spermatozoa and affect DNA compaction and spermatozoa stability and motility. Zinc, folate, and GSH may provide protection of cells against oxidative and ROS-mediated electrophilic stress that would lead to damage to DNA, protein, or cell membranes. Collectively, folate, zinc, thiols and ROS influence apoptosis and are imperative for sperm release (66). A better understanding of these nutritional and biochemical pathways might serve to improve diagnosis and management of male factor infertility.

Patient treatment with oral antioxidant vitamins has become standard practice for male infertility. Numerous controversial studies have assessed the ability of antioxidants to treat male infertility, with most agreeing that these therapies result in only partial success (6, 81). A recent randomized, double-blind, placebo-controlled study recorded a statistically significant increase in viable pregnancy rate with use of orally administered antioxidant therapy in IVF/ICSI (230). This effect is thought to be attributed to reductions in sperm DNA damage caused by OS (81).

Menezo *et al*. (141) investigated the efficacy of an antioxidant vitamin associated with zinc and selenium. Oral intake of vitamins was shown to reduce DNA fragmentation, but it also increased sperm decondensation considerably. A high degree of sperm decondensation may prevent IVF/ICSI success, as it may lead to asynchronous chromosome condensation and cytoplasmic fragments in the embryo (141). Antioxidant treatment for improving semen quality in subfertile men should be carefully considered and contraindicated in men whose semen samples show a high degree of decondensation. Otherwise, when sperm decondensation is not an issue of concern, the benefits of antioxidant therapy may override risk.

OS is becoming increasingly implicated in the pathology of various female conditions, many of which precipitate subfertility and pregnancy loss. It has been demonstrated that OS results in luteolysis and that oral antioxidant supplementation is useful in preventing luteal phase deficiency and results in a higher pregnancy rate (48, 96). Other investigations have failed to report this beneficial effect (82). A meta-analysis of the effect of vitamin C supplementation on pregnancy outcome was inconclusive (187). Another meta-analysis using the fixed effects model for women taking any of the vitamin supplements starting prior to 20 weeks gestation failed to show a reduction in total fetal losses or in early and late miscarriage (188). There is a potential role for well-balanced nutrition and medicinal herbs to achieve adequate levels of antioxidants *in vivo* and the prevention and treatment of reproductive diseases such as preeclampsia and ovulatory disorder infertility (45, 194). Ongoing trials continue to assess the safety and effectiveness of therapies that modulate ROS to improve fetal and maternal outcomes.

E. Recurrent pregnancy loss

Vitamins including vitamins B6, B12, and folic acid are reported to reduce the risk of pregnancy loss and prevent subfertility (66, 184). Though it is a proven fact that folic acid supplementation prevents neural tube defects, the role of vitamins in the prevention of spontaneous abortion and recurrent pregnancy loss (RPL) has yet to be demonstrated (78, 245). It has been shown that addition of vitamins C and E may strengthen antioxidant defenses in *in vitro* media, but oral antioxidant supplementation in human studies does not provide definite, conclusive evidence. Evidence of increased teratogenicity associated with high dose vitamin A does exist in the literature, but adequate dietary vitamin consumption may not be harmful.

Patients with homocysteinemia have increased incidence of multisystem disorder, especially affecting the cardiovascular system. Homocysteine has been reported to have oxidant properties (177) and thus may contribute to vascular pathology and functional derangements of the placenta that have been reported to lead to uteroplacental insufficiency and intrauterine growth restriction. Homocysteine metabolism is closely linked to folate and vitamin B6 levels, and dietary deficiency of these leads to hyperhomocysteinemia. Levels of vitamin B6 have been documented to be lower in patients with spontaneous RPL, and supplementation of vitamin B6 in these patients resulted in an increase in live birth rate (79, 177, 184).

Selenium status and its association with OS-induced RPL have been investigated. Selenium is co-factor for the antioxidant enzyme GSH peroxidase. While a few studies showed no significant differences in selenium levels between patients and controls (17), some studies reported significantly lower selenium levels in patients with RPL (129, 153). The contradictory findings, possibly due to studies encompassing small sample sizes, fail to provide conclusive evidence of potential benefit from selenium supplementation.

F. Endometriosis

Samples incubated with TNF-α and Infliximab (a monoclonal antibody that neutralizes the toxic effects of TNF-α by binding both its soluble and membrane forms) were seen to have

higher sperm motility and membrane and chromatin integrities than samples treated with TNF-α only. These results advocate the potential use of Infliximab to treat female infertility caused by endometriosis in patients with elevated levels of TNF-α in their peritoneal fluid. Intake of Vitamins E and C, zinc, and selenium assessed by a questionnaire correlated inversely with the severity of the disease in women with endometriosis (142).

Pentoxifylline, a 3′5′-nucleotide phosphodiesterase inhibitor, is another drug being studied for its potential to treat infertility secondary to endometriosis. Pentoxifylline has strong immunomodulatory actions and has been observed to significantly depress the embryotoxic effects of H_2O_2 (250)

G. Unexplained infertility

Higher ROS levels in patients with unexplained infertility suggest reduced levels of antioxidants such as vitamin E and GSH, which would ultimately reduce ROS-scavenging ability and prevent the neutralization of toxic ROS effects (239). This assertion is supported by a study in which concentrations of antioxidants in idiopathic infertility patients were found to be significantly reduced compared to those of fertile patients. These differences suggest that antioxidant supplementation to treat elevated ROS levels in patients with unexplained infertility might be of potential benefit.

H. Preeclampsia

Based on recent data that suggest placental OS contributes to the endothelial dysfunction that causes preeclampsia, antioxidants have been contemplated as a potential preventive measure (182), although they may be ineffective once the disease is evident. The use of vitamin C and vitamin E supplementation to reduce OS, limit the injury of endothelial cells, and prevent or reduce disease severity has been assessed (116). Poston *et al*. (174) carried out a placebo-controlled trial in a diverse group of high risk women that demonstrated antioxidant supplementation did not reduce the risk of preeclampsia; rather, it was associated with a significantly higher incidence of complications, including low birth weight, fetal academia, gestational hypertension, and the need for intravenous antihypertensive and magnesium sulfate therapies (174). The risk of these complications was shown to be especially increased in women with diabetes.

In a randomized study, Rumbold *et al*. (188) reported no significant differences between the vitamin group and placebo group in the risks of preeclampsia, infant mortality/morbidity, or delivery of a low birth weight infant (188). A beneficial secondary outcome relating antioxidant therapy with a significant reduction in the risk of respiratory distress syndrome was illustrated. However, the supplementation also was associated with increased risk of hospitalization due to hypertension in women and the use of antihypertensive therapy. The group concluded that the study results did not support the routine use of antioxidant vitamins by nulliparous, low-risk pregnant women to prevent preeclampsia or to improve perinatal morbidity. As the trial was powered to detect only a reduction of risk for preeclampsia by 50% or more, the possibility of a smaller benefit of antioxidant therapy cannot be ruled out. The fact that some potentially harmful adverse maternal outcomes were more common in the antioxidant group than in the placebo group might be explained by chance alone. However, certain adverse outcomes were similar to those reported in another recent study involving pregnant women at high risk for preeclampsia (174).

It is not clear whether some subgroups of women may be at increased risk for adverse effects from supplementation with antioxidants and whether a role for antioxidant therapy in lowrisk women still exists. Until more information is available, antioxidant therapy should not be prescribed as part of routine practice.

XIII. CONCLUSIONS AND FUTURE DIRECTIONS

A delicate balance exists between free radicals and antioxidants in the female reproductive process that maintains redox homeostasis. Free radicals and OS have an important role in modulating many physiological functions in reproduction, as well as in conditions such as infertility, endometriosis, abortion, hydatidiform mole, embryopathies, and pregnancy complications such IUGR and preeclampsia. The evaluation of *in vivo* OS is difficult. The minimum safe 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 OS during assisted reproduction. Future studies must elucidate a better understanding of the role of metabolism in controlling the intracellular redox potential of oocyte and pre-implantation embryos. There is ongoing debate over the role of antioxidant supplementation in both male and female infertility. Only a few reports of antioxidant therapy in female infertility exist, and antioxidant therapies have not been successful in ameliorating infertility conditions or in modifying the outcomes of many of the diseases investigated. It is not clear whether some subgroups of women may be at increased risk for adverse effects from supplementation with antioxidants and whether a role for antioxidant therapy in low-risk women still exists. Until evidence of benefit is available from ongoing trials, antioxidant therapy should not be prescribed as part of routine practice.

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ABBREVIATIONS

ATP, adenosine 5′-triphosphate; Ad5BP, antibody to Ad4-binding protein; aPL, antiphospholipid; ASK1, apoptosis signaling-regulation kinase; CN, cellulose nitrate; Cu, copper; cGMP, cyclic guanosine monophosphate; COX, cyclo-oxygenase; CSH, cysteamine; Cu,Zn-SOD; Cu, Zn-superoxide dist-

mutase; ET-1, endothelin-1; EDTA, ethylenediamine tetraacetic acid; GFH, glutathione; GSSG, GSH disulphide; GPx, glutathioneperoxidase; H_2O_2, hydrogen peroxide; $HO^{\cdot}$, hydroxyl; IUGR, intrauterine growth retardation; Fe, iron; LDL, low-density lipoprotein; MDA, malondialdehyde; MAPK, mitogen activated protein kinases; Mn-SOD, manganese superoxide dismutase; NO, nitric oxide; NOS, nitric oxide synthase; NADH, nicotinamide adenine dinuceotide phosphate; PRDX, peroxiredoxins; PCD, programmed cell death; ROS, reactive oxygen species; RNS, reactive nitrogen species; RPL, recurrent pregnancy loss; $O_2^{-\cdot}$, superoxide.

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