Invited review

Significance of oxidative stress in human reproduction

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Submitted: 10 October 2008
Accepted: 24 November 2008

Arch Med Sci 2009; 5, 1A: S28–S42

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Abstract

The aim of this review is to summarize the role of free radicals and oxidative stress in the pathophysiology of human reproduction. As our knowledge of human reproduction and factors contributing to infertility continues to expand at a phenomenal rate, it is evident that oxidative stress (OS) has been implicated in both male and female infertility. Reactive oxygen species function *via* proinflammatory cytokines and this mechanism has been proposed as a common underlying factor for endometriosis, hydrosalpinx and various other pathologies affecting the female reproductive process. This review highlights the role of OS in the above mentioned female pathologies as well as pre-eclampsia, hydatidiform mole, unexplained infertility, premature ovarian aging and recurrent pregnancy loss. Oxidative stress, sperm DNA damage and apoptosis have been implicated in male infertility, as seen in male pathologies highlighted in this review – varicocele, cryptorchidism and infection. Substantial evidence provides a positive correlation between elevated reactive oxygen species and poor fertility outcome. The literature provides evidence suggesting treatment with antioxidant supplementation provides an effective therapeutic modality for OS in human reproduction, however further studies are warranted to provide answers on their safety and effectiveness.

Key words: reactive oxygen species, male infertility, female infertility, antioxidants, free radicals.

Introduction

Infertility is defined as the inability to conceive after 1 year of unprotected intercourse by couples of reproductive age. Although the national survey of family growth conducted in 1995 by the Centers for Disease Control and National Institutes of Health suggests male factor fertility is the causative factor, in approximately 40% of the 2 million infertile young married couples in the United States, female infertility remains a major contributing factor. As our knowledge of human reproduction and infertility continues to expand at a phenomenal rate, it is becoming evident that oxidative stress (OS) plays a major role. This review is designed to explain oxidative stress and its role related to both male and female reproduction.

Role of oxidative stress in male reproduction

Infertility affects approximately 13-18% of couples. Increasing evidence from different studies indicates an increase in incidence of male reproductive problems [1]. The pathology of the male reproductive system can be classified as congenital (cryptorchidism, sickle cell disease,

Klinefelter's syndrome, etc.) and acquired (varicocele, torsion, infection, etc.). Regardless of classification, all these disorders can cause temporary or permanent impairment in spermatogenesis and diminish infertility [2, 3]. Sperm migration leads to poor morphology and negatively impacts sperm function. The result is spermatozoal dysfunction and infertility. In the era of evidence-based medicine, identifying causes of pathologies and implementing specific treatments is necessary for all reversible male pathologies. Numerous studies have been conducted to provide insight into the relationship male infertility and male reproductive pathologies that lead to male infertility. Thus this review will discuss the role of OS in the reversible causes of male reproductive pathologies, focusing on varicocele, cryptorchidism, and infection.

Physiological role of reactive oxygen species in the male reproductive system

Reactive oxygen species (ROS) are required for normal spermatozoal function and maturation. Extensive research suggests a basal level of ROS is essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation [4, 5]. Under normal circumstances, capacitation of spermatozoa is a process that prepares the spermatozoa for fertilization [6, 7]. During the process, levels of intracellular calcium, ROS, and tyrosine kinase are elevated, leading to increased formation of cyclic adenosine monophosphate. Cyclic adenosine monophosphate, in turn, leads to hyperactivation of spermatazoa, a state in which the spermatozoa acquires high motility [8, 9].

Once spermatozoa are capacitated and exhibit hyperactivation, the ability to undergo the acrosome reaction ensues, resulting in spermatazoa's ability to fertilize [10]. Studies have demonstrated the role of ROS in promoting sperm capacitation. The role of ROS in facilitating sperm-oocyte interaction has also been demonstrated. Lipid peroxidation alters the plasma membrane, creating a favorable environment for sperm adhesion to the oocyte [11]. Although documentation supports the role of ROS in normal sperm function leading to fertilization, the exact levels and/or duration of normal exposure has yet to be established.

Sources of excess oxidative stress

Every human sperm ejaculate serves as a potential source of ROS, due mainly to the fact that it contains different types of cells, i.e. mature and immature spermatozoa, round cells from different stages of spermatogenesis, leukocytes, and epithelial cells [12].

Of these different types of cells, the major sources of ROS in semen are immature spermatozoa and seminal leukocytes [13]. Reactive oxygen species generated by abnormal spermatozoa occur at the level of the plasma membrane and mitochondria *via* the NADPH oxidase system and NADPH-dependent oxidoreductase system, respectively [14]. They include radicals, non-radicals, and oxygen derivatives. Reactive nitrogen species (RNS) are free nitrogen species and are classified as a subclass of ROS. In addition to immature spermatozoa and seminal leukocytes as major sources of ROS, dead spermatozoa also contribute. Dead spermatozoa liberate the enzyme amine oxidase, which acts on spermine and spermidine to produce hydrogen peroxide and other amine compounds [15].

Mechanism of oxidative stress-induced injury

Substantial evidence demonstrates that high levels of ROS cause damage to cellular components in testis, including the DNA of mature spermatozoa. An oxidative DNA damage adduct, 8-hydroxy-2-deoxyguanosine (8-OHdG), serves as the key biomarker of oxidative stress [16]. Oxidative stress has been shown to cause extensive deleterious effects on the DNA of mature spermatazoa, including abnormal DNA denaturation, DNA base-pair oxidation, chromatin cross-linking, and chromosome microdeletions [17-21]. Furthermore, ROS has been demonstrated to cause various types of gene mutations, such as deletions, point mutations, or polymorphisms, resulting in decreased semen quality [22, 23]. Reactive oxygen species facilitates various other mechanisms that induce damage and include lipid peroxidation [21], apoptosis and altered mitochondrial function *via* decreasing mitochondrial membrane potential.

Spermatozoa have the ability to self-repair damaged DNA; however, this is in the case of minimal damage. In cases of extensive damage, apoptosis occurs. Normally, apoptosis functions to control overproduction of male gametes and eliminates abnormal spermatozoa [24]. In ROS-induced apoptosis, some cells labeled for programmed cell death escape, a process known as abortive apoptosis. The result is a large population of abnormal spermatozoa that failed to be eliminated [25].

Role of oxidative stress in male pathologies

Varicocele

Varicocele is defined as abnormal tortuosity and dilatation of veins of the pampiniform plexus in the spermatic cord [26]. The etiology behind varicocele development remains unclear; however, various theories currently are being circulated. The most commonly accepted explanation is that Leydig cells and germinal cells are damaged due to tissue

hypoxia caused by venous stasis and small vessel occlusion [27]. Further explanations include retrograde flow of toxic metabolites from adrenal or renal origin, impairment of the hypothalamic-gonadal axis leading to suppressed gonadotropin or androgen secretion and elevation in scrotal temperature [28, 29]. Whatever the patho-physiology, varicocele has long been associated with male infertility.

Numerous studies attest to oxidative stress as a cause of male infertility. An imbalance between reactive oxygen species and seminal antioxidants in the semen leads to oxidative stress and subsequent spermatozoa damage [30]. Agarwal et al. performed various studies demonstrating high levels of seminal oxidative stress in men with varicocele and suggested sperm damage in men with varicocele is attributed to oxidative stress. Under normal physiological conditions, spermatozoa possess an enzymatic antioxidant system to protect from potential oxidative stress damage. When the redox equilibrium between oxidants and antioxidants is disturbed, oxidant DNA damage in spermatozoa leads to male infertility [31].

In evaluating the relationship between varicocele and oxidative stress, studies have identified various markers to assess the extent of oxidative stress. Sakomoto et al. created a study to assess oxidative stress markers, antioxidant capacity and cytokines in seminal plasma from patients with varicocele [32]. In the study, particular focus was on two biomarkers of oxidative stress: 8-OHdG and hexanoyl-lysine (HEL). Formation of 8-OHdG may have potential diagnostic value in evaluating sperm function and male fertility; whereas HEL levels indicate early stages of lipid peroxidation by oxidative modification. To assess the integrity of the enzymatic antioxidant system, levels of superoxide dismutase (SOD) were assessed. Finally, since several studies suggest cytokines enhance oxidative stress, levels of IL-6, IL-8 and TNF-α were assessed as well [32].

The study evaluated semen samples of infertile men, further subcategorized into three groups: (1) men with azoospermia, (2) men with oligospermia, and (3) men with normospermia and a varicocele. A control group comprised normozoospermic healthy men. Study results showed significantly higher HEL concentration and SOD activity in seminal plasma from men with azoospermia, oligospermia, and varicocele. Oligospermic males with varicocele also had significantly higher IL-6 levels in seminal plasma. Study results were consistent with demonstrating excessive oxidative stress occurring in seminal plasma of patients with varicocele and further strengthened the association between DNA damage seen in varicocele patients and oxidative stress [32].

To better understand the mechanisms of sperm damage attributed to male infertility seen in varicocele, Smith et al. designed a study to determine the extent of sperm nuclear DNA damage and its relationship to oxidative stress [33]. Semen samples from patients diagnosed with grade II and III clinical varicocele were collected; the control group consisted of normozoospermic healthy patients. Standard semen analysis on samples was performed, assessing sperm parameters. Semen samples from the patients with varicocele were classified as having normal or abnormal sperm parameters based on World Health Organization (WHO) criteria and Kruger's strict criteria. Sperm DNA damage was evaluated by sperm chromatin structure assay (SCSA) and by TUNEL assay. In addition, measurements of ROS levels and total antioxidant capacity (TAC) were determined. Results indicated a positive association between varicocele and high levels of DNA-damaged spermatozoa even in the presence of a normal semen profile. Furthermore, results suggested oxidative damage is associated with sperm DNA damage in these patients.

Cryptorchidism

Cryptorchidism has been shown to induce germ cell apoptosis resulting in increased OS production. Nitric oxide (NO) has been associated with a decrease in TAC and contributes to increased germ cell apoptosis seen in cryptorchidism. Ishikawa et al. conducted a study investigating the relationship between endothelial NO synthase (eNOS) and germ cell apoptosis in testes of transgenic mice [34]. The results provided evidence that eNOS plays a functional role in mouse spermatogenesis in cryptorchidism apoptosis. This gives way to the potential for a new therapeutic strategy for male infertility associated with cryptorchidism, focusing on eNOS reduction. Ishikawaka et al. evaluated this potential treatment modality. Tetrahydrobiopterin (BH4) is an essential cofactor of NOS. Activation of the immune system has been shown to stimulate an increase in the BH4 rate-limiting enzyme GTP cyclohydrolase I (GTPCH1) activity, resulting in an increase in intracellular BH4 and NO levels. The study demonstrated that treatment with BH4 causes a decrease in the GTPCH1 mRNA and BH4 levels, with a simultaneous decrease in endothelial NOS protein levels, nitrotyrosine levels, and NO levels, resulting in a decrease in testicular damage. Thus, supplementation with BH4 could provide a new therapeutic intervention for the cyptorchid patients [35].

Kumagai et al. also have shown that xanthine oxidase inhibitors suppress testicular germ cell apoptosis induced by experimental cryptorchidism in

rats [36]. Xanthine oxidase is found abundantly in microvascular endothelium. It converts hypoxanthine to xanthine and xanthine to uric acid with simultaneous production of superoxide anion. Thus it acts as a source for ROS in testicular injury by reperfusion or toxic chemicals. Allopurinol, a competitive xanthine oxidase inhibitor, reduces OS injury in several organs ($P<0.01$ vs. control) [36]. A study by Vigueras-Villasenor et al. [37] found that administration of allopurinol decreases ROS ($P<0.05$) and reduces histopathological changes and apoptotic death of germ cells, thus increasing the epithelial area. They also found an increase in the proliferation index (PI) in cryptorchidism rats treated with allopurinol in comparison with rats without allopurinol, but no stimulation of proliferation was noted since the contralateral testes did not show any rise in PI. They confirmed that the overproduction of reactive oxygen species plays an important role in cryptorchid testes.

Many studies have been conducted to prove the role of OS in the apoptosis induced in cryptorchism and the protective role of various antioxidants in directly or indirectly reducing apoptosis in cryptorchism. Gao et al. showed that nitric oxide synthase inhibitor (L-NAME) could protect the germ cell from apoptosis in experimentally induced cryptorchidism in rats ($P<0.01$). L-NAME acts by reducing the activity of NOS and reducing the levels of NO in the testis [38]. Fei et al. showed that a decrease in antioxidant enzyme activity is closely related to germ cell apoptosis in cryptorchidism ($P<0.01$).They showed that the activity levels of antioxidant enzymes SOD and catalase were significantly decreased in germ cell apoptosis with no change in glutathione peroxidase (GSHPx) activity and increased levels of malonic diethyl aldehyde (MDA) in the rats with cyptorchidism [39].

Infection

Considerable evidence provides an association between bacterial infections and an increase in ROS production. The associated bacteria include *Shigella*, *Entamoeba histolytica*, *Rickettsia rickettsii*, and *Salmonella typhi*, to name a few [40-43]. Leukocytes are responsible for generating the extracellular ROS seen in infectious states [44, 45].

During infection, leukocytes levels increase [45]. Cytokines activate the leukocytes, resulting in an increase in ROS production and subsequent spermatozoa damage [46]. Leukocytes generate ROS *via* enzymes as a means of defense against the pathogen. It has been suggested that the high levels of activated leukocytes seen during infection produce ROS that leak out of the cell membrane causing damage to spermatozoa, leading to infertility [47]. Depuydt et al. conducted a study demonstrating that leukocytospermia and infection of the male accessory gland negatively affect sperm parameters, thereby reducing fertility [48]. Additional studies demonstrated that patients with leukocytospermia exhibit abnormal DNA integrity [49].

Role of oxidative stress in female reproduction

Many studies have proven the presence of ROS and antioxidants in the female reproductive tract. ROS and antioxidants have been detected in follicular fluid [50, 51], tubal fluid, oocytes, embryo [52], and endometrium. ROS in ovaries are produced by multiple cells like macrophages, parenchymal steroidogenic cells, and endothelial cells [53]. Genes for SOD and glutathione peroxidase are expressed in oocytes, embryos, and oviducts. Superoxide dismutase has been detected in the endometrium through the menstrual cycle. High SOD concentrations were detected in decidual cells during early pregnancy [54]. The ROS levels in the microenvironments associated with follicular, hydrosalpingeal, and peritoneal fluid have a direct bearing on oocyte quality, sperm-oocyte interaction, sperm-mediated oocyte activation, implantation, and early embryo development.

Physiological role of reactive oxygen species and antioxidants in the female reproductive tract

A number of studies have shown that controlled OS plays a modulatory role in a variety of physiological processes in the female reproductive system like oocyte maturation, physiological follicular atresia, ovulation, fertilization, luteal regression, and luteal maintenance in pregnancy [55].

Role of oxidative stress in ovaries

Reactive oxygen species aids in oocyte maturation by inducing the resumption of meiosis I (MI) at puberty [56, 57], and antioxidants can inhibit this [58]. Glutathione (GSH) plays a critical role in oocyte maturation, particularly in the cytoplasmic maturation required for pre-implantation development and formation of the male sperm pronucleus [59]. In bovine models, β-carotene has been shown to play a role in cytoplasmic maturation [60]. As already mentioned, antioxidants inhibit the progression of MI, but are helpful for meiosis II (MII). This suggests a complex role for antioxidants and ROS in the ovarian environment.

Low ROS levels induce theca interna cell proliferation [61]. Behl and Pandey (2001) [62] studied the changes in the concentration of the antioxidants catalase and estradiol (E2) in ovarian follicular cells with changes in the levels of FSH. They found a concomitant increases in catalase and E2 in response to FSH. From this the authors suggested that catalase may play a role in follicle selection and prevention of apoptosis.

Vascular changes and the proteolytic cascade are responsible for mammalian ovulation [63]. The interaction between these two is governed by cytokines, vascular endothelial growth factor, and free radicals (both ROS and RNS). Oxidative stress and cytokines are proposed to be interlinked and act as intercellular and intracellular messengers in the ovary. Interleukin-1β causes nitrite to accumulate in rat ovaries, demonstrating the close interaction between cytokines and NOS [64].

Reactive oxygen species also are believed to play a role in ovarian steroidogenesis. Superoxide dismutase is present in the theca interna cells of the antral follicles as evidenced by immunohistochemistry staining [65]. 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. Thus, it controls steroidogenesis in the ovaries. The correlation between Ad4BP and SOD expression suggests an association between OS and ovarian steroidogeneis [65]. Antioxidants like ascorbate and α-tocopherol present in the follicular fluid (FF) of mature follicles help in preventing oxidative damage to lecithin-cholesterol acyltransferase (LCAT) [66], which plays an important role in reverse cholesterol transport and follicular estrogen synthesis. By protecting LCAT, they help in ovarian steroidogenesis.

The presence and expression of eNOS in human corpora lutea has been reported in the early and mid- luteal phase and to a lesser extent in the late luteal [67]. Nitric oxide inhibits steroidogenesis in the corpus luteum and has luteolytic action mediated through increased prostaglandins and by apoptosis [67, 68]. Nitric oxide also plays a role in physiological follicular atresia by causing apoptosis. Besides NO, ROS also cause luteolysis by stimulating apoptosis in luteal cells, thereby inhibiting their function at an appropriate time of the menstrual cycle. Ovarian E2 plays a role in pregnancy-mediated luteal rescue by acting as an ROS scavenger [69]. Glutathione along with FSH also helps in protecting against apoptosis in cultured pre-ovulatory rat follicles [70].

Role of oxidative stress in endometrial cycle

Reactive oxygen species plays a regulatory role in the endometrial cycle. According to Sugino et al. [71] ROS may cause shedding of endometrium by causing the secretion of PGF2α *via* NFκβ activation. Late secretory phase human endometrium has been demonstrated to have elevated lipid peroxide concentrations and decreased SOD concentrations. Serviddio et al. [72] found a hormonal pattern to be involved in maintaining optimal redox balance in the endometrium during the endometrial cycle through regulating GSH levels and metabolism. Estrogen and progesterone withdrawal in endometrial cells cultured *in vitro* results in a decrease in SOD activity, leading to increased, unhindered levels of ROS, which leads to a cascade of events and facilitates the endometrial processes of shedding and/or implantation. Reduced expression of SOD leads to failed pregnancy. Reactive oxygen species are involved in the intracellular signaling between hypoxia and the angiogenic response by acting as signal inducers [73] or intracellular messengers and thus help in angiogenesis [74]. Angiogenesis is required for endometrial regeneration after menstruation.

Expression of endothelial NOS mRNA has been demonstrated in the mid- and late secretory phase of the endometrial cycle [75]. Endothelial NOS is thought to produce physical changes that prepare the endometrium for implantation. It acts as a regulator for implantation [52] and may also contribute as an anti-platelet agent during implantation [76, 77]. Higher concentrations of NO also are associated with implantation failure, resulting in lower pregnancy rates.

Effect on tubal motility and uterine contraction

An endogenous NO system exists in the fallopian tubes [78]. Nitric oxide has a relaxing effect on smooth muscles, and it has similar effects on tubular contractility. Nitric oxide deficiency may lead to tubal motility dysfunction, resulting in retention of the ovum, delayed sperm transport, and infertility. Increased NO levels in the fallopian tubes are cytotoxic to the invading microbes and also may be toxic to spermatozoa [78]. In addition, NO might participate in the regulation of uterine contraction [79]. In normal fertile woman, contractions increase throughout the proliferative and periovulatory phases and decrease in the secretory phase. It is thought that NO acts synergistically with progesterone in the secretory phase and may cause uterine relaxation in a paracrine fashion. In support of this hypothesis a study conducted by Bansal et al. (1997) [80] showed that expression of inducible NOS (iNOS) was highest in patients with preterm pregnancy and not in patients in term labor. The expression of these enzymes decreased by 75% at term and was barely detectable in preterm patients. In contrast to this, Seyffarth et al. (2004) [81] have demonstrated increased iNOS expression in fetal membranes in labor and in *in vitro* studies. Their study showed higher concentrations of NO metabolites in amniotic fluid collected from women in labor than in non-laboring patients, both at term and preterm.

Sources of reactive oxygen species

The exact source of ROS in the female reproductive tract is still under debate. Reactive oxygen species

can arise from exogenous or endogenous sources. Oxidative phophorylation, NADPH oxidase, and xanthine oxidase are predominant sources of ROS generation in oocytes and embryos. Oxidative phosphorylation is a process necessary for the generation of ATP to meet embryo energy requirements, and it results in ROS production. In the inner mitochondrial membrane, electron leakage during the electron transport chain allows various spontaneous molecular interactions to occur. Specifically, excess electrons transfer to oxygen molecules and result in an unpaired electron located in oxygen's outer orbital. Other metabolic pathways like glycolysis, pentose phosphate shunt, etc. are also a source for ROS production. Other sources are macrophages, neutrophils, and granulose cells in the graffian follicles, etc.

Exogenous sources for the generation of ROS include visible light, metallic ions like Cu and Fe, a hyperoxic environment (increases enzymatic activity for the generation of the superoxide radicals), hypoxia, smoking (causes decrease in β-carotene levels) and alcohol ingestion (affects vitamin E levels).

Role of reactive oxygen species in female infertility

Mechanism of reactive oxygen species-induced cellular injury

Reactive oxygen species are known to cause peroxidative damage to the macromolecules of the cell such as lipid, proteins, and nucleic acid. Metallic ions like Fe and Cu potentiate this effect. In this section we discuss the effects of ROS specific to the female reproductive system. Metallic ions like Fe and Cu potentiate the effect of ROS.

a. Perioxidative damage to lipids

Polyunsaturated fatty acids are targets for ROS action. Peroxidation of the lipids in the biological membranes leads to a change in the activity of membrane enzymes and ion channels and thus may affect the normal cellular mechanisms required for fertilization. Peroxidation of lipids is a self-propagating process unless it is counteracted by antioxidants.

b. Mitochondrial alterations

Reactive oxygen species affects the mitochondria in oocytes and embryos [82]. Mitochondrial DNA lacks histone, making them more prone to oxidative injury. Being the main site of ROS production, they are the first to be affected by ROS-mediated injury, and once damaged they release a large amount of ROS into the surrounding environment [83]. Cell cycle arrest and cell death occur due to decreased ATP production by the damaged mitochondria.

c. Perioxidative DNA damage

Perioxidative damage to deoxyribonucleic acid bases and phosphodiester backbones leads to the formation of altered nitrogenous bases, which affects replication and transcription processes leading to mutations and altered gene expressions [84]. 8-hydroxy-2-deoxyguanosine, which results from reaction between hydroxyl radical and guanine, is a commonly used marker for oxidative DNA damage.

Effect on oocytes and follicles

The alteration of spindle structure and chromosomal alignment of MII mouse oocytes by OS has been reported [85].

Our group conducted a prospective study to examine the effect of exogenous exposure of hydrogen peroxide and TNF-α on mouse metaphase II oocyte spindle structure and to examine the potential benefits of supplementing the culture media with vitamin C. Microtubule changes and chromosomal alignment were observed by epifluorescence microscopy. The MII oocyte was selected because the ovulated oocyte is arrested at this stage. The authors concluded that ROS causes dose- and time-dependent meiotic spindle damage and chromosomal alterations leading to low oocyte quality. This effect is irreversible, in contrast to cryopreservation damage. It also leads to augmentation of the effects of TNF-α. Since the microtubule is the most important structure during fertilization, the results from this study explain the failure of these occytes to fertilize and thus provide an explanation for the results of the study conducted by Takahashi et al., who showed that a single, brief exposure of fresh oocytes to OS significantly reduced fertility outcomes. The authors also suggested that this alteration may be the root cause for poor fertility outcomes in patients with endometriosis or may even account for aneuploidy, simulating the effects of aging on oocyte quality [85, 86].

TNF-α, an inflammatory mediator, has been found in increasing concentrations in the peritoneal fluid, and its receptors have been identified in human oocytes [87], including the primordial follicles [88]. A relationship exists between TNF-α and a reduced number of ovarian follicles [89]. Higher levels of TNF-α are reported in poor quality oocytes [90].

High ROS levels inhibit theca interna cell proliferation in the secondary follicles [61].

Effect on the embryo

Excessive levels of ROS adversely impact embryo quality and competence by acting on the cellular molecules of the embryo [15, 52]. Reactive oxygen species causes aggregation of cytoskeletal

components, condensation of endoplasmic reticulum in the embryos, and loss of membrane fluidity due to peroxidation of membrane lipids. Embryo cleavage depends partly on microtubules and membrane fluidity; any disturbance in these factors can arrest embryo development [83], and ROS have been shown to cause these disturbances. Reactive oxygen species probably are implicated in regulating the speed of pre-implantation embryo development [91]. Reactive oxygen species have been demonstrated to cause arrest of the zygote at the one-cell stage. Exposure of the zygote to 200 mm H_2O_2 for 15 min led to complete inhibition of cleavage and caused arrest at the one-cell stage [48]. In support of this Fatehi et al. [50] also reported oocyte death and a complete block of first cleavage when denuded oocytes were exposed to H_2O_2 (10 and 50 mm). High ROS levels were associated with lower fertilization and blastocyst rates and embryo fragmentation [18].

Besides causing arrest at the one-cell stage, ROS can also cause two-cell embryo block [92, 93] due to xanthine, the product of purine metabolism. Addition of hypoxanthine causes a decrease in ROS production [94]. The changing energy needs of developing embryos induce the two-cell block. A shift in metabolic pathways from oxidative phosphorylation to glycolysis occurs due to increased energy requirements. No such effect was observed in embryos collected *in vivo* [92].

Oxidative stress plays an important role in apoptosis at controlled levels; however, when OS increases, apoptosis may occur pathologically and damage tissues. Luteal regression may occur even in the presence of pregnancy. A high concentration of H_2O_2 can lead to embryo fragmentation [95]. Nitric oxide also plays a role as a bioregulator [96] in apoptosis. Low concentrations of NO may prevent apoptosis; however, pathologically high concentrations of NO, as well as increased superoxide generation by NO synthase due to lack of arginine, may promote cell death by peroxynitrite generation [78], resulting in embryo fragmentation. Apoptosis results in fragmented embryos, which have limited potential to implant and therefore result in poor fertility outcomes [97].

Reactive oxygen species also cause a decrease in blastomere numbers and pre-implantation embryonic death. Embryos have different sensitivities to ROS at different developmental stages. Nine- to sixteen-cell bovine embryos are more resistant to exogenous H_2O_2 than zygotes and blastocysts [98]. These different sensitivities are due to variations in defense mechanism thresholds.

Effect on implantation

It has been proposed that ROS can lead to implantation failure by decreasing the expression of genes that are expressed before and during implantation e.g. human claudin 4 gene, which peaks during the implantation window; or GP × 3 and solute carrier family 1 member 1 (SLC1A1), which peak during the mid- and late luteal phase [99]. It is also possible that OS, by increasing the levels of PGF 2α, causes endometrial shedding and consequently leads to implantation failure [100]. Higher concentrations of NO also are associated with implantation failure, resulting in lower pregnancy rates.

Role of oxidative stress in gynecological diseases

Preeclampsia

Preeclampsia is known to affect 5-10% of pregnancies and is a major cause of maternal and fetal morbidity and mortality [101-106]. Increased OS in these patients results from a combination of decreased antioxidant levels, early onset of uteroplacental circulation, and leukocyte free radical production [107, 108]. Women with preeclamsia are found to have slightly higher numbers of leucocytes. Decreased SOD levels in maternal blood as well as in the placenta have been found in this condition [109-111]. Mehendale et al. (2008) [112] found lower levels of vitamin C and E in these women. Oxidative stress leads to uncontrolled lipid production in these patients, which is responsible for vascular endothelial damage, ultimately leading to hypertension and proteinuria. The role of antioxidants in preeclampsia is controversial. Although some studies have shown beneficial effects of antioxidant supplementation [113, 114], many have failed to demonstrate any significant effect [114-118]. One of the studies even suggested that vitamin C supplementation during pregnancy may increase the risk of preterm birth [119]. Recently, Rumiris et al. (2006) [105] have suggested that antioxidant supplementation given earlier in pregnancy may be effective by preventing abnormal placentation.

Hydatidiform mole

Hydatidiform mole (H. mole) is responsible for 1 in 41 cases of early pregnancy loss. Studies have shown that these patients are exposed to increased OS, which may play a role in the pathogenesis of the disease. Decrease in the TAS [120], increased concentrations of NO [121], and placental oxidative stress (from abnormal placentation) all have been linked to H. mole. Oxidative stress leads to increased DNA damage [122] and apoptosis. Bcl-2 expression was found to be significantly increased in patients with complete hydatidiform mole (CHM) [123-125]. Harma et al. also demonstrated a dramatic increase in the level of caspase-3 activity in CHM patients.

Endometriosis

Endometriosis, which affects 10-20% of women in the reproductive age group (195), is responsible for 21-41% of all infertility cases [126-128]. Whether OS is related to the pathogenesis of endometriosis is still not clear. Studies have shown increased levels of ROS [129-131] and decreased levels of peritoneal fluid antioxidants in these women [71, 131-135]. Abnormal expressions of antioxidant enzymes, like glutathione peroxidase and xanthine oxidase, also are found in women with endometriosis [129, 136]. Contrary to these findings, some authors have found no significant difference in the total antioxidant status (TAS) of these women as compared with controls [103, 137, 138]. Increased numbers of macrophages and the presence of metallic ions such are iron (from heme) in the peritoneal cavity, as well as the ongoing inflammatory process are responsible for increased ROS production [139-142]. Increased ROS and decreased antioxidant levels lead to OS in these patients.

Along with this, elevated levels of NOS, NO [143], and a higher expression of NOS have been seen in these patients. Altered NOS expression has been implicated in the pathophysiology of endometriosis. It facilitates the development of ectopic endometrial glands by inducing endometrial angiogenesis [143]. Oxidative stress contributes to angiogenesis by increasing vascular endothelial growth factor (VGEF) production [144]. Oxidative stress also causes increased expression of glycodelin, a glycoprotein that also causes increased VEGF expression [144]. Altered NOS expression also may hinder embryo implantation by affecting the endometrial receptivity.

Abnormally high levels of TNF-α have been demonstrated in the peritoneal fluid of these women and has been correlated with disease progression [145, 146]. TNF-α is a major pro-inflammatory cytokine and is known to play a role in endometrial proliferation and shedding. It impairs GSH production by several mechanisms, creating an environment conducive to OS development. Oxidative stress 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. In an *in vitro* study, incubation with TNF-α decreased spermatozoa quality in a dose- and time-dependent manner [147]. This may be one of the reasons for infertility in these women.

Oxidative stress is thought to damage the mesothelial cells in the peritoneal cavity, facilitating the adhesion of ectopic endometrial cells [148]. It also may lead to infertility by having a direct effect on reproductive stages such as reproductive cell viability, sperm motility, oocyte fecundity, and implantation [149-151]. Interestingly, it has been hypothesized that OS may be responsible for the generation of autoantibodies in these patients. Lipid peroxidation by OS leads to the generation of malonaldehyde (MDA), which the body's defense system identifies as a foreign body. Antibodies are formed against MDA, which lead to red blood cells, endometrial cells, and peritoneal cells, stimulating more mononuclear phagocytes, thus perpetuating a cycle of oxidative damage [140-142, 152].

While many studies suggest a role of OS in endometriosis, others have demonstrated no significant association between OS and this condition [138, 146, 148, 153].

Trials elucidating the preventive role of antioxidants in these women have been conducted by many investigators. Many investigators believe that their use in women with the disease may be effective in reducing the severity of symptoms and complications [154]. Daily supplementation of vitamin E and C for a period of 4 months was associated with decreased lipid hydroperoxides and MDA in the peripheral blood of these women, however, no significant difference in the pregnancy rates was found between patients and controls [154]. Injection of antioxidant enzymes such as SOD or catalase into the peritoneal cavity in animal models can prevent the formation of intraperitoneal adhesions at typical endometriotic implantation sites [155]. Treatment with infliximab, a monoclonal antibody that neutralizes the toxic effects of TNF-α, has been advocated for treatment of infertility in these patients. Pentoxifylline is another drug being studied for its potential to treat infertility secondary to endometriosis. Pentoxifylline is a 3'5'-nucleotide phosphodiesterase inhibitor with strong immunomodulatory actions and has been shown to significantly depress the embryotoxic effects of H_2O_2 [156].

Hydrosalpinx

Hydrosalpinx, which is defined as a blocked, dilated, and fluid-filled salpingeal tube, is associated with a higher risk of spontaneous abortions as well as low pregnancy and implantation rates. Oxidative stress in hydrosalpinx fluid (HSF) is thought to be responsible for the embryotoxicity of the HSF. But the study conducted by Chanr et al. (2004) [157] is in disagreement with this hypothesis. Apart from this, HSF decreases endometrial integrins by interfering with their expression [158, 159] leading to failure of implantation. Hydrosalpinx fluid also interferes with the secretion of cytokines like IL-1, IL-1β, LIF and CSF-1, which are recognized as having a function in implantation [160]. There is a notion that HSF may mechanically flush the embryo from the uterus [161, 162] or may alter the character of endometrial peristalsis (reflux phenomenon

opposing cervix to fundus peristalsis) [163], thereby resulting in reduced implantation and pregnancy rates. Hydrosalpinx fluid also may act as a physical barrier to implantation [161, 162, 164, 165]. Laproscopic salpingectomy followed by *in vitro* fertilization (IVF) is the only effective treatment for these patients. Removal of the diseased part of the salpingeal tube results in restitution of the endometrial integrins. Tubal reconstructive surgery should be used as a primary treatment of hydrosalpinges with preserved mucusa [166].

Unexplained infertility

Higher levels of ROS [138], NO [167] and low antioxidant status [168] have been found in the peritoneal fluid of women suffering from unexplained infertility. Howard et al. (1994) [169] demonstrated that oral selenium (cofactor for glutathione peroxidase) supplementation was able to normalize red blood cell glutathione peroxidase levels and increase the pregnancy rate. N-acetyl-cysteine (NAC), a powerful antioxidant, also has been investigated in these patients for potentially improving the ovulation in women with polycystic ovary syndrome. Badawy et al. (2006) [170] showed that NAC was ineffective in inducing or augmenting ovulation in these women.

Premature ovarian aging

Oxidative stress occurs around menopause because of the decrease in estrogen concentration, which has antioxidant effects, as well as reduced expression of antioxidant genes [171, 172]. Oxidative stress causes derangement of the intracellular calcium homeostasis, ultimately leading to a decline in oocyte quality and premature oocyte aging [173-175]. Decreased intracellular ATP levels, which are seen in aged oocytes, also are caused by OS-induced mitochondrial damage [176, 177]. Reduced mitochondrial ATP is responsible for the disassembly of MII oocyte spindles [177], thereby leading to low quality oocytes. Tarin et al. (1998) [178] also showed that OS causes disturbance in the chromosomal distribution in the MII spindle of mouse oocyte.

Tarin et al. have demonstrated the beneficial effects of antioxidant supplementation in preventing oocyte aging [178]. They have proposed early onset of antioxidant supplementation [179]. Oral antioxidants also may protect the hypothalamic-pituitary system from free radical damage, thus maintaining baseline FSH and LH levels for a longer period of time [179]. Antioxidants like vitamin C, E, SOD, and NAC also decreases the number of follicles undergoing atresia by inhibiting granulose cell apoptosis [180, 181]. Hormone replacement therapy also is helpful in overcoming the damaging effects of OS as it antagonizes the decrease in SOD activity that is known to occur after menopause. Lastly, NO has been shown to prevent oocyte aging by acting as an atypical antioxidant [182]. Culture media in assisted reproductive technologies can be supplemented with NO to prevent oocyte aging and improve fertility and reproductive outcomes with these techniques.

Recurrent pregnancy loss

Recurrent pregnancy loss (RPL), defined as three or more consecutive pregnancy losses before 20 weeks gestation, affects 0.5-3% of women in the reproductive age group. The causative factors include but are not limited to 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. Although multiple causative factors have been identified, the etiology behind 50-60% of the cases still remains unclear. Higher production of ROS [183] and decreased levels of β-carotene and vitamin E [184] have been found in patients with RPL. In these women, OS is the result of abnormal placentation. Early onset of maternofetal circulation and resulting placental oxidative stress cause damage to syncytiotrophoblasts that lack adequate antioxidant defenses at that time. Oxidative stress also plays a role in the pathophysiology of antiphospholipid (aPL) syndrome and hyperhomocysteinemia and causes oxidative damage to sperm DNA. In aPL syndrome, OS causes oxidation of LDL. Ox-LDL is responsible for changing the antigenic properties of many oxidized phospholipids, making them more susceptible to aPL antibody attack. Homocysteine generates ROS such as H_2O_2 that may induce OS when homocysteine levels are elevated. Normally, plasma homocysteine levels decrease at the time of pregnancy, but this is not the case with hyperhomocysteinemia. The resulting OS in hyperhomocystenemia is responsible for endothelial dysfunction as well as increased apoptosis, ultimately leading to early pregnancy loss. The use of folate, vitamin B_6, and B_{12} has been suggested by many authors to normalize homocysteine levels [185-188] and further improve pregnancy rates. Selenium is a co-factor for the enzyme GSH peroxidase, and its deficiency has been linked to the depletion of GSH peroxidase. Whether selenium supplementation is beneficial is still controversial.

Role of oral antioxidant supplementation

Antioxidants (AOs) are the main defense parameter against OS. Antioxidants are categorized as either preventative or scavenger AOs. Preventative AOs function to inhibit production

of new ROS; scavenger AOs remove existing ROS. Dietary products provide excellent sources of antioxidants, such as lycopene, vitamin C, and E.

Treatment with oral antioxidant vitamins currently is under evaluation for male fertility. Antioxidants have been shown to not only prevent reduction in sperm motility but also increase sperm motility. Evidence shows oral supplementation of 2-3 g/day of carnitines for >2 months improved sperm concentration and motility [189]. Antioxidants also have been shown to decrease DNA fragmentation caused by OS. Many studies can attest to this. It was demonstrated that vitamin C and E supplementation reduced the number of TUNEL-positive spermatozoa [190]. Furthermore, the addition of vitamin E or C to sperm preparation media during density gradient separation protected sperm from DNA damage [191].

Although AO supplementation proves to be beneficial in male infertility, whether it should be given in the case of female infertility is a matter of debate. Oral AO supplementation has been shown to be effective in preventing luteal phase deficiency, thus increasing the pregnancy rate [192, 193]. In contrast, a study conducted by Griesinger et al. [194] failed to report this beneficial effect [195]. The role of antioxidant supplementation in some of the female gynecological disorders has been discussed earlier in this article.

Conclusions

Our knowledge of human reproduction has shown phenomenal growth in the past decade. Our understanding of OS and its role in pathologies has given rise to several new treatment modalities, now being investigated to improve both male and female infertility. Although many new antioxidants are available to improve infertility, a major concern about their usage remains due to lack of scientific evidence supporting their effectiveness. Evaluation of OS status and the use of antioxidants warrant further studies.

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