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SPERMATOZOA - A UNIQUE REPRESENTATION OF OXYGEN-ANTIOXIDANT PARADOX

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Infertility has become a common occurrence in males. Oxidant stress has become one of the most common causes of infertility. X-irradiation, or exposure to environmental toxicants and the physical conditions of varicocele and cryptorchidism have been demonstrated to increase testicular oxidative stress. The resultant oxidant stress may lead to an increase in germ cell apoptosis and subsequent hypospermatogenesis. This may result in changes in the dynamics of testicular microvascular blood flow, endocrine signaling, and germ cell apoptosis. Oxidative stress, therefore, becomes a major and the most probable finding associated with male infertility. This raises a possibility of application of antioxidant therapy that could help alleviate the reduced spermatogenesis. *Acta Medica Medianae 2010;49(1):48-53.*

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Introduction

Contemporary lifestyle requires the efficient processing of energy to perform work where only the oxidation of carbohydrates, starches, and fats can deliver the necessary efficiency. However, the presence of oxygen within cells forms free radicals (atoms or groups of atoms with odd numbers of electrons). Once formed, these free radicals can launch chain reactions that quickly damage DNA, membranes, and proteins, resulting in either severely damaging or killing the cells.

In order to prevent free radicals damage, the cells of advanced life must possess a system of antioxidants. Antioxidants are molecules that safely interact with free radicals inside the cell to stop the chain reactions before any significant damage can occur.

This results in the oxygen-antioxidant paradox. One of the cells which seem to be a part of this paradox is spermatozoa. Reactive oxygen species (ROS) were exclusively considered toxic to human spermatozoa. However, substantial evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (1-4). Low levels of ROS have been shown as essential for fertilization, acrosome reaction, hyperactivation, motility and capacitation (4-5).

The male factor is considered a major contributory factor to infertility. Apart from the conventional causes of male infertility such as varicocoele, cryptorchidism, infections, obstructive lesions, cystic fibrosis, trauma, and tumours, a new and important cause has been identified: oxidative stress. Oxidative stress is a result of the imbalance between ROS and antioxidants in the body. It is a powerful mechanism that can lead to sperm damage, deformity and eventually, male infertility (6).

ROS are small, oxygen-based molecules that are highly reactive because of unpaired electrons (7). The most prominent ROS are the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl ion ($OH^{\bullet}$).

ROS can be produced in large amounts not only by macrophages and neutrophils, but also by spermatozoa and other cell types under pathologic conditions. Superoxide anions are largely generated as a result of redox reactions within the mitochondria, but in most situations superoxide is quickly converted to hydrogen peroxide by the enzyme superoxide dismutase SOD (8). Hydrogen peroxide can undergo reactions with heavy metals like Fe^{++} or Cu^{++} to form ferric or cupric ions and hydroxyl ions or can be detoxified through the glutathione/ glutathione peroxidase (GPX) pathway to yield water and reduced glutathione. Hydrogen peroxide can also be reduced by catalase to produce oxygen and water. Hydroxyl ions are not only produced from hydrogen peroxide but can also can be generated by other reactions, including the reaction of ionizing radiation with water. Hydroxyl ions have nanosecond half-lives, and are damaging inside the cell because they can cause the covalent cross-linking of a variety of biological molecules

as well as the propagation of other free radicals through more complex reactions (9).

Any oxidizing radical is a potential agent of oxidative stress. Some are highly reactive with short half-lives, such as hydroxyl radicals, whereas others are less reactive but with longer half-lives, such as hydrogen peroxide (not a free radical, but an ROS, nonetheless). A consequence of a longer half-life is the potential for a greater diffusion distance, which can allow the reactive species to do damage even if remote from their source. Oxidative damage can occur to many classes of molecules, including lipids, proteins, nucleic acids, and sugars. This means that cell, nuclear, and mitochondrial membranes, structural and cytoplasmic proteins, complex carbo-hydrates, RNA, and DNA are all potential victims of oxidative stress. In a tissue, such as the testis, with its high rates of metabolism and cell replication, oxidative stress can be especially damaging, which makes the antioxidant capacity of the tissue very important (8,10).

The major antioxidant enzymes in mammals are SOD, catalase, and GPX, the latter necessitating a number of other enzymes, such as glutathione reductase, glutathione-S-transferase, and -glutamyl transpeptidase, required for the recycling or elimination of glutathione. All of these antioxidant enzymes are expressed in the testis . SOD exists in cytosolic, mitochondrial, and extracellular forms, all of which catalyze the dismutation of superoxide anion by successive oxidation-reduction of the transition metal at the enzyme's active site. Catalase exists in only one form and is a highly efficient, intracellular enzyme converting hydrogen peroxide to hydrogen and water. GPX exists in 5 different forms, with the predominant form depending on the tissue. GPX IV, also known as phospholipid hydroperoxide GPX, is the predominant form in the mouse testis, whereas GPXs III and V are predominant in the mouse epididymis (11). This glutamate-based system is a major defense against oxidative stress .A number of nonenzyme factors also function as anti-oxidants in the testis. Among them, vitamin C, vitamin E, resveratrol (a botanical antioxidant), and melatonin have each proven efficacious in reducing testicular oxidative stress under different circumstances (12-15). Lipocalins like prostaglandin D2 synthase may also have a protective function, because in some systems they sequester lipid peroxidation products and reduce oxidative damage (16).

Nitrosylated Oxygen Radicals — NO, a potent vasodilator and cell-signaling molecule, can play its own role in amplifying testicular injury, but through interaction with superoxide radicals it forms peroxynitrite ($ONOO^-$), another potent oxidizing agent. NO can also react with CO_2 to form nitrogen dioxide ($^{\bullet}NO2$), a radical of less activity than peroxynitrite but of longer diffusion distance. Peroxynitrite can modify proteins with thiol groups to generate nitrosothiols, which can disrupt metal-protein interactions and lead to the generation of other metal-derived free radicals (17).

NO is synthesized by nitric oxide synthase (NOS), which exists in 3 known forms: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS. The latter appears to exist only in a truncated form in the testis and is likely inactive there. NOS and/or NO have been found to be up-regulated in a number of experimental conditions known to induce testicular oxidative stress, such as cryptorchidism, testicular torsion, obstructive azoospermia and varicocele (18).

Various causes that induce Testicular Oxidative Stress

Testicular oxidative stress plays a role in a number of conditions known to be detrimental to male fertility. These conditions vary from toxicant exposure to aging and from varicocele to testicular torsion. The involvement of oxidative stress in these and other pathologic conditions is briefly summarized below.

Toxicant Exposure — Multiple studies have shown that environmental toxicants can cause oxidative stress in the testis with resulting disturbance in spermatogenesis. This review cannot cover all these compounds, but several examples will serve. Rats exposed to the pesticide hexachlorocyclo-hexane, for example, exhibit a significant increase in testicular oxidative stress leading to an increase in damaged and apoptotic germ cells. Other industrial pollutants such as 1,3-dinitrobenzene or nonylphenol have the same effect. Methoxyethanol, a glycol ether used in paints, brake fluids, and other industrial products, along with its primary metabolite, methoxyacetic acid, also causes an increase in oxidative stress with subsequent testicular atrophy (19-20). Other industrial toxicants such as 2,4,6-trinitrotoluene from explosives manufacturing and sulfur dioxide from the burning of petroleum products and coal also have a pro-oxidant effect in the testis. 2,5-hexanedione, another organic toxicant known to induce germ cell apoptosis (GCA) is an example of a compound that may produce its effect via oxidative stress, but other routes to apoptosis are possible. We have reviewed the effects of xenobiotics, generally, on male reproduction (21).

Exposure to high concentrations of certain metals has also been shown to cause oxidative stress. For example, high iron doses increase oxidative damage and deplete antioxidants in the testes of rats. Cadmium also increases testicular oxidative stress and high lead exposures decrease rat testicular sperm output, increase epididymal sperm ROS production, and decrease epididymal sperm motility as well as lowering antioxidant capacity of the testis and increasing lipid peroxi-dation (22-24).

Finally, lifestyle choices such as excessive alcohol consumption or cigarette smoking increase free-radical production in all tissues and have on multiple occasions been associated with male infertility or with conditions contributing to that infertility.

Chemotherapy — Cancer chemotherapy is gonadotoxic). That effect may result from factors ranging from endocrinopathy) to generalized cell-

stress responses mediated by heat shock proteins, but it is widely recognized that many chemotherapy agents like doxyrubicin), cyclophosphamide), and cisplatin (25) induce oxidative stress in a variety of tissues and cell types. Although studies of chemotherapy agents on oxidative stress in the testis, specifically, have not been found, given the acknowledged sensitivity of the testis to the effects of oxidative stress, it is likely an important factor in the loss of testis function after chemotherapy (26).

Ionizing Radiation — The testis is very sensitive to x-irradiation, which induces oxidative stress and results in GCA . Not all cells in the testis are equally sensitive to irradiation, however, with Sertoli and Leydig cells being relatively radiation-resistant. This may be caused by the increase in antioxidants also noted in those cells after irradiation (27).

Orchitis/Inflammation — Localized infections or systemic inflammations may have transient or even permanent effects on male fertility, but because not all infections/inflammations are the same, how they impact male fertility can vary. In the laboratory setting, testicular inflammation has been associated with a significant decrease in testosterone production, a disruption of spermatogenesis, and an increase in GCA. For example, using a rat model of systemic inflammation, Reddy et al., 2006, (28) noted a rise in testicular iNOS, interleukin (IL)-1β, and cyclooxygenase-2, which occurred contemporaneously with a decrease in antioxidant enzymes and germ cells. Allen et al., 2004, (29) reported that a single injection of the inflammatory agent lipopolysaccharide in mice resulted in an increase in lipid peroxidation of Leydig cell membranes, a marked reduction in mitochondrial membrane potential, and a reduction in steroidogenesis, which is, itself, associated with GCA. Interestingly, a recent microarray analysis of human testicular gene expression in infertility patients demonstrated an increase in the expression of inflammatory-response genes in those testes. Those data suggest that inflammation or inflammatory-like conditions with its associated oxidative stress is a common underlying factor in male infertility (30).

Varicocele — Varicocele, or dilation of the spermatic vein, typically occurs on the left side only and is associated with an increase in male infertility. Experimental left varicocele bilaterally increases testicular blood flow and temperature in lab animals and causes a reduction in testicular sperm output). The unilateral lesion in humans also bilaterally increases testicular temperature and establishes a trend toward increased blood flow. Both the increased blood flow and the increased temperature may play a role in the oxidative stress evidenced in the testes and semen of varicocele patients. Varicocele is also associated with a decrease in antioxidant capacity of the rat testis and human semen. Interestingly, NO has been linked to an increase in lipid peroxidation in both human varicocele patients and rats with experimental varicocele. This implies a role for peroxynitrites in the oxidative stress of varicocele. Although much remains to be understood about the basic pathobiology of varicocele, it does appear that testicular oxidative stress is an associated factor (31-32).

Cryptorchidism — The increases in testicular temperature implicit in cryptorchidism have long been associated with increases in testicular oxidative stress. Li et al, 2006, (33) examined ROS production and gene expression patterns after the induction of cryptorchidism in adult mice. Those investigators reported that cryptorchidism induced an increase in ROS, which was correlated with increased GCA and alterations in the expression of a number of genes associated with energy and lipid metabolism, stress response, and redox reactions. Testis tissue under the increased temperature in vitro also shows an increased susceptibility to oxidative stress and GCA (34-35). The increase in ROS during cryptorchidism has also been correlated with a decline in testosterone, and oxidative stress, specifically; an increase in NO subsequent to eNOS overexpression, however, has been linked to germ GCA in a mouse model of cryptorchidism.

Aging — Aging results in eventual decline in steroidogenesis , which has been suggested to be caused by an increase in testicular oxidative stress. Further, Cao et al. and Luo et al. (36-37) have shown that Leydig cells from aged rats show a reduced expression of key enzymatic and nonenzymatic antioxidants, which leads to enhanced oxidative damage. Elements of the glutathione-dependent antioxidant system are also reduced in the aged rat testis. These results from the testis are consistent with what is known about increased oxidative stress and aging, generally, but the detailed relationships between aging, oxidative stress, and testis function remain to be clarified.

Testicular Torsion — The incidence of testicular torsion has been estimated to be as high as 1 in 158 males who by the age of 25 with >35% have poor ejaculate quality. Numerous studies have reported increases in oxidative stress in the testis after repair of testicular torsion; (36,37) and all have reported its adverse effects on testicular function, including germ cell loss and disruption of the seminiferous epithelium. As might be expected, inhibitors of oxidative stress provide significant testicular salvage after torsion repair and reperfusion of the organ. Thus, testicular torsion, when repaired before infarction and necrosis, causes an ischemia-reperfusion (IR) injury that is a classic inducer of oxidative stress.

In many of the conditions or exposures mentioned in previous sections, such as toxicant exposure, cryptorchidism, or varicocele, it has been established that testicular oxidative stress occurs. Commonly, however, little research has been done on the chemical and cellular events that cause the oxidative events or the tissue, cell, or molecular consequences of those events. A number of laboratories have used testicular torsion as a model of acute oxidative stress and have evaluated testicular cell and molecular responses ranging from the microvascular endothelium to the seminiferous epithelium. This

multilevel approach has allowed a broad understanding both of what happens in the testis under oxidative stress and how alterations of one cell type may influence others. The following discussion borrows extensively but not exclusively from those studies.

Vascular Events and Testicular Oxidative Stress that may contribute to or result from

In the normal rat testis, variation in microvascular blood flow is caused by vasomotion or cyclic vascular contraction/relaxation under complex regulation. Vasomotion is altered after testicular IR and only returns days later (38). As mentioned previously and in further detail below, intratesticular testosterone declines under the oxidative stress induced by IR and reduced testosterone concentrations have been shown to eliminate vasomotion. Vasomotion may also be influenced by the vascular relaxing effects of NO, which increases in the testis after IR. NO is also active in cell processes other than those inducing vascular relaxation and may participate in other events leading to testicular injury. As a case in point, NO has been reported to be a regulator of the expression of cell adhesion molecules (CAMs) on the luminal surface of the vascular endothelium (39). CAMs play a key role in IR injury in the testis as well as in other tissues because they are key modulators of leukocyte recruitment. The recruitment of leukocytes is the forerunner of much of the subsequent IR pathology in organs, generall and in the testis, specifically; thus, it would be of interest to know more about the role of NO and its key regulatory molecules, iNOS and eNOS, in modulating vasomotion and endothelial CAMs during periods of testicular oxidative stress (40-41).

Previous studies have indicated that testicular testosterone production is acutely reduced in a number of conditions associated with ROS production and oxidative stress in the testis. Examples are cryptorchidism, aging, and IR injury It is also true that steroidogenesis itself produces ROS, largely from mitochondrial respiration and the catalytic reactions of the steroidogenic cytochrome P450 enzymes. The ROS produced by spermatogenesis, if unchecked by intracellular antioxidants, can also damage mitochondrial membranes and contribute to the inhibition of subsequent steroid production. In the average male, the oxidative damage from steroidogenesis may be more of a chronic than an acute factor and has been hypothesized to be important in the declining testosterone production seen in the aging testis (42).

Increased NO from a variety of stresses also decreases testosterone secretion). This might come in part from the formation of peroxynitrites, but in other tissues, both NO and ischemia increase the transcription factor hypoxia inducible factor (HIF)-1α (43).

Oxidative Stress and Germ Cell Response

Apoptosis results from the activation of an intracellular program that leads to cell death without the induction of an inflammatory response. GCA is a significant process even in conventional spermatogenesis, but it is clear that the process is up-regulated in a number of the stress conditions already mentioned, such as toxin exposure, cryptorchidism, and testicular torsion. With the IR injury caused by testicular torsion of sufficient duration, for example, the seminiferous epithelium undergoes a catastrophic induction of GCA and that induction coincides with the increase in testicular oxidative stress (44). Although the details of apoptosis induction have not been elaborated in all causes of oxidative stress, in the case of testicular IR it is caused by a cytokine-induced stress-kinase stimulation of E-selectin expression in the testicular vascular endothelium, which leads to testicular neutrophil recruitment and an increase in intratesticular ROS. ROS, in turn, cause peroxidative damage to cell membranes and the initiation of GCA (Any severe induction of GCA increases the requirement that Sertoli cells engulf large numbers of dying germ cells. This may overwhelm usual Sertoli cell processes and initiate a switch-on of cytokine expression involving nuclear factor kappa B or cytokines like IL-1 and IL-6. How Sertoli cells handle the demand for increased engulfment and phagocytosis of germ cells when faced with a large increase in GCA remains an unexplored facet of testicular oxidative stress.

There are numerous molecular pathways to apoptosis, depending on proximate causes and the specific tissue involved. There is a prominent role for the so-called mitochondrial pathway to GCA after IR injury to the testis in both rats and mice. The primary effect of oxidative stress is on the mitochondrial membrane, where associations between proapoptotic and antiapoptotic members of the Bcl-2 family (45) (eg, Bax and Bcl-XL or Bcl-2 and BclW, respectively) are altered allowing the release of cytochrome c and the eventual activation of a caspase cascade, which ultimately results in the fragmentation of a cell's DNA. Consistent with this pathway, Bax is the predominant proapoptotic molecule in the rat testis, where it exhibits increased expression after IR-induced oxidative stress. Not all testicular stresses activate the mitochondrial pathway as primary oxidative stress does. For example, Boekelheide and associates have shown that certain organic toxicants induce GCA through a pathway involving Fas ligand (FasL) and Fas, members of the TNF superfamily of ligands and receptors (46). FasL is secreted by Sertoli cells; its receptor, Fas, is on the germ cell membrane. Fas-FasL binding initiates the intracellular "death domain" pathway, which, like the mitochondrial pathway, eventually leads to DNA degradation via the caspase cascade. In theory, this Sertoli cell–induced GCA can be selective to particular germ cells, especially for the GCA that occurs during conventional spermatogenesis.

Non-Enzymatic Antioxidants

Vitamin E and selenium supplementation lead to a significant decrease in MDA concen-

trations and improved sperm motility. Disparate results in sperm quality and quantity were observed in those reports using doses of vitamin E below 400mg. In contrast, patients taking vitamin B showed no change in sperm motility, but a small decrease in the MDA concentration was observed (14).

Selenium could potentially self-protect against oxidative DNA damage in human sperm cells. A significant inverse correlation was observed between 8-OHdG and selenium concentration in seminal plasma ($r=-0.40$, $p<0.01$), but the experience with this trace element is scarce. (23)

Vitamin C (ascorbate) is another important chain breaking antioxidant contributing up to 65% of the antioxidant capacity of the seminal plasma. Dietary supplementation protects human sperm from endogenous oxidative DNA damage, thereby decreasing the risk of genetic defects, particularly in populations with low vitamin C levels, such as smokers (12).

Glutathione is the most abundant non-thiol protein in mammalian cells. A glutathione deficiency can lead to instability of the mid-piece, resulting in defective motility. It protects plasma membrane from lipid peroxidation, scavenges superoxide, and prevents O_2 formation. In a study of infertile men with unilateral varicocele or genital tract inflammation, glutathione led to significant improvement in sperm quality. Molecules such as N-acetyl L-cysteine, carotenoids, coenzyme Q10 and carnitines provide excellent antioxidant support. N-acetyl L-cysteine is a precursor of glutathione that improves sperm motility and reduces ROS-induced DNA damage (47).

Conclusion

Approximately 15% of couples attempting to conceive are clinically infertile, and male-factor infertility is involved in fully one-half of those cases. Conditions like varicocele, cryptorchidism, testicular torsion, or endocrinopathy, all of which are associated with testicular oxidative stress, are strongly associated with testicular disfunction; in fact, approximately 45% of male infertility patients have at least 1 of these indications. Further, approximately 25% of male infertility patients have abnormal semen analyses in the absence of any recognized cause. The proportion of these men experiencing testicular oxidative stress is unknown, but such stress could come from unappreciated factors in patient's history such as excessive alcohol consumption, drug use (including steroids), unsuspected toxicant exposure, or even excessive exercise. Thus, from both known and unknown conditions, testicular oxidative stress likely plays a larger rather than appreciated role in male infertility. Such a conclusion suggests that the development of new, more efficient antioxidant therapies may be important for the treatment of hypospermatogenesis.

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