The mechanisms by which Oxidative Stress and Free Radical Damage produces Male infertility

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Abstract: In a healthy body, ROS (reactive oxygen species) and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress (OS) occurs. OS results from an imbalance between prooxidants (free radical species) and the body's scavenging ability (antioxidants). ROS are a double-edged sword - they serve as key signal molecules in physiological processes but also have a role in pathological processes. The production of ROS is a normal physiological event in various organs including the testis. Overproduction of ROS can be detrimental to sperm and being associated with male infertilities. The excessive generation of ROS by abnormal spermatozoa, contaminating leukocytes and by a various type of pollutants has been identified as detrimental etiologies for male infertilities Free radicals are substances with one or more unpaired electrons, which are formed as a results of many physiological and pathological cellular metabolic processes, especially in mitochondria. Enzymatic (Catalase, superoxide dismutase) and non enzymatic (vitamins A and E) natural antioxidant defense mechanisms exist; however, these mechanisms may be overcome, causing lipid peroxidation to take place. For example, breakdown in the cells results in the formation of molecules whose further metabolism in the cell leads to ROS production. Thus increased OS stimulates the activity of enzymes called cytochrome P450, which contribute to ROS production. . Oxidative stress index (OSI) was calculated as ([TOS/TAS] x 100). TOS and OSI were significantly higher and PON-1 activity and TAS were significantly lower in subfertile male with abnormal semen parameters than in male with idiopathic subfertility and fertile donors. PON-1 activity was also strongly correlated with sperm concentration, motility, and morphology in the overall group. The receiver operating characteristic curve analysis revealed a high diagnostic value for PON-1 activity with respect to male-factor sub fertility. ROS may cause infertility by two principal mechanisms, first ROS damage the sperm membrane which in turn reduces the sperm motility and ability to fuse with the oocyte secondly, and ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. Oxidative stress due to excessive production of ROS, impaired antioxidant defense mechanisms, or both precipitates a range of pathologies that are currently believed to negatively affect the male reproductive function. Oxidative stress-induced damage to sperm may be mediated by lipid peroxidation of the sperm plasma membrane, reduction of sperm motility, and damage to the DNA in the sperm nucleus, as the production of ROS is one of the principal mechanisms by which neutrophils destroy pathogens, it is not surprising that seminal leukocytes have the potential to cause oxidative stress. Despite the established role of OS in the pathogenesis of male infertility, there is a lack of consensus as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to utilize the OS test in clinical practice is related to the lack of a standard protocol for assessment of seminal OS. Antioxidants are powerful and there are few trials investigating antioxidant supplementation in male reproduction. Several researches indicate that the diagnostic and prognostic capabilities of the seminal OS test are beyond those of conventional tests of sperm quality and function. The OS test can accurately discriminate between fertile and infertile male and identify male with a clinical diagnosis of male-factor infertility that are likely to initiate a pregnancy when followed over a period of time. We strongly believe that incorporating such a test into the routine andrology workup is an important step for the future of the male infertility practice. The resulting state of the cell, known as (OS) can lead to cell injury. ROS production and Lipid peroxidation, free radical and oxidative stress in relation to fertility are the aim of this review

[Magda M El-Tohamy. **The mechanisms by which Oxidative Stress and Free Radical Damage produces Male infertility.** Life Science Journal 2012; 9(1):674-688]. (ISSN: 1097-8135). http://www.lifesciencesite.com. 98

Key Words: oxidative stress; free radicals; reactive oxygen species; proteins; DNA; lipids; glutathione peroxidase.

Introduction:

Reactive oxygen species (ROS), defined as including oxygen ions, free radicals and peroxides are generated by sperm and seminal leukocytes within semen and produce infertility by two key mechanisms. First, they damage the sperm membrane, decreasing sperm motility and its ability to fuse with the oocyte. Second, ROS can alter the sperm DNA, resulting in the passage of defective DNA.

The ability of sperm to produce ROS inversely correlates with their maturational state. During spermatogenesis, there is a loss of cytoplasm to allow the sperm to form its condensed, elongated form.

Immature teratozoospermic sperm are often characterized by the presence of excess cytoplasmic residues in the mid-piece. These residues are rich in the enzyme glucose-6-phosphate dehydrogenase, an enzyme which controls the rate of glucose flux and intracellular production of b-nicotinamide adenine dinucleotide phosphate (NADPH) through the hexose monophosphate shunt.

NADPH is used to fuel the generation of ROS via NADPH oxidase located within the sperm membrane**(Fisher and Aitken, 1997; Gomez *et al.*, 1998; Said *et al.*, 2005).** As a result, teratozoospermic sperm produce increased amounts of ROS compared with morphologically normal sperm.

The generation of ROS in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and function. ROS are highly reactive oxidizing agents belonging to the class of free radicals **(Aitken, 1997)**. A free radical is defined as "any atom or molecule that possesses one or more unpaired electrons" **(Warren *et al.*, 1987)** reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile male **(de Lamirande *et al.*, 1995; Padron *et al.*, 1997)**. However, strong evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities **(Aitken, 1999).**

Spermatozoa, like all cells living in aerobic conditions, constantly face the oxygen (O_2) paradox: O_2 is required to support life, but its metabolites such as ROS can modify cell functions, endanger cell survival, or both **(de Lamirande and Gagnon, 1995)**. Hence, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. It is not surprising that a battery of different antioxidants is available to protect spermatozoa against oxidants **(Sies, 1993).**

Seminal oxidative stress (OS) develops as a result of an imbalance between ROS generating and scavenging activities **(Sharma and Agarwal, 1996; Sikka, 2001).** Spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (**Alvarez and Storey, 1995)** and their cytoplasm contains low concentrations of scavenging enzymes **(Aitken and Fisher, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996).** In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection afforded by the seminal plasma **(Zini *et al.*, 1993)**. Oxidative stress attacks not only the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus **(Aitken, 1999).**

Even though OS has been established as a major factor in the pathogenesis of male infertility, there is a lack of consensus as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to use an OS test in clinical practice may be the lack of a standard protocol to assess seminal OS. The main objective of this review in this area was to transfer this important knowledge from the research bench to clinical practice by designing studies with the following aims: 1) to understand the precise mechanism by which OS develops in semen, which we thought, could help and develop strategies to overcome the problem, 2) to establish assays for accurate and reliable assessment of seminal OS, and 3) to identify the clinical significance of testing seminal OS in the male infertility practice. In this review, we summarize the efforts of some studies to explore the role of OS in male infertility.

Reactive Oxygen Species (ROS) and Sperm Physiology

ROS were exclusively considered toxic to spermatozoa. The idea that limited amounts of ROS can intervene in a physiological manner in the regulation of some sperm functions was first evoked in a study by **Aitken *et al.* (1989).** The authors found that low levels of ROS can enhance the ability of spermatozoa to bind with zonal pellucida, an effect that was reversed by adding vitamin E. Other studies have found that incubating spermatozoa with low concentrations of hydrogen peroxide (H_2O_2) stimulates sperm capacitation, hyperactivation, and the ability of the spermatozoa to undergo the acrosome reaction and oocyte fusion **(de Lamirande and Gagnon, 1993; de Lamirande *et al.*, 1995; Kodoma *et al.*, 1996; Aitken, 1997).** ROS other than H_2O_2, such as nitric oxide and superoxide anion ($O_2^{\bullet}$), have also been shown to promote sperm capacitation and the acrosome reaction **(Zini *et al.*, 2000).**

Mechanism of Antioxidant Protection in Semen

It is interesting that seminal plasma is well endowed with an array of antioxidant defense mechanisms to protect spermatozoa against OS **(Sikka, 1996; Armstrong *et al.*, 1998)**. These mechanisms compensate for the deficiency in cytoplasmic enzymes in sperm **(Donnelly *et al.*, 1999).** Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase (SOD) **(Alvarez *et al.*, 1987),** the glutathione peroxidase/glutathione reductase (GPX/GRD) system **(Chaudiere *et al.*, 1984)**, and catalase **(Jeulin *et al.*,**

1989). In addition, seminal plasma contains a variety of nonenzymatic antioxidants such as ascorbate **(Fraga *et al.*, 1991)**, urate **(Thiele *et al.*, 1995), -** tocopherol **(Aitken and Clarkson, 1988; Moilanen *et al.*, 1993)**, pyruvate **(de Lamirande and Gagnon, 1992),** glutathione **(Lenzi *et al.*, 1994),** taurine, and hypotaurine **(Alvarez and Storey, 1983).**

It has been reported that seminal plasma from fertile male has a higher total antioxidant capacity (TAC) than seminal plasma from infertile male **(Lewis *et al.*, 1995)**. However, pathological levels of ROS detected in semen from infertile male are more likely a result of increased ROS production rather than reduced antioxidant capacity of the seminal plasma **(Zini *et al.*, 1993).** Antioxidant defense mechanisms include three levels of protection: 1) prevention, 2) interception, and 3) repair **(Sies, 1993)**.

The term oxidative stress is applied when oxidants out-number antioxidants **(Sies, 1993)**, peroxidation products develop **(Spitteler, 1993),** and when these phenomena cause pathological effects **(Janssen *et al.*, 1993).** Oxidative stress has been implicated in numerous disease states such as cancer, connective tissue disorders, aging, infection, inflammation, acquired immune deficiency syndrome, and male infertility **(Aitken *et al.*, 1992, 1995).**

In the context of human reproduction, a balance normally exists between ROS generation and scavenging in the male reproductive tract. As a result, only a minimal amount of ROS remains, which is needed to regulate normal sperm functions such as sperm capacitation, acrosome reaction, and sperm-oocyte fusion **(Gagnon *et al.*, 1991; Griveau and Le Lannou, 1997).** Excessive ROS production that exceeds critical levels can overwhelm all antioxidant defense strategies of spermatozoa and seminal plasma causing OS **(Sharma and Agarwal, 1996; de Lamirande *et al.*, 1997; Sikka, 2001).** All cellular components including lipids, proteins, nucleic acids, and sugars are potential targets for OS. The extent of OS-induced damage depends not only on the nature and amount of ROS involved but also on the moment and duration of ROS exposure and on extracellular factors such as temperature, oxygen tension, and the composition of the surrounding environment including ions, proteins, and ROS scavengers.

Lipid Peroxidation of Sperm Plasma Membrane

Lipid peroxidation is broadly defined as "oxidative deterioration of PUFA" (ie, fatty acids that contain more than two carbon-carbon double bonds **(Halliwell, 1984)**. The LPO cascade occurs in two fundamental stages: initiation and propagation. The hydroxyl radical ($OH^{\bullet}$) is a powerful initiator of LPO **(Aitken and Fisher, 1994).** Most membrane PUFAs have unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene group makes the methylene C-H bonds weaker and, therefore, hydrogen is more susceptible to abstraction. Once this abstraction has occurred, the radical produced is stabilized by the rearrangement of the double bonds, which form a conjugated diene radical that can then be oxidized. This means that lipids, which contain many methylene-interrupted double bonds, are particularly susceptible to peroxidation **(Blake *et al.*, 1987).** Conjugated dienes rapidly react with O_2 to form a lipid peroxyl radical (ROO•), which abstracts hydrogen atoms from other lipid molecules to form lipid hydroperoxides (ROOH). Lipid hydroperoxides are stable under physiological conditions until they contact transition metals such as iron or copper salts. These metals or their complexes cause lipid hydroperoxides to generate alkoxyl and peroxyl radicals, which then continue the chain reaction within the membrane and propagate the damage throughout the cell **(Halliwell, 1984).** Propagation of LPO depends on the antioxidant strategies employed by spermatozoa. One of the by-products of lipid peroxide decomposition is malondialdehyde. This by-product has been used in biochemical assays to monitor the degree of peroxidative damage in spermatozoa **(Aitken and Fisher, 1994).** The results of such an assay exhibit an excellent correlation with the degree to which sperm function is impaired in terms of motility and the capacity for sperm-oocyte fusion **(Aitken *et al*., 1993; Sidhu *et al*., 1998).**

Impairment of Sperm Motility

The increased formation of ROS has been correlated with a reduction of sperm motility **(Agarwal *et al*., 1994; Armstrong *et al*., 1999).** The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion **(de Lamirande and Gagnon, 1995).** Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase **(Aitken *et al*., 1997)**. Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized

glutathione and reduced glutathione. This can reduce the antioxidant defenses of the spermatozoa and increase peroxidation of membrane phospholipids **(Griveau *et al*., 1995).**

Impaired sperm function

Impaired sperm function is a general cause of male infertility and sub -fertility. A balanced and controlled generation of ROS is associated with normal sperm physiological capacitating and acrosome reaction **(de Lamirande and Gngon, 1993).** These finding stresses the importance of a balance between ROS scavenging and small, physiologic levels of ROS that are necessary for normal sperm function. An imbalance, excessive production of ROS and decreased level of antioxidant enzymes caused decreased sperm motility and viability and increased sperm defects by imitating an oxidative chain damaging protein, lipid and DNA. Seminal plasma has antioxidant system that seems to be very relevant to the protection of sperm. Seminal plasma contains small molecular mass free radicals scavenger such as vitamin C, E and tyrosine. Also, contains antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase that are active in scavenging ROS. Spermatozoa are particularly vulnerable to such stress because their plasma membranes are so enriched with unsaturated fatty acids, particularly decosohexaenoic acid.

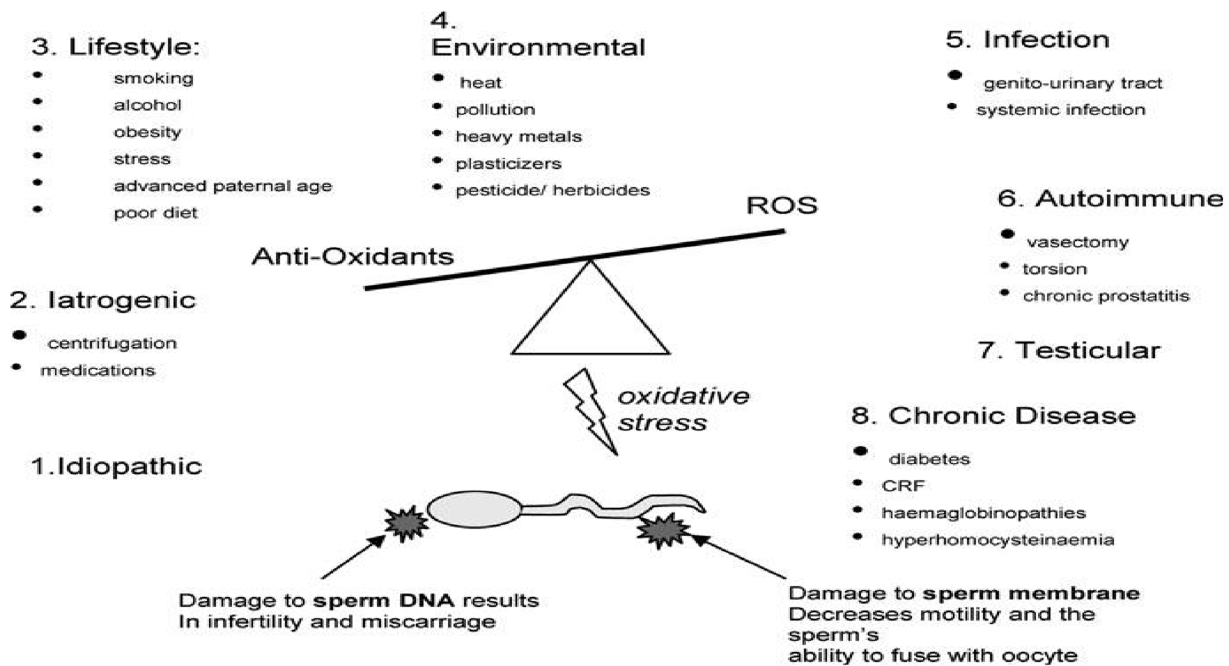

Figure 1: Origin of oxidative stress **(Aitken, 1999) .**

Origins of oxidative stress

The origins of sperm oxidative stress are summarized in **Fig. 1**. While pathologies such as genitourinary tract infection and varicocele are well established causes of oxidative stress, others such as hyper-homocysteinaemia and diabetes are only now just becoming recognized as possible causes. It is hoped that this review will stimulate further research in these less well established potential Causes of male oxidative infertility.

Oxidative stress

Oxidative stress is the presence of active oxygen species in excess of the available antioxidant buffering capacity. These products, ROS can damage proteins, lipids and DNA, altering the organism's structure and function. Not surprisingly, the organism has an efficient system to buffer these products and also not surprisingly, it will occasionally not be able to do so **(James *et al*., 2004).** When this happens, whether because of reduced antioxidant enzymes, insufficient intake of dietary antioxidants or with excessive production of ROS, oxidative stress ensues.

Oxidative stress may be defined as an imbalance between prooxidant and antioxidant forces resulting in an overall prooxidant insult. Pregnancy is a physiological state accompanied by a high energy demand of many bodily functions and an increased oxygen requirement. Because of levels of oxidative stress would be expected. Arguments for a role of oxidative stress /oxidative lipid derivatives in the pathogenesis of preeclampsia are documented in many papers other conditions such as toxic substance exposure induces oxidative stress. The oxidized lipid

products generated as a consequence of these conditions are highly reactive and cause damage to cells and cell membranes. Thus increased oxidative stress accompanied by reduced endogenous defenses may play a role in the pathogenesis of a number of diseases in the new born **(Gitto *et al*., 2002).**

Oxidative Stress-Induced DNA Damage

Two factors protect sperm DNA from oxidative insult: the characteristic tight packaging of the DNA and the antioxidants present in seminal plasma **(Twigg *et al*., 1998a).** Studies in which the sperm was exposed to artificially produced ROS resulted in a significant increase in DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links, and chromosomal rearrangements **(Duru *et al*., 2000).** Oxidative stress has also been correlated with high frequencies of single and double DNA strand breaks **(Twigg *et al*., 1998a; Aitken and Krausz, 2001).**

Measurement of TOS in Seminal Plasma

The TOS of semen samples was determined by using a new automated colorimetric measurement method **(Erel, 2005).** The assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in acidic medium and the measurement of the ferric ion by xylenol orange. Within- and between-batch precision values were lower than 3%. The results were expressed as µmol hydrogen peroxide (H_2O_2) equivalent per liter **(Verit *et al*., 2006).**

Measurement of TAS in Seminal Plasma

TAS of semen samples was determined by using a novel automated measurement method developed by **Erel. (2004).** In this method, the hydroxyl radical, the most potent radical, is produced via Fenton reaction and consequently the colored dianisidinyl radical cations, which are also potent radicals, are produced in the reaction medium of the assay. Antioxidant capacity of the added sample against these colored potent free radical reactions measured the total antioxidant capacity. The assay has excellent precision values; within- and between-laboratory precision values are lower than 3%. The results were expressed as millimoles of Trolox equivalent per liter **(Verit *et al*., 2006).**

Measurement of PON-1 Activity in Seminal Plasma

PON-1 activity was determined by using paraoxon as a substrate and measured by increases in the absorbance at 412 nm because of the formation of 4-nitrophenol as already described **(Verit *et al*., 2008).** Briefly, the activity was measured at 25°C by adding 50 µL of seminal plasma to 1 mL Tris-HCl buffer (100 mM at pH 8.0) containing 2 mM $CaCl_2$ and 5.5 mM of paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm. Enzymatic activity was calculated by using the molar extinction coefficient 17 100 M–1 cm–1.

Oxidative Stress Index

The percentage ratio of TOS to TAS gave the oxidative stress index (OSI), an indicator of the degree of oxidative stress ([TOS/TAS x 100] (**Verit *et al*., 2006).** Oxidative stress (OS), as one of HS-induced response, has long believed to influence male reproductive function. Although OS was suggested as an important factor in disruption of sperm function over 50 years ago, the importance of OS has gained recently a wider understanding. Reactive oxygen species (ROS) are normal physiological event in various organs including the testis. Paradoxically, the production of ROS is both essential and detrimental to life; hence, numerous studies indicate that ROS play an important role in normal sperm function and that an imbalance in ROS production (over-production) and/or degradation (under-scavenging) by antioxidants may have serious adverse effects on sperm (**Akiyama, 1999).**

Protocol for Measurement of Total Antioxidant Capacity in Semen by Enhanced Chemiluminescence Assay— Total antioxidant capacity in the seminal plasma can be measured using an enhanced chemiluminescence assay **(Kolettis *et al*., 1999).** Frozen samples of seminal plasma are thawed at room temperature and immediately assessed for TAC. Seminal plasma is diluted 1:20 with deionized water (dH_2O) and filtered through a 0.20-µm filter (Allegiance Healthcare Corporation, McGaw Park, Ill). Signal reagent is prepared by adding 30 µL of H_2O_2 (8.8 molar/L), 10 µL of para-iodophenol stock solution (41.72 µM), and 110 µL of luminol stock solution (3.1 mM) to 10 mL of Tris buffer (0.1 M pH 8.0). Horseradish peroxidase working solution is prepared from the HRP stock solution by making a dilution of 1:1 of dH_2O. Light emission occurs when the chemiluminescent substrate luminol is oxidized by H_2O_2 in a reaction catalyzed by HRP. HRP catalyzes the reaction between a hydrogen acceptor (oxidant) and a hydrogen donor. Under normal circumstances, this reaction produces low-intensity light emission that may decay rapidly. The characteristics of the reaction can be altered substantially by the addition of para-iodophenol as an enhancer that gives a more intense, prolonged, and stable light emission. The continuous light output depends on the constant production of free radical intermediates derived from para-iodophenol (enhancer), luminol (substrate), and H_2O_2 (oxidizer)

in the presence of HRP. ROS production from spermatozoa has been measured by chemiluminescence in the two fractions of a Percoll gradient column (47 and 90%). Chemiluminescent signals were recorded in each fraction after the addition of luminol and horse-radish peroxidase (basal state), and after stimulation with formyl-methionyl-leucyl-phenylalanine and phorbol ester (PMA). Oligozoospermic samples show a higher rate of ROS production than the normozoospermic samples in both fractions of Percoll. Also, ROS were generated at a higher rate by asthenozoospermic samples in the 90% Percoll fraction than by normal samples after stimulation with PMA. Data confirm that fact of white blood cells play a major role in the production of ROS, even after purification on a Percoll gradient. Immunological cases were also found to be associated with an increased production of ROS, which may be caused by the same underlying pathological condition responsible for the production of the antibodies. Repeated centrifugation of the samples triggers a burst of ROS in excess of that produced after Percoll preparation **(Kolettis *et al*., 1999).** In addition, superoxide dismutase activity was found to be significantly increased in cases with an elevated production of ROS. It is concluded that measuring the ROS generation by semen may yield useful information on the functional capacity of spermatozoa, which may be used to improve the success of male infertility management. These findings explain the importance of a balance between ROS scavenging and small, physiologic levels of ROS that are necessary for normal sperm function.

In biological systems, a diversity of antioxidant defense systems operates to control levels of ROS. Some antioxidants synthesized within the cells themselves (endogenous) and others need to be provided in the diet (exogenous). These ROS scavengers have an important protective action on the membrane integrity and lipid stability in both seminal plasma and spermatozoa. Only a few reports have analyzed the nutritional requirement (additives) of rabbit bucks to improve both fertility and resistance to the hazards of HS.

There are many sources of oxygen radicals in cells that make them ubiquitous species. The role of oxygen radicals in physiological processes is now far from being completely understood and depends in the first place on the ability of oxygen radicals to interact with substances, drugs, proteins, lipids, enzymes, DNA and other cellular compounds. However the effect of oxygen radicals is not confirmed to these processes their action on such cell components as mitochondria microsomes plasmolemma, sarcoplasmic reticulum etc and on the cells themselves is also of great importance it is common knowledge that oxygen radicals are able to damage cells and cellular components, being possibly a primary factors of many pathologies **(Afanas 1991).**

A free radical is any molecule capable of independent usually brief existence that contains one or more unpaired electrons. Most free radicals in biology fit within the broader category of ROS which include not only oxygen centered radicals such as superoxide anion radical (O_2) Hydroxyl radical (OH), or nitric oxide (NO), but also some potentially dangerous non-radical derivatives of oxygen such as HO peroxynitrite anion (ONOO) and hypochlorous acid (HOCL) **(Halliwell, 1993).**

In healthy individual the generation of ROS appears to be counterbalanced by the antioxidant defense, although there may be some cumulative oxidative damage that contributes to the aging process and age related diseases **(Ames, 1989).** An imbalance between ROS and antioxidant defenses in favor of the former creates oxidative stress. This can happen either if the antioxidant levels are depleted and/or if the formation of ROS is high. The antioxidant defense is recruited either from endogenous systems or from the diet **(Halliwell, 1997).**

Free radicals are so reactive and short-lived that direct measurement is usually not possible. However, hundreds of biomolecules are known to be derived from the interaction of free radicals with biomolecules. Assays for some of these oxidative stress biomarkers, as well as assays for several of the body's antioxidant defense mechanisms, have been widely used. Although there are numerous tools on the market, a small number of oxidized lipids, as well as byproducts of DNA and protein oxidation, have withstood the test of time. Our major goal is to provide straightforward, reliable assays for oxidative stress biomarkers and for antioxidant capacity of biological fluids

Peng (2000) found that antioxidant supplementation decreased serum (MDA) and protein carbonyls in healthy non pregnant subject. Some of the antioxidants such as vitamin E, vitamin C, beta carotene, bilirubin and albumin trap radicals and prevent chain reactions **(Ryter, 2000).** Especially, vitamin E and beta carotene are potent antioxidant nutrients and may counteract free radical attack and thereby protect cell membranes against free radical mediated lipid and protein oxidation **(Zhang, 2000).**

Protection against ROS toxicity

Because ROS production is a naturally occurring process, a variety of enzymatic and nonenzymatic mechanisms have evolved to protect cells against ROS **(Yu, 1994).** At least some of these mechanisms are impaired after long term of consumption and may

therefore contribute to damage to organs. Enzymes involved in the elimination of ROS include superoxide dismutases (SOD), catalase and glutathione peroxidase.

Nonenzymatic mechanisms; Because of all its functions, GSH is probably the most important antioxidant present in cells. Therefore, enzymes that help generate GSH are critical to body's ability to protect itself against oxidative stress. Mitochondria cannot synthesize GSH but import it from the cytosol using a carrier protein embedded in the membrane surrounding the mitochondria **(Fernandez *et al*., 1997).**

Male germ cells may be susceptible to oxidative stress because of high concentration of polyunsaturated fatty acids and low antioxidant capacity which are associated closely with free radical generating phagocytic Sertoli cells. Decrease in sperm concentration and total sperm output may be due to direct interaction of ROS with the sperm cell membrane resulting in impairment of membrane fluidity and permeability and damage of germ cells ,spermatozoa and mature sperms **(Sarkar *et al*., 2003).** The effect of heavy metals on acrosome integrity may be attributed to the high lipid peroxidation in epididymes as a result of elevated oxidative stress, which alters the stability of plasma membrane that surrounds the acrosome through the effect on its content of polyunsaturated fatty acids and lipoproteins **(Zini *et al*., 2000).**

Decrease in swollen coiled sperm percentage may be due to direct interaction of ROS with polyunsaturated fatty acids in the cell membrane leading to a chain of chemical reactions results in the formation of various oxidatively modified products, which are toxic to cells. The spermatozoal membrane contains large amounts of polyunsaturated fatty acids, which maintain its fluidity. Peroxidation of these fatty acids leads to the loss of membrane flexibility and a reduction in the ability to swell and expand covering the tail when exposed to hypoosmotic solutions **(Amorim *et al*., 2009).**

The beneficial effects of antioxidants on sperm may be due to the decrease in the levels of TBARS present as a consequence of environmental pollution and cellular metabolism **(Castellini *et al*., 2003, 2006).** Antioxidant reduced the formation of radicals in seminal and blood plasma and different tissues. Antioxidant normalizes the activities of enzymes by protecting free radicals responsible for oxidative stress and production of ROS, prevents oxidative damage to cell membrane induced by radicals in the aqueous environment **(Arrigsoni and De Tullio, 2002).**

The decrease in MDA might be explained by enhancing the activities of antioxidant enzymes **(Reddy and Lokesh, 1994),** inhibiting the generation of ROS and mitochondrial release of cytochromes or counteracting the depletion of antioxidant enzymes **(Nagar, 2004).** The antioxidant mechanism might be attributed to scavenging or neutralizing of free radicals, interacting with oxidative cascade and preventing its outcome, quenching oxygen and making it less available for oxidative reaction, and inhibiting oxidative enzyme like cytochrome P450 and chelating and disarming oxidative properties of metal ions such as iron **(Sreejayan and Rao, 1994).**

Evidence now suggests that reactive oxygen species (ROS)-mediated damage to sperm is a significant contributing pathology in 30–80% of cases (Iwasaki and Gagnon, **(Agarwal *et al*., 2006).** ROS, defined as including oxygen ions, free radicals and peroxides, cause infertility by two principal mechanisms. First, ROS damage the sperm membrane which in turn reduces the sperm's motility and ability to fuse with the oocyte. Secondly, ROS directly damage sperm DNA, compromising the paternal genomic contribution to the embryo. Despite the common association between compromised sperm quality and oxidative damage, men are rarely screened for oxidative stress nor treated for this condition. Instead they are usually offered 'mechanical' treatments such as intracytoplasmic sperm injection (IVF-ICSI) or intrauterine insemination (IUI). This is less than Optimal as oxidative damage to sperm DNA is not directly ameliorated by either IVF-ICSI or IUI treatment. In addition, direct treatment of oxidative stress may allow for natural conception, thereby conserving scarce medical resources. This review will provide an Overview of who is at risk of oxidative stress, the mechanisms by which oxidative stress produces infertility and the methods available for its diagnosis and treatment within semen there are two principal sources of production of free radicals; leukocytes and sperm. The vast majority of semen specimens contain leukocytes, with neutrophils being the predominant leukocyte type **(Aitken and Baker, 1995).** As the production of ROS is one of the principal mechanisms by which neutrophils destroy pathogens, it is not surprising that seminal leukocytes have the potential to cause oxidative stress. However, a link between the presence of leukocytes in semen and male oxidative infertility is still under debate **(Wolff, 1995).**

Several researchers have reported a positive correlation between seminal leukocyte numbers and ROS production **(Sharma *et al*., 2001).** However, other studies have failed to find a significant difference in seminal leukocyte concentration between fertile and infertile men **(Tomlinson *et al*., 1993)** and the activation state of leukocytes must also

play an important role in determining final ROS output. This is supported by the observation of a positive correlation between seminal ROS production and pro-inflammatory seminal plasma cytokines such as interleukin IL-6 **(Rajasekaran *et al*., 1995; Martinez *et al*., 2007).**

The non-enzymatic antioxidants present within semen include ascorbic acid (Vitamin C), a-tocopherol (Vitamin E), glutathione, amino acids (taurine, hypotaurine), albumin, carnitine, carotenoids, flavenoids, urate and prostasomes. These agents principally act by directly neutralizing free radical activity chemically.

However, they also provide protection against free radical attack by two other mechanisms. Albumin can intercept free radicals by becoming oxidized itself, thereby sparing sperm from attack **(Twigg *et al*., 1998).** Alternatively, extracellular organelles (prostasomes) secreted by the prostate have been shown to fuse with leukocytes within semen and reduce their production of free radicals **(Saez *et al*., 1998).** A substantial number of researchers have reported a significant reduction in non-enzymatic antioxidant activity in seminal plasma of infertile compared with fertile men **(Song *et al*., 2006).**

Effect of Sperm Morphology on ROS Production

Gomez *et al* (1998) have indicated that levels of ROS production by pure sperm populations were negatively correlated with the quality of sperm in the original semen. The link between poor semen quality and increased ROS generation lies in the presence of excess residual cytoplasm (cytoplasmic droplet). When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective **(Huszar *et al*., 1997).** Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme G6PD (Figure 2) **(Aitken, 1999).**

It is hoped that this review will stimulate further research in these less well established potential causes of male oxidative infertility.

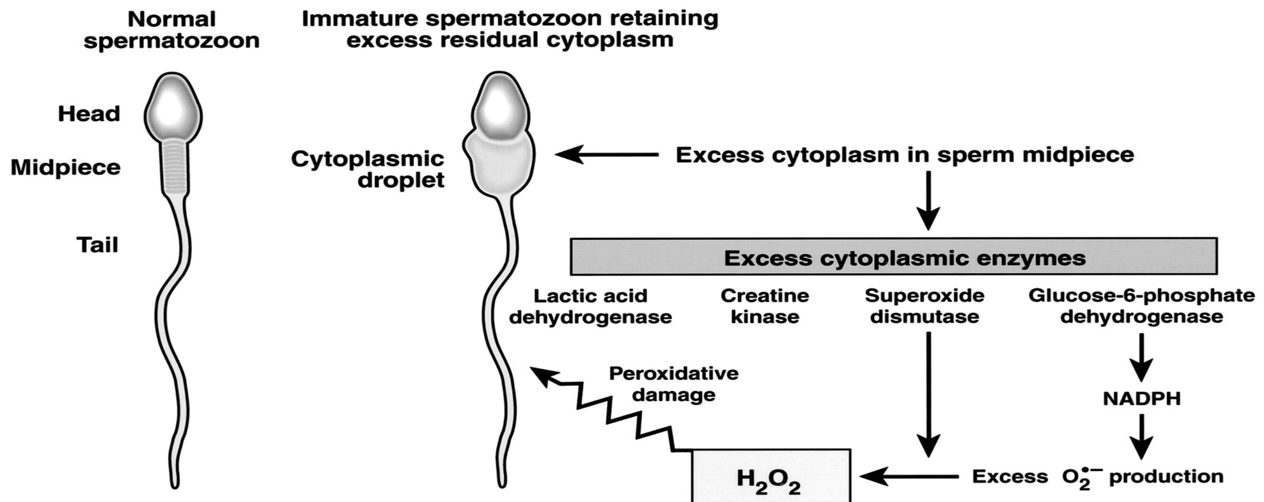

Figure 2. Mechanism of increased production of ROS by abnormal spermatozoa (spermatozoa with cytoplasmic retention).

Several environmental pollutants have been linked with testicular oxidative stress. Pesticides such as lindane **(Chitra *et al*., 2001)**, methoxychlor **(Latchoumycandane *et al*., 2002)** and the herbicide dioxin-TCDD **(Latchoumycandane *et al*., 2003)** have all been linked with testicular oxidative stress in rodent models. The commonly used preservative sulfur dioxide has also been shown to produce testicular oxidative stress in laboratory animals **(Meng and Bai, 2004).** Air pollutants such as diesel particulate matter act as potent stimuli for leukocyte ROS generation **(Alaghmand and Blough, 2007).** While no study has directly linked airborne pollutants with testicular oxidative stress, it is possible that this oxidative insult is responsible for the increase in sperm DNA damage seen following periods of airborne pollution **(Rubes *et al*., 2005).** Heavy metal exposure has been conclusively linked with sperm

oxidative damage **(Eltohamy *et al*., 2004).** Both cadmium and lead are linked with an increase in testicular oxidative stress **(Acharya *et al*., 2003)** and a resultant increase in sperm DNA oxidation **(Naha and Chowdhury, 2006).** The increase in infertility and miscarriage observed in the partners' of welders and battery/paint factory workers **(Bonde, 1993)** may be due to oxidative damage to sperm DNA initiated by the inhalation of metal fumes.

Direct methods

These assays measure damage created by excess free radicals against the sperm lipid membrane or DNA. As oxidative stress is the result of an in balance between ROS production and total antioxidant capacity (TAC), direct tests reflect the net biological effect between these two opposing forces. The most widely used method of assessing sperm membrane peroxidation is the measurement of MDA levels in sperm or seminal plasma with the thiobarbituric acid assay. MDA levels in sperm are quite low and therefore require the use of sensitive high-pressure liquid chromatography (HPLC) equipment **(Li *et al*., 2004; Shang *et al*., 2004)** or the use of iron-based promoters and spectrofluometry measurement **(Aitken *et al*., 1993).** Seminal Plasma levels of MDA are 5–10-fold higher than sperm, making measurement on standard spectrophotometers possible **(Tavilani *et al*., 2005).**

Measurement of MDA appears to be of some clinical relevance since its concentration within both seminal plasma and sperm is elevated in infertile men with excess ROS production, compared with fertile controls or normozoospermic individuals **(Tavilani *et al*., 2005; Hsieh *et al*., 2006).** Furthermore, in vitro impairment of motility, sperm DNA integrity and sperm–oocyte fusion capacity by ROS is accompanied by an increase in MDA concentration **(Aitken *et al*., 1989, 1993).** Other direct tests of sperm membrane lipid peroxidation such as measurement of the isoprostane 8-Iso-PGF2a **(Khosrowbeygi and Zarghami, 2007)** and the c11-BODIPY assay **(Aitken *et al*., 2007; Kao *et al*., 2007)** are showing promise but are not yet in common usage It is well recognized that oxidative stress is one of the major causes of sperm DNA damage **(Aitken *et al*., 1998; Oger *et al*., 2003; Saleh *et al*., 2003a, b).** However, measurement of sperm DNA damage by TUNEL or SCSA is an imperfect assessment of oxidative stress as sperm DNA can be damaged by nonoxidative mechanisms such as aberrant apoptosis and incomplete sperms protamination **(Ozmen *et al*., 2007).** The best direct assessment of sperm DNA oxidative damage is the measurement of the oxidized deoxynucleoside, 8-oxo-7, 8-dihydro 20 deoxyguanosine (8-OHdG). This can be measured in sperm or seminal plasma by HPLC **(Fraga *et al*., 1991; Loft *et al*., 2003),** enzyme-linked immunoabsorbent assay **(Nakamura *et al*., 2002)** or directly within sperm using immunoflurorescence **(Kao *et al*., 2007).** Since a large prospective study has reported that chances of natural conception is inversely correlated with sperm 8-OHdG levels **(Loft *et al*., 2003)**, measurement of this direct marker of sperm oxidative stress appears to have some clinical utility. A large number of round cells within semen may suggest the presence of oxidative stress as they may represent seminal leukocytes **(Sharma *et al*., 2001).** However, round cells may also be immature sperm rather than leukocytes, so formal identification of leukocytes requires ancillary tests such as the peroxidase test,CD45 staining or measurement of seminal elastase **(WHO manual, 1999; Zorn *et al*., 2003; Kopa *et al*., 2005).** Finally, poor sperm membrane integrity assessed by the hypo-osmolarswelling test has been linked with the presence of sperm oxidative stress **(Dandekar *et al*., 2002).**

Role of oxidative stress in male infertility

While a role for oxidative stress in male infertility is now established, many unanswered questions still remain. First, there is a clear need to develop inexpensive assays to identify sperm oxidative stress that can be easily conducted in any andrology laboratory. Secondly, large RCTs are needed to confirm the effectiveness of surgical interventions (varicocelectomy, testicular biopsy) in the management of oxidative stress.

Antioxidant defense play an important role in neutralizing various ROS. superoxide dismutase dismutase the superoxide anion (O_2^-) to H_2O_2. The enzymatic defense against H_2O_2 include catalase and family of glutathione peroxidase **(Yu, 1994)** Seminal plasma possesses antioxidant system that seems to be very relevant to the protection of spermatozoa. The sperm oxidative defense enzymes include superoxide dismutase, glutathione peroxidase and catalase **(Sikka *et al*., 2001).**

Antioxidant Effects

Antioxidants are important in maintaining the oxidant-antioxidant balance in tissues. Among the well-known biological antioxidants, superoxide dismutase, catalase, and the glutathione peroxidase/reductase system have a significant role in protecting the sperm against peroxidative damage **(De Lamirande and Gagnon, 1993; Sharma and Agarwal, 1996).** Depressed seminal antioxidant capacity has been implicated in male subfertility. TAS levels have been shown to be lower in the semen of subfertile male as compared with fertile

male **(Lewis *et al*., 1995, 1997).** More specifically, **Raijmakers *et al.* (2003)** reported significantly higher seminal plasma thiol glutathione concentrations in fertile male compared with subfertile male. In accordance with this finding, it has been reported that ascorbate levels were significantly reduced in seminal plasma of asthenozoospermic subfertile male **(Lewis *et al*., 1997).** Furthermore, studies have suggested that subfertile male empirically treated with antioxidants have demonstrated improved semen characteristics, fertilization in vitro, and higher pregnancy rates in the treatment group **(Lenzi *et al*., 1993; Geva *et al*., 1996).** In study, TAS was significantly decreased in subfertile male with abnormal semen parameters, but not in the idiopathic subfertile group.

Phenolic antioxidants from processed honey increased antioxidant activity. These effects of honey might be attributed to its antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities (**Schramm *et al.*, 2003**). At a dose of 1.2 g/kg b.wt./day for 2 weeks, honey increased antioxidant agents such as blood vitamin C concentration, β-carotene, uric acid, and glutathione reductase (GRx) (**Al-Waili, 2003**). These compounds reduce the lipid peroxidation level (**Hegazi and Abd El Hady, 2009**).

Honey increases the antioxidant parameters of the liver and kidney glutathione reduced (GSH), oxidized glutathione (GSSG) content and also decrease in (GPx) and (SOD) caused by Ochratoxin A- induced hepatotoxicity and nephrotoxicity in rats. The level of (MDA) -as lipid peroxidation marker- was also significantly decreased (**El-Khayat *et al.*, 2009).**

Honey has protective effects against oxidative stress (attenuate free radical scavenging enzymes and reduce lipid peroxidation in kidney and pancreas of streptozotocin-induced diabetic rats. The combination of two hypoglycemic drugs; glibenclamide (0.6 mg/kg b.wt) and metformin (100 mg/kg b.wt), and honey (1.0 g/kg b.wt) for 4 weeks revealed a marked increases the activities of catalase (CAT) and GRx, and the levels of total antioxidant status (TAS) and GSH in diabetic kidney (**Erejuwa *et al.*, 2011a).** The combination of glibenclamide, metformin, and honey significantly up regulated CAT activity and downregulated GPx activity while MDA levels were significantly reduced. Honey also restored SOD and CAT activities. (**Erejuwa *et al.*, 2010**).

Antioxidant activities in honey were represented by increased TAS, GSH, GSH)/GSSG ratio, GPx, and GRx in diabetic spontaneously hypertensive rats (**Erejuwa *et al.*, 2011b**) and suppressed the lipid peroxidation (**Hegazi and Abd El Hady, 2009**).

HS increased oxygen radicals, possibly by the disruption of the electron transport assemblies of the membrane. **Bruskov *et al*. (2002)** discussed that heat-induced reactive oxygen species (ROS) formation may be an additional factor that provides molecular changes in DNA, proteins, lipids and other biological molecules that may contribute to low fertility. Several studies have suggested that heat exposure could result in oxidative stress, which in turn lead to cytotoxicity **(Lord-Fontaine and Averill-Bates, 2002).**

Nichi *et al*. (2006) suggested other hypothesis to explain heat-induced ROS formation; in the absence of increased blood flow, the testicular parenchyma becomes hypoxic. Hypoxia probably increases production of ROS through the ischemia-reperfusion mechanism, thus **Filho *et al*. (2004)** had considered it a higher index of testicular oxidative stress.

There is growing evidence that oxidative stress significantly impairs sperm function, and plays a major role in the etiology of defective sperm function. This may lead to the onset of male infertility via mechanisms involving the induction of peroxidative damage to the plasma membrane; **(Griveau and Le Lannou, 1997).** Both spermatozoa and seminal plasma possess antioxidant systems capable of counteracting the harmful effects of ROS. Studies have demonstrated that infertile animals are more likely than fertile ones to have depressed total antioxidant capacity (TAC) and lower levels of individual antioxidants **(Lewis *et al*., 1995).**

Antioxidants and Fertility

Since ROS has both physiological and pathological roles, an array of antioxidants maintains a steady state 3 of ROS in the seminal plasma. Antioxidants act as free radical scavengers to protect spermatozoa against ROS. These antioxidants are SOD, catalase, and glutathione peroxidase (GPX). In addition, semen contains a variety of non-enzymatic antioxidant molecules such as vitamin C, vitamin E, pyruvate, glutathione, and carnitine **(Aitken, 2004)**. These antioxidants compensate for the loss of sperm cytoplasmic enzymes as the cytoplasm is extruded during spermiogenesis, which, in turn, diminishes endogenous repair mechanisms and enzymatic defenses. **Agarwal *et al.* (2006)** in an exhaustive review of the literature, found a total of 57 studies related to antioxidants and fertility – 10 studies were randomized controlled trials, 16 were controlled studies, and 31 were uncontrolled studies.

PON-1 is an antioxidant enzyme that is highly effective in preventing lipid peroxidation of LDL **(Mackness *et al*., 1993).** It is principally responsible for the breakdown of lipid peroxides before they accumulate on LDL **(Mackness *et al*., 1993).** PON-1 can also destroy H_2O_2; a major ROS produced under

oxidative stress during atherogenesis **(Aviram *et al*., 1998),** and increase the LDL clearance **(Shih *et al*., 2000).**

PON-1 also protects HDL against lipid peroxidation **(Mackness *et al*., 1993; Aviram *et al*., 1998; Rozenberg *et al*., 2003)**. Inhibition of HDL oxidation by PON-1 preserves the antiatherogenic effects of HDL in reverse cholesterol transport **(Aviram *et al*., 1998).** The antioxidant effect of HDL is also assumed by PON-1 **(Aviram and Rosenblat, 2004).**

The association between PON-1 activity and male infertility is unknown. PON-1 activity was significantly lower in male-factor subfertile patients compared with idiopathic subfertile male and fertile donors in the present study. There were also significant positive correlations between PON-1 activity and semen parameters such as concentration, motility, and morphology. We suggest that decreased PON-1 activity must be related to enhanced production of ROS. In addition, it has been previously shown that PON-1 activity was decreased in some diseases because of ROS pathogenesis under oxidative stress and inflammation conditions such as diabetes, coronary artery disease, and endometriosis **(Ayub *et al*., 1999; Verit *et al*., 2008).**

In conclusion the results showed that TOS was significantly higher and TAS and PON-1 activity were significantly lower in male-factor subfertility, but not in an idiopathic subfertile group. Reduced PON-1 activity may play a role in the pathogenesis of male subfertility. Therefore, both protection from oxidative stress and increases in PON-1 activity could be used as a powerful tool for the prevention of subfertility.

Future Research

Further research is also required to determine what combination and dose of antioxidant supplement provides sperm with maximal protection against oxidative stress. Finally, the development of new sperm culture media that can better protect sperm from the ravages of ROS damage is clearly required.

Although research already have gained substantial insight into the mechanisms and consequences of alcohol induced oxidative stress, additional studies are required to further clarify how alcohol produces oxidative stress in various tissues. More detailed information is needed on the mechanisms involved in some of the major proposed pathways. Finally, it still is unclear how alcohol induced oxidative stress is produced in tissues where only limited alcohol mechanism occurs. Many of these issues can study using animal models, however extrapolation of findings from animal to human will be a difficult task because ROS production and antioxidant status in human as affected by numerous .nutritional, environmental and drug influences that are difficult to reproduce in animals. To date, scattered data suggest that blood of human alcohol can contain lipids modified by radicals and other reactive molecules as well as immune molecules targeted at such modified lipids and proteins. Other questions that should be addressed in future research.

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