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Clinical Relevance of Oxidative Stress and Sperm Chromatin Damage in Male Infertility: An Evidence Based Analysis

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ABSTRACT

Oxidative stress (OS) in the reproductive tract is now a real entity and concern due to the potential harmful effects of high levels of reactive oxygen species (ROS) on sperm number, motility, quality, and function including damage to sperm nuclear DNA. Evaluation of OS related damage to non-functional sperm is highly relevant as intracytoplasmic sperm injection (ICSI) technique, an effective therapy for severe male factor infertility, bypasses the majority of reproductive tract deficiencies. Despite the controversial findings in the existing literature, there is now enough evidence to show that sperm DNA damage is detrimental to reproductive outcomes. In addition, spermatozoa of infertile men are suggested to carry more DNA damage than do the spermatozoa from fertile men. Besides impairment of fertility such damage is likely to increase the transmission of genetic diseases during the assisted reproductive procedures. Standardization of protocols to assess reactive oxygen species and DNA damage is very important in introducing these tests in such clinical practice. Thus evaluation of seminal ROS levels and extent of sperm DNA damage especially in an infertile male may help develop new therapeutic strategies and improve success of assisted reproductive techniques (ART).

***Key words**: free radicals; oxidative stress; sperm; DNA; antioxidants; male infertility*
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INTRODUCTION

A large population of apparently normal males have problem impregnating their partners even when their fertility status by routine semen analysis is considered normal. These cases are classified as idiopathic infertility. Men with idiopathic infertility generally present with significantly higher seminal ROS levels and lower antioxidant potential than healthy fertile controls (1). In addition, high ROS levels have been detected in the semen samples of 25% to 40% of infertile men (2,3).

In the context of human reproduction, a balance called oxidative stress status (OSS) normally exists between ROS production and antioxidant scavenging system in the male reproductive tract (4). Small physiological levels of ROS are essential for the regulation of normal sperm functions such as sperm capacitation, the acrosome reaction, and sperm-oocyte fusion (5,6). However, production of excessive

amounts of ROS in semen especially during leukocytospermia can overwhelm the antioxidant defense mechanisms of spermatozoa and seminal plasma resulting in oxidative stress. Studies suggest that ROS attack the integrity of DNA in the sperm nucleus by causing base modifications, DNA strand breaks, and chromatin cross-linking (7,8). Spermatozoa have limited defense mechanisms against oxidative attack on their DNA mainly due to the complex packaging arrangement of DNA. In vivo, such damage may not be the cause for concern because the collective peroxidative damage to the sperm membrane ensures that spermatozoa susceptible to oxidative stress are unable to participate in the fertilization process. However, these safeguards are circumvented during the course of ICSI and some spermatozoa with significant DNA fragmentation may be used that will produce adverse unfavorable results.

The assessment of sperm DNA damage appears to be a potential tool for evaluating semen samples prior to their use in ART. Testing DNA integrity may help andrologists to select spermatozoa with intact DNA or with the least amount of DNA damage for use in assisted reproduction possibly increasing the success rate. In addition, interest in the physiologic and pathologic effects of ROS on male fertility is growing. Therefore, it is essential for urologists and fertility specialists to understand free radical sources, their generation, sperm damage mechanisms that may affect male reproductive system. In addition, it has been postulated that protective agents against ROS e.g., antioxidants, may be useful for treating male factor infertility. For this reason, deciphering the levels and sources of excessive ROS production in human semen may be useful in developing therapeutic strategies for use in male infertility uses.

This article will discuss in detail about the clinical relevance of oxidative stress in human semen, how excessive ROS damages sperm nuclear DNA as well as how such DNA damage contributes to male infertility and assisted reproductive techniques.

Design: A thorough literature survey was performed using the Medline, EMBASE, BIOSIS and Cochrane databases. We restricted the survey to clinical publications between 1985 and 2006 that were relevant to male infertility with emphasis on oxidative stress and DNA damage.

WHAT ARE REACTIVE OXYGEN SPECIES AND OXIDATIVE STRESS?

Reactive oxygen species (ROS) known as free radicals are oxidizing agents generated as a result of metabolism of oxygen and have at least one unpaired electron that make them very reactive species. Normally, free radicals attack the nearest stable molecule, which becomes a free radical itself, beginning a cascade of chain reaction. These can very rapidly oxidize biomolecules that they encounter in their vicinity thus exerting either a positive or a negative influence on normal cell function (9).

Normal aerobic metabolism is related to optimal levels of ROS because a balance exists between ROS production and antioxidants activity. Oxidative stress (OS) is the term applied when oxidants outnumber the antioxidants due to excessive generation of reactive oxygen species and when antioxidants cannot scavenge these free radicals (10). Such phenomena cause pathological effects, damaging cells, tissues and organs (11).

REACTIVE OXYGEN SPECIES AND SEMINAL OXIDATIVE STRESS

Spermatozoa produce small amounts of ROS that play a significant role in many of the sperm physiological processes such as capacitation, hyperactivation, and sperm-oocyte fusion (12,13). However, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. Excessive generation of ROS in semen can cause damage to spermatozoa due to its exclusive structural composition. During the maturation process the spermatozoa extrudes cytoplasm, which is the major source of antioxidants. Once this process is slowed down, residual cytoplasm forms a cytoplasmic droplet in the sperm mid region. These spermatozoa carrying cytoplasmic droplets are though to be immature and functionally defective (14). The residual cytoplasm contains high concentration of certain cytoplasmic enzymes (G6PDH, SOD), which are also a source of ROS (15). Lack of cytoplasm results in decreased antioxidant defense. This process is the link between poor sperm quality and elevated ROS.

Human ejaculate consists of different types of cells such as mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and epithelial cells. Of these, peroxidase-positive leukocytes and abnormal spermatozoa that produce free radicals continuously (16,17). Spermatozoa are also particularly susceptible to the damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA), which readily experience lipid peroxidation by ROS, resulting in a loss of membrane integrity (18,19). There are two major systems of ROS production in sperm. One is the nicotinamide adenine dinucleotide-dependent oxidase system at the level of the sperm plasma membrane and the other is NADH-dependent oxido-reductase (diphorase) system at the mitochondrial level (20). There is a strong positive correlation between immature spermatozoa and ROS production, which in turn is negatively correlated with sperm quality (21). Furthermore, it has been noticed that as the concentration of immature spermatozoa in the human ejaculate increases, the concentration of mature spermatozoa with damaged DNA rises (22) (Figure-1).

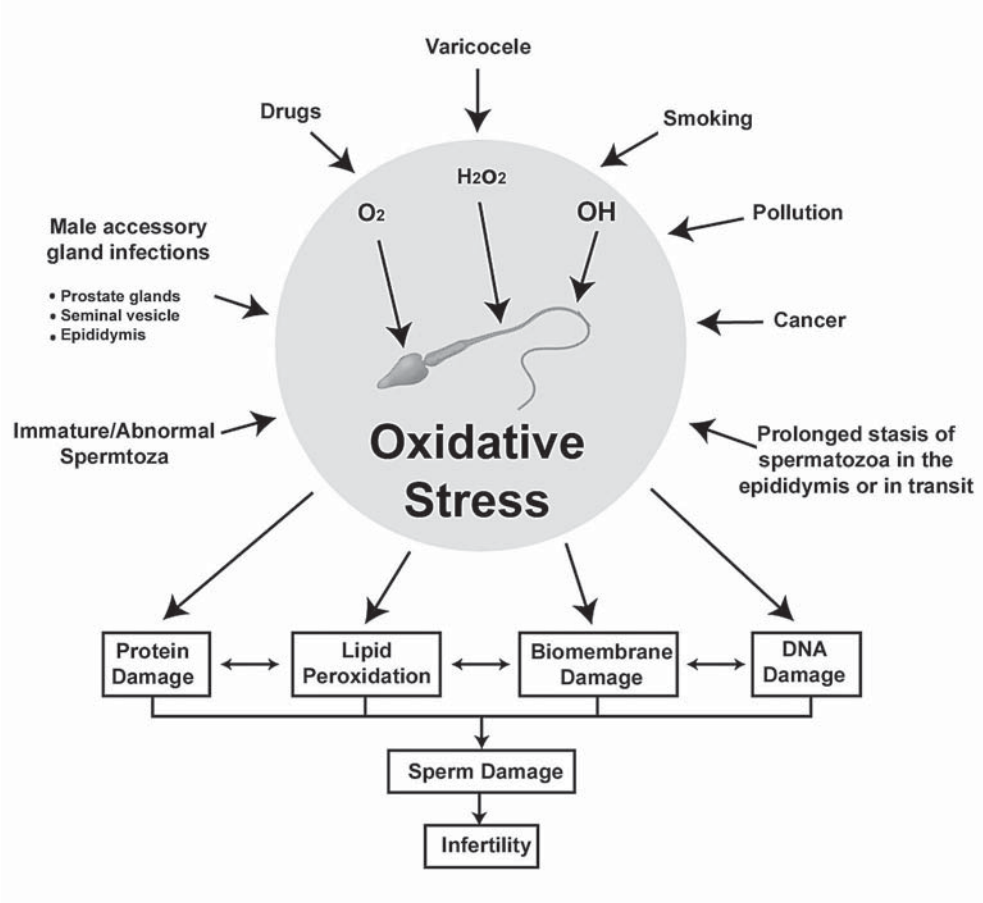

Figure 1 – *Association of increasing reactive oxygen species (ROS) production with infertility.*

OXIDATIVE STRESS AND EFFECT ON SPERM MOTILITY

Seminal ROS levels, when present in excess, possess potentially toxic effects on both sperm quality and function (23,24). Elevated seminal ROS production has been associated with decreased sperm motility, defective acrosome reaction, and loss of fertility (25). Sperm cell dysfunction, a result of ROS damage, is dependent on the nature, amount, and duration of exposure to ROS. The extent of ROS damage is also dependent upon surrounding environmental factors such as oxygen tension and temperature as well as the concentrations of molecular components such as ions, proteins, and ROS scavengers (5).

As reported by Aitken et al., low hydrogen peroxide concentrations do not influence sperm motility, but do suppress human sperm competence during oocyte fusion (26). Possibly ROS levels are not high enough to affect standard seminal parameters but can cause defects in other processes that are required for fertilization, such as sperm-oocyte interaction. These findings suggest an explanation why patients with normal semen parameters can experience idiopathic infertility. Decreased motility is a result of cascade of events including lipid peroxidation (LPO) of sperm plasma membrane that ultimately affect an axonemal protein phosphorylation and sperm immobilization (2).

CLINICAL DIAGNOSIS AND ASSESSMENT OF SEMINAL OXIDATIVE STRESS

Spinal Cord Injury

Recent studies report the detection of increased ROS levels in the semen of 25% to 40% of infertile men (2,3). Padron et al. documented that in men with spinal cord injury, elevated seminal ROS levels are associated with poor sperm motility and morphology. These associations are independent of both ejaculation method and specimen type (3).

Varicocele

The role of ROS in varicocele has been previous reported by our center and others (17,27,28). Excessive nitric oxide release within dilated spermatic veins has been identified in subfertile males with varicocele. This nitric oxide release may cause spermatozoal dysfunction (27,29). Allamaneni et al. report a positive correlation between seminal ROS levels and varicocele grade in which significantly higher levels of seminal ROS are seen in men with varicocele grades 2 and 3 versus men with varicocele grade 1 (30). Varicocele patients also present low seminal plasma TAC levels and increase 8-hydroxy-2'-deoxyguanosine levels, indicating a deficient pro-oxidant defense system and oxidative DNA damage, respectively (17,31). According to a recent meta-analysis, varicocele patients as compared with normal sperm donors have significantly increased oxidative stress parameters such as ROS and lipid peroxidation as well as significantly decreased antioxidant concentrations (32). Antioxidant supplementation may therefore be beneficial to this infertile population with varicocele.

Mostafa et al. first reported that varicocelectomy reduces the seminal plasma ROS levels of infertile men associated with increased seminal plasma concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and vitamin E of infertile men (33). Daitch et al. reported that couples who do not achieve pregnancy following varicocelectomy might significantly increase their pregnancy and live birth rates after undergoing intrauterine insemination, despite failing to show improvements in semen parameters (34). It is therefore suggested that pregnancy rate improvement following varicocelectomy may be due to functional factors such as seminal oxidative stress and the spermatozoal DNA integrity not routinely tested during standard semen analysis (34).

Leukocytospermia

ROS in the human ejaculate originate mainly from seminal leukocytes. Leukocytospermia is characterized by abnormally high seminal leukocyte, polymorphonuclear neutrophils, and macrophages (35). Seminal leukocyte ROS production induces spermatozoal damage during ART procedures (1,36). Patients with accessory gland infection demonstrate both leukocytospermia and elevated ROS levels (37). In these patients, sperm function defects are resultant

of abnormal lipid peroxidation, stimulated by the high ROS levels (38).

Genito-Urinary (GU) Tract Infection

During GU infection, the presence of leukocytes in semen has been associated with decreased sperm motility and fertilization capacity (39-41). However, El-Demiry et al. reported no association between standard seminal parameters and leukocyte concentration in human semen (42). This dilemma may be partially due to the different techniques used to determine leukocyte concentration in semen as well as the lack of agreement on the lower leukocyte concentration responsible for sperm damage (43-45). Infections located in the testis and epididymis produce ROS that are particularly harmful to spermatozoa due to its lack of a pro-oxidant defense system. Sperm function may also be indirectly affected by an infection stimulating the presence of ROS in the prostate gland, and seminal vesicles. An association between prostatitis and male infertility has been reported, but the responsible mechanism is still poorly understood (46). Prostatitis is associated with the presence of granulocytes in prostatic fluid. Irrespective of leukocytospermia status, increased seminal oxidative stress is reported in men with chronic prostatitis and prostatodynia (46). Such findings support the controversial prostatitis-infertility relationship debate. Multiple hypotheses discuss male genital tract infections and their relationship with ROS. Specifically, the leukocytes stimulate human spermatozoa to produce ROS. The mechanisms responsible for such stimulation are unknown, but may include the direct contact of sperm and leukocytes or may be regulated by leukocyte release of soluble products (1,47).

Environmental Factors

An association between cigarette smoking and reduced seminal quality has been identified (48). Harmful substances including alkaloids, nitrosamines, nicotine, cotinine and hydroxycotinine are present in cigarettes and produce free radicals (49). In a prospective study, Saleh et al. compared infertile men who smoked cigarettes with nonsmoker infertile men (50). Smoking was associated with a significant increase (approximately 48%) in seminal leukocyte concentrations, a 107% ROS level increase, and a 10 point decrease in ROS-TAC score. The authors concluded that infertile men who smoke cigarettes present higher seminal OS levels than infertile nonsmokers, possibly due to significant increase in leukocyte concentration in their semen. An earlier study also reported an association between cigarette smoking in infertile men and increased leukocyte infiltration in the semen (51). Significantly higher levels of DNA strand breaks in men who smoke have also been identified. DNA strand breaks may be resultant from the presence of carcinogens and mutagens in cigarette smoke (52). In recent decades evidence suggestive of the harmful effects of occupational exposure chemicals known as endocrine disruptors on the reproductive system has gradually accumulated (53). Environmental pollution is a major source of ROS production and has been implicated in the pathogenesis of poor sperm quality (54). In a study conducted by De Rosa et al., tollgate workers with continuous environmental pollutant exposure had inversely correlated blood methaemoglobin and lead levels to sperm parameters in comparison to local male inhabitants not exposed to comparable automobile pollution levels. These findings suggest that nitrogen oxide and lead, both present in the composition of automobile exhaust, adversely affect semen quality (55). In addition, the increase of industrialization has resulted in an elevated deposition of highly toxic heavy metals into the atmosphere. Paternal exposure to heavy metals such as lead, arsenic and mercury is associated with decreased fertility and pregnancy delay according to recent studies (56,57). Oxidative stress is hypothesized to play an important role in the development and progression of adverse health effects due to such environmental exposure (58).

FREE RADICALS AND ASSISTED REPRODUCTIVE TECHNIQUES (ART)

Numerous conditions associated with male infertility, e.g., microdeletions of the Y chromosome, sperm maturational arrest, meiotic defects, aneuploidies, defective centromeres and defects in oocyte activation still lack a specific treatment. However, advances in ART have helped in improving

treatment of male factor infertility (35). Currently, ICSI is the most common ART method, although it is associated with the highest number of miscarriages. One of the explanations can be the poor selection of sperm that are possibly damaged by free radicals during ART procedures.

ROS are produced during ART mainly by oocytes, embryos, cumulus cells and immature spermatozoa (59). Sperm preparation techniques can be used to decrease ROS production to enhance and maintain sperm quality after ejaculation (35). The most common sperm preparation techniques used to preserve and optimize sperm quality after ejaculation is density gradient centrifugation, migration-sedimentation, glass wool filtration, and conventional swim-up (60). The first three preparation techniques are more effective in reducing levels of free radicals than the conventional swim-up technique (60). However, repeated centrifugation causes mechanical injury to spermatozoa and increases ROS production (61). Currently use of antioxidants and other substances to prevent ROS generation during sperm preparation processes are under evaluation.

Aitken et al. reported that men with elevated ROS levels in semen have a sevenfold reduction in conception rates when compared with men having low ROS (47). Also high ROS levels are associated with decreased pregnancy rate following IVF or ICSI and arrested embryo growth. Based on a recent meta-analysis, which included all of the available evidence from the literature, our group found that there is a significant correlation between ROS levels in spermatozoa and the fertilization rate after IVF (estimated overall correlation 0.374, 95% CI 0.520 to 0.205) (62). Thus, measuring ROS levels in semen specimens before IVF may be useful in predicting IVF outcome and in counseling selected patients with male factor or idiopathic infertility.

LABORATORY EVALUATION OF OXIDATIVE STRESS IN INFERTILITY PRACTICE

ROS Measurement

For clinical purposes, it is essential to have a reliable and reproducible method of ROS measurement. Numerous methods are available to measure ROS levels in semen. Direct methods such as electron-spin resonance spectroscopy, also known as electron paramagnetic resonance, have been utilized mainly for research purposes since these are relatively expensive technologies that require fresh samples, and great technical expertise (63,64). This method is used to detect electromagnetic radiation being absorbed in the microwave region by paramagnetic species that are subjected to an external magnetic field. This technique is the only analytical approach that permits the direct detection of free radicals and reports on the magnetic properties of unpaired electrons and their molecular environment (64). However, short life span of ROS makes the application of these techniques difficult.

Indirect techniques, e.g., chemiluminescence method are commonly used for measuring ROS produced by spermatozoa (65,66). This assay quantifies both intracellular and extracellular ROS depending on the probe used. Chemiluminescence determines the amount of ROS, not the level of the sperm-damaging ROS present at any given time. Also, it can differentiate between the production of superoxide and hydrogen peroxide by spermatozoa depending on which probe is used (66). Two probes may be used with the chemiluminescence assay: luminol and lucigenin. A luminol-mediated chemiluminescence signal in spermatozoa occurs when luminol oxidizes at the acrosomal level. Luminol reacts with a variety of ROS and allows both intracellular and extracellular ROS to be measured. Lucigenin, however, yields a chemiluminescence that is more specific for superoxide anions released extracellularly (67,68).

The number of free radicals produced is counted as photons per minute. Presence of leukocytes as a confounding factor and the need of fresh semen samples with high sperm count ($>1 \times 10^6$/mL) are the limitations of this technique (66). Also other multiple factors that affect chemiluminescence include the concentration of reactants, sample volume, reagent injection, temperature control, instrument sensitivity, and background luminescence (69).

A diversity of luminometers is available to measure the light intensity resulting from the

chemiluminescence reaction. Single/double tube luminometers are sensitive and inexpensive but can measure only one or two samples at a given time, which are suitable for small research laboratories. On the other hand multiple tube or plate luminometers are more expensive since they can measure multiple samples at the same time and are suitable for centers that are engaged in regular research work on chemiluminescence (66).

ROS-TAC Score

Since oxidative stress is caused by an imbalance between levels of ROS produced and antioxidant protection at any given time, it is a conceivable that measurement of oxidative stress can be made either by assessment of ROS or total antioxidant capacity (TAC). The TAC is measured by enhanced chemiluminescence assay or colorimetric assay (10,70). Sharma et al. described a ROS-TAC score for assessment of seminal oxidative stress that showed to be superior to ROS or TAC alone in discriminating fertile and infertile population (10). This score minimizes the variability of the individual parameters (ROS or TAC) of oxidative stress. The ROS-TAC score was based on a group of normal healthy fertile men who had very low levels of ROS. Men with male factor or idiopathic infertility had significantly lower seminal ROS-TAC scores compared to normal controls, or the men with initial male factor that eventually were able to initiate pregnancy. The average ROS-TAC score for fertile healthy men was 50 ± 10, which was significantly higher ($p \leq 0.0002$) compared to infertile patient (35.8 ± 15). The probability of successful pregnancy is estimated at < 10% for values of ROS-TAC < 30, but increased as the ROS-TAC score increased.

Leukocyte Evaluation

Since lower leukocyte levels are sometimes associated with significant ROS levels in semen it is important to determine the exact source of ROS in semen because the clinical implications of infiltrating leukocytes are quite different from those of pathological conditions in which spermatozoa themselves are the source of ROS (36,45,71). Methods that are currently used for assessment of seminal OS, such as chemiluminescence assays, do not provide information on the differential contribution of spermatozoa and leukocytes to ROS production in semen. Nitroblue tetrazolium test (NBT) can be used for assessment of seminal oxidative stress, and the differential contribution of cells to ROS generation, and to determine the state of activation of seminal leukocytes. ROS levels measured by chemiluminescence assay are strongly correlated with the results of NBT staining. Also, the NBT reduction test is commonly available, easily performed, inexpensive and has high sensitivity (72).

Oxidative Stress Status (OSS)

Currently there is no consensus regards to the inclusion of ROS measurement as part of the routine clinical evaluation of male infertility mainly because there is a lack of standardization of ROS analytical methods, equipment, and range of normal levels of ROS in semen. Some investigators have defined the basal levels of reactive oxygen species in neat semen specimens of normal healthy donors (45,73). Measurement of ROS levels in neat semen after liquefaction in the presence of seminal antioxidant protection proved to be a better test to evaluate oxidative stress status. The ROS levels for fertile donors with normal genital examination and normal standard semen parameters were 1.5×10^4 cpm/20 million sperm/mL. Using this value as a cutoff, infertile men can be classified as either OS-positive ($> 1.5 \times 10^4$ cpm/20 million sperm/mL) or OS-negative ($\leq 1.5 \times 10^4$ cpm/20 million sperm/mL), irrespective of their clinical diagnosis or results of standard semen analysis (73). Assessing ROS directly in neat semen showed diagnostic and prognostic capabilities identical to those obtained from ROS-TAC score (73).

Earlier studies have shown that sperm washing procedures like multiple centrifugation, resuspension, and vortexing artificially elevate ROS levels (61,74,75). The antioxidant activity of seminal plasma is removed during sperm washing steps, which also results in elevated ROS levels (74). Excessive washing and manipulation including duration of centrifugation was found to be more important than the force of centrifugation for ROS formation by human spermatozoa (76). Therefore procedures that

minimize multiple centrifugation, resuspension, and vortexing should be used for the preparation of spermatozoa for ART (61).

Conflicting studies make it difficult to establish the clinical value of ROS measurement in medical practice since there is no clear evidence whether high ROS levels are a cause or an effect of abnormal semen parameters and sperm damage (77). However, a more recent study reported high levels of ROS as an independent marker of male factor infertility, irrespective of whether these patients have normal or abnormal semen parameters (78). These findings suggest that ROS measurement should be used as a diagnostic tool in infertile men especially in cases of idiopathic infertility and that the reference values of ROS in neat semen can be used to define the pathologic levels of ROS in infertile men and may guide in better therapeutic interventions.

STRATEGIES TO REDUCE SEMINAL OXIDATIVE STRESS

Given the major role of oxidative stress in the pathogenesis of male infertility, treatment strategies with the goal of reducing levels of seminal oxidative stress are necessary for natural as well as assisted reproductive technologies. Spermatozoa produce small amounts of ROS that must be continuously inactivated to keep only the necessary amount to maintain normal physiologic cell function. The pathologic levels of ROS detected in the semen of infertile men are more likely caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma (13). The body has a number of mechanisms to minimize free radical induced damage. Unfortunately, spermatozoa are unable to repair the damage induced by oxidative stress, because they lack the required cytoplasmic enzyme systems to perform the repair (79). Antioxidants are the most important defense mechanisms against OS induced by free radicals. Metal chelators and metal binding proteins that block new ROS formation are classified as preventative antioxidants. Scavenger antioxidants, such as vitamins E and C, beta-carotene and other antioxidant dietary supplements, glutathione and enzymes, act via removing ROS already generated by cellular oxidation.

Many clinical trials have demonstrated the beneficial effect of antioxidants in treating selected cases of male infertility (80-85), whereas others failed to report the same benefits (86-88). Pregnancy, the most relevant outcome parameter of fertility, was reported in only a few of them (80,84,89-91). The majority of the studies analyze multiple antioxidant combinations, different dosages and durations. Also the patient's selection is another important aspect because oxidative stress can not be considered the cause of male infertility in all patients. Recently, Agarwal et al. in an extensive review of literature concluded that many studies suffer from the lack of placebo-controlled, double-blind design, making the effectiveness of antioxidant supplementation in infertile patients still inconclusive (79).

Antioxidants may not be very effective depending on the etiology of infertility (79). Primarily, specific therapeutics directed against the etiological causes of elevated ROS should be attempted. Once the primary cause of infertility have been treated or no specific etiology is identified (idiopathic infertility) patients can be advised to take optimal doses of antioxidants supplementation.

ORIGIN OF DNA DAMAGE IN SPERMATOZOA

Sperm genetic material is structured in a special manner that keeps the nuclear chromatin highly stable and compact. The normal DNA structure is capable of decondensation at appropriate time transferring the packaged genetic information to the egg without defects in the fertilization process. The cause of DNA damage in sperm can be attributed to various pathological conditions including cancer (92), varicocele (93), high prolonged fever (94), advanced age (95) or leukocytospermia (96). Also a variety of environmental conditions can be involved as radiation (97), air pollution, smoking (8), pesticides, chemicals, heat and ART prep protocols (52,97,98). Most of these agents not only disrupt hormone levels but may also

Table 1 – *Etiological factors associated with increased human sperm DNA damage.*

Etiology	Reference	Study Population	DNA Assay	Conclusion
Pollutants	De Rosa et al. 2003 (55)	85 men employed at motorway tollgates 85 controls	Acridine orange	Higher sperm DNA damage in men exposure to pollutants. Nuclear DNA damage was inversely correlated with methaemoglobin levels.
Varicocele	Saleh et al. 2003 (93)	31 infertile men 16 fertile controls	SCSA	Infertile men with varicocele showed significant higher DNA damage that appears to be related to high OS.
Leukocytospermia	Erenpreiss et al. 2002 (96)	187 men	Acridine orange	Normal semen has low DNA integrity and resist to leukocytospermia. Leukocytes increasing primary or provoking potential DNA damage.
	Alvarez et al. 2002 (123)	56 infertile patients 18 healthy fertile men	SCSA	Significant increase in sperm DNA damage in leukocytospermic samples compared to normal controls.
Smoking	Potts et al. 1999 (52)	35 fertile smokers 35 fertile non-smokers	SCSA	Higher sperm DNA damage in smokers compared to non-smokers.
	Singh et al. 2003 (95)	40 infertile men 26 healthy men	Comet	Significant higher DNA damage in men > 36 years old.
Advanced age	Moskovtsev et al. 2006 (124)	1125 infertile men	Acridine orange	DNA damage is significantly higher in men over 40 years old
	Trisini et al. 2004 (125)	252 infertile men	Comet	Significant high DNA damage in men older than 35 years.

Table 1 – *Etiological factors associated with increased human sperm DNA damage. (continued)*

Etiology	Reference	Study Population	DNA Assay	Conclusion
Cancer	Fossa et al. 1997 (126)	39 patients with testicular cancer	SCSA	Sperm DNA damage is higher in men with cancer even before cancer therapy.
		18 healthy controls		Recovery of spermatogenesis is higher when normal SCSA is found before adjuvant treatment.
	Spermon et al. 2006 (127)	22 patients with testicular cancer treated with cisplatin-based chemotherapy	CMA3 TUNEL	High sperm DNA damaged in these patients. Improvement in sperm chromatin packaging after chemotherapy.
	O'Donovan et al. 2005 (92)	8 men with leukemia 12 men with testicular cancer 3 men with lymphoma	Comet	Detrimental effect on chromatin condensation and DNA integrity of cancer and as its treatment.
ROS	Said et al, 2005 (8)	28 infertile men	TUNEL	Increased ROS production showed a positive correlation with sperm DNA damage in a time-dependent manner.
	Henkel et al, 2005 (43)	63 infertile men	TUNEL	DNA fragmentation was strongly positively correlated with intrinsic ROS production, whereas this correlation was weaker for extrinsic ROS production.

DFI = DNA fragmentation index, SCSA = sperm chromatin structure assay; ROS = reactive oxygen species; OS = oxidative stress. TUNEL = terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate (dUTP)-nick end-labeling.

induce oxidative stress, which could damage sperm DNA (99) (Table-1).

The extent of sperm DNA damage has been closely associated with impaired sperm function as well as male infertility (7). However the precise mechanism(s) responsible for chromatin abnormalities in human spermatozoa is/are most likely to be multi factorial and are not accurately understood at this time (100) (Figure-1). The most important theories proposed as molecular mechanism of sperm DNA damage are: (a) defective chromatin packaging, (b) reactive oxygen species (ROS) (8,101,102), (c) apoptosis mainly during spermatogenesis (7,103), and (d) DNA fragmentation induced by endogenous endonucleases (104).

ROLE OF OXIDATIVE STRESS IN SPERM DNA DAMAGE AS RELATED TO MALE INFERTILITY

Excessive generation of ROS in the reproductive tract not only affect the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus. DNA bases are susceptible to oxidative damage resulting in base modification, strand breaks, and chromatin cross-linking. Oxidative stress-induced DNA damage causes pro-mutagenic change, which in its most severe form affects the quality of the germ line and prevents fertilization. When there is less oxidative damage, fertilization can occur, but the oocyte must repair the DNA strand breaks before the initiation of the first cleavage. Apoptosis and OS are involved in mediating DNA damage in the germ line (105) (Figure-2). The Y chromosome is particularly vulnerable to DNA damage, due to its genetic structure as well as it cannot correct double-stranded DNA deletions.

Fertile healthy men with normal seminal parameters almost consistently have low levels of DNA breakage, whereas infertile men, in particular those with abnormal seminal parameters, have higher fraction of sperm DNA damage (106). Idiopathic infertile men may present normal routine seminal

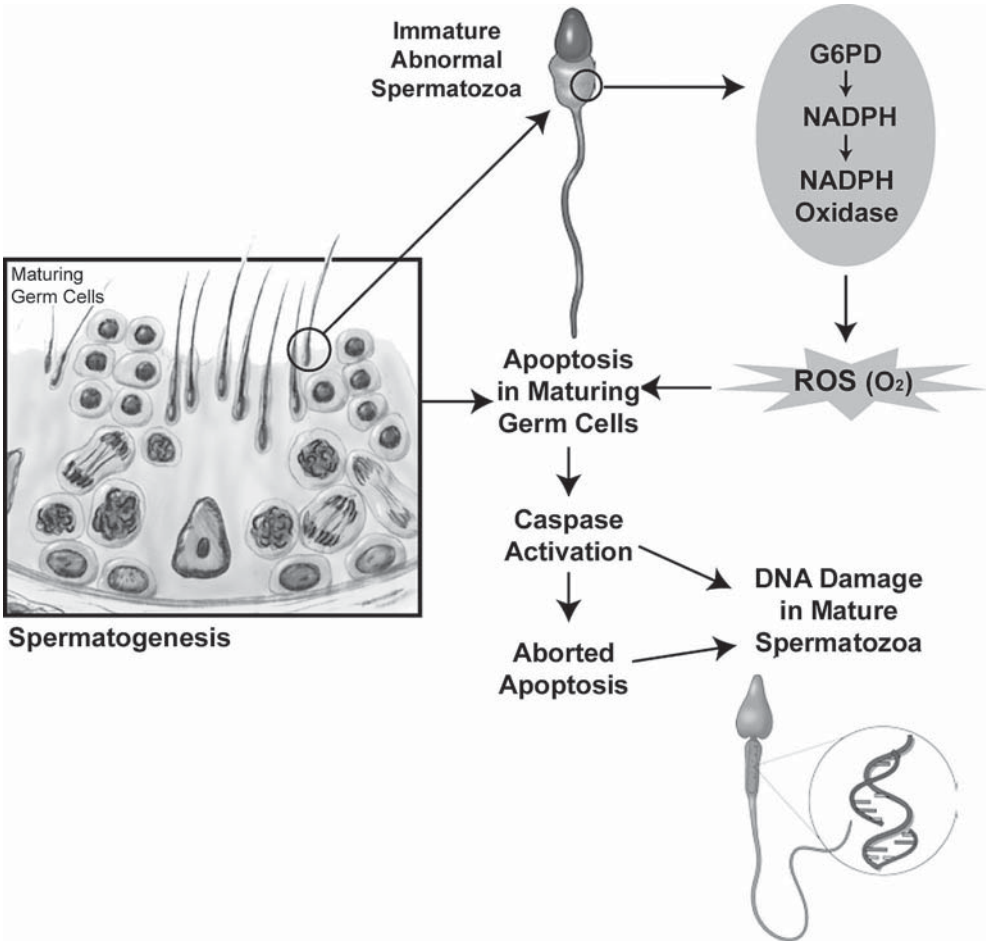

Figure 2 – *Mechanistic pathway showing sperm DNA damage due to oxidative stress.*

parameters (concentration, motility, and morphology) with abnormal DNA integrity (83,106,107). It is of great concern that the most efficient ART techniques used to treat male factor infertility with high degree of sperm DNA damage. During ICSI, it is always desirable to select spermatozoa with normal morphology that reduces the risk of introducing spermatozoa with strand breaks (108). This is sometimes not always true since the traditional sperm parameters such as sperm count, motility and morphology have been proven to be poorly correlated to DNA damage status (109,110). Moreover, this has significant clinical implications because in vitro fertilization using spermatozoa with damaged DNA may lead to paternal transmission of defective genetic material with adverse consequences for embryo development. These findings suggest that an estimate of the percentage of DNA damaged spermatozoa in fertile and infertile men may be important and a future challenge will be to develop methods to identify and select spermatozoa with intact DNA during the IVF/ ICSI procedures.

Recently sperm from infertile men with varicoceles have been associated with significantly high levels of DNA damage (93). The finding of high seminal OS in patients with varicoceles may indicate that OS plays an important role in the pathogenesis of sperm DNA damage in patients with this condition. Although Zini et al. reported that varicocelectomy can improve human sperm DNA integrity in infertile men with clinical varicoceles (28), a limited number of studies has examined potential treatments to reduce sperm DNA damage. Therapeutic conditions have been suggested that avoidance of gonadotoxins (52) (smoking, mediations) and hyperthermia (94) (saunas, hot tubes) may reduce sperm DNA damage. Treatment of GU infection can also be helpful based on the evidence that leukocytospermia induce ROS production and possibly DNA damage (44). Studies suggested that sperm DNA damage can be reduced with oral antioxidants administered during a relatively short time period (111). However, these recommendations have been based on small, uncontrolled studies and to date no treatment for abnormal DNA integrity has been shown to have successful clinical results (107).

ASSESSMENT OF SPERM CHROMATIN INTEGRITY

Several techniques can measure DNA defects in human spermatozoa and the ability of these techniques to accurately estimate sperm DNA damage depends on many technical and biological aspects. However, to establish a threshold level between the fertile population and the lowest sperm DNA integrity required for achieving pregnancy remains extremely challenging. Currently both direct (fragmentation, oxidation) and indirect (sperm chromatin compaction) methods are available to evaluate the integrity of sperm DNA. Direct methods for detecting DNA breaks include (a) the single-gel electrophoresis assay ("Comet assay") and (b) terminal deoxynucleotidyl transferase-mediated 2`-deoxyuridine 5`-triphosphate (dUTP)-nick end-labeling (TUNEL) assay (106,112). Indirect methods mainly sperm chromatin integrity assays (SCSA) for assessing DNA damage uses chromatin and/or DNA intercalating dyes such as acridine orange to differentiate single-stranded and double-stranded DNA (106,109,110).

Less frequent clinical tests for DNA damage include the sperm chromatin dispersion test (SCD) using the Halosperm kit which allow to simultaneously perform DNA fragmentation and chromosomal analyses in the same sperm cell (113), liquid chromatography that detect oxidized DNA nucleotide residues (83) and evaluation of nuclear protein (protamine/histone ratio) levels in sperm samples.

All methods currently lack a threshold, except for the sperm chromatin structure assay (SCSA), which assesses the ability of the DNA to resist denaturation by acid or heat and uses DNA flow cytometry approach. The sperm DNA damage is expressed as the DNA fragmentation index (DFI) (114) that can distinguish fertile and infertile population in clinical practice (115).

DNA DAMAGE AND REPRODUCTIVE OUTCOME

Sperm DNA damage is critical in the context of success of assisted reproductive techniques

(99,116). The main nuisance of ART is that they bypass the natural defense barrier present throughout female reproductive tract responsible for selecting the best spermatozoa for oocyte fertilization. Normally oocytes are capable of repairing partial DNA damage. However, when the damage is severe, embryo death and miscarriages are more likely to happen. Probably that explains why miscarriage rate is higher after ICSI compared to classic IVF (117).

Standard semen parameters do not identify subtle defects in sperm chromatin architecture, which after the advent of ICSI has become more important parameter of sperm functional quality than count, motility or morphology. The emphasis on evaluation of genomic integrity has recently increased due to reports that correlate the degree of DNA damage with various fertility indices including rates of fertilization, embryo cleavage, implantation, pregnancy and live birth (118-120).

Sperm DNA integrity is an essential requirement to achieve pregnancy in natural conception (110) as well as for IVF outcomes where the natural process of fertilization is circumvented (121). A high degree of sperm DNA damage has been found in couples presenting with unexplained recurrent pregnancy loss (117). All male partners of couples who achieved a pregnancy during the first 3 months attempting to conceive had < 30% sperm with fragmented DNA (109), whereas, 10% of the couples who achieved pregnancy in months 4-12 and 20% of couples who never achieved a pregnancy had > 30% sperm with fragmented DNA. Moreover 84% of the men who initiated pregnancy before 3 months had sperm DNA damage levels of < 15%.

Bungum et al. reported that for IUI, there was a significantly higher chance of pregnancy/ delivery in the group with DFI < 27% and HDS (highly DNA stainable) of < 10% than in patients with DFI > 27% and HDS > 10%. Although, no statistical difference between the outcomes of IVF versus ICSI was observed in the group with DFI < 27%, ICSI had significantly better results than those of IVF in patients with DFI > 27%. The authors concluded that combining the two SCSA parameters, DFI and HDS is a useful method for prediction of IUI outcomes.

Henkel et al. reported that even though sperm DNA fragmentation did not correlate with the fertilization and embryo fragmentation rates, patients with a high percentage of TUNEL positive spermatozoa (> 36.5%) showed a significantly lower pregnancy rate compared to those patients with lower than 35.5% TUNEL-positive sperm (118). The decision to incorporate a new test into clinical practice depends on the volume and quality of reports that favor or refute such claims. Although multiple studies have analyzed the relationship between the degree of DNA damage and the fertilization rate, embryo cleavage rate, implantation rate, pregnancy rate, and live birth rate of offspring, existing data on the relationship between abnormal DNA integrity and reproductive outcomes are limited and not analyzed systematically (122). The Practice Committee of the American Society for Reproductive Medicine summarizes the current understanding of the impact of abnormal sperm DNA integrity on reproductive outcomes (107). This Committee concluded that current methods for evaluating sperm DNA integrity alone do not predict pregnancy rates achieved with intercourse, IUI, or IVF and ICSI.

Before sperm DNA damage analysis is introduced routinely in clinical practice, studies with adequate sample size must be conducted evaluating outcomes and role of such tests in the management of male infertility (122).

TAKE HOME MESSAGE

Limited amount of free radicals and oxidative stress have an important role in modulating many physiological functions in reproduction. ROS are being constantly produced in small controlled amounts in the reproductive tract and by a variety of semen components. Many scavenging enzymes and molecules (antioxidants) control the damaging effects of ROS to keep the normal physiological balance. However, when ROS production exceeds the scavenging capacity of the antioxidants a state referred to as oxidative stress is generated that becomes toxic to sperm. High levels of ROS and OS in reproductive tract and semen are associated with sperm dysfunction and damage to sperm nuclear

DNA. Although routine semen analysis remains the backbone of evaluating male infertility, determining the levels and sources of excessive ROS generation in semen may be useful in developing future therapeutic strategies for male infertility.

Current evidence suggests the use of systemic antioxidants for the management of selective cases of male infertility as well as in vitro supplements during various sperm preparation techniques. However, a definitive conclusion cannot be drawn from the available studies, as oxidative stress is not the only cause of male infertility.

Sperm DNA damage is more common in infertile men and has been correlated with poor reproductive outcomes. Although ART is able to compensate for the impairment of sperm chromatin integrity, transmission of abnormal genetic material through ART needs further investigations in order to reduce sperm DNA damage. Current methods for evaluating sperm DNA integrity are not standardized and are not routinely used in clinical laboratories. Also to date no treatment for abnormal DNA integrity has proven to be of clinical value.

A significant percentage of couples, even after extensive infertility evaluation, show no apparent male or female factor and are still unable to conceive. Increased oxidative stress and DNA damage may be responsible for the poor fertility in these patients. Although assisted reproduction provides opportunity to these couples with unexplained infertility, the potential medical risks entailed by multiple-gestation pregnancies and the associated costs are significant. It is important to further decipher the molecular basis of male infertility in order to thoroughly understand the effects of abnormal spermatozoa on fertilization and embryo development. With this understanding, the success of ART and ICSI can be improved significantly.

CONFLICT OF INTEREST

None declared.

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