Chapter 13

Significance of Oxidative Stress and Sperm Chromatin Damage in Male Infertility

Ashok Agarwal

Director, Andrology Laboratory and Sperm Bank, and Director, Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Urological Institute and Department of Obstetrics-Gynecology, Cleveland Clinic Foundation, Cleveland, OH 44195

Abstract

Researchers studying the causes of male infertility have recently focused on the role played by reactive oxygen species (ROS)—a highly reactive oxidizing agent belonging to the class of free radicals. ROS are produced by a variety of semen components, and antioxidants in the seminal fluid keep their levels in check. Small amounts of ROS help spermatozoa acquire their necessary fertilizing capabilities. However, when ROS production exceeds the scavenging capacity of the antioxidants—a state referred to as oxidative stress (OS)—ROS become toxic to sperm. Research suggests that ROS attack the integrity of DNA in the sperm nucleus by causing base modification, DNA strand breaks, and chromatin cross-linking. DNA damage induced by excessive levels of ROS may accelerate the process of germ cell apoptosis, leading to the decline in sperm counts associated with male infertility. This chapter will discuss in more detail the cellular origins of ROS in human semen, how ROS damages sperm nuclear DNA, and how such DNA damage contributes to male infertility. Research highlights from the Cleveland Clinic, including the novel ROS-TAC (ROS total antioxidant capacity) score for assessing OS, will also be presented.

Introduction

Defective sperm function is the most prevalent cause of male infertility and is difficult to treat (1). The mechanisms underlying abnormal sperm function are still poorly understood. This may be due to a lack of basic knowledge about biochemical and physiological processes involved in spermatogenesis (2).

One possible mechanism that is currently being studied is the generation of reactive oxygen species (ROS) in the male reproductive tract. ROS are highly reactive oxidizing agents that belong to the class of free radicals. A free radical is defined as "any atom or molecule that possesses one or more unpaired electrons" (3). When produced in large amounts, ROS have potentially toxic effects on sperm quality and function. Recent reports have indicated that high levels of ROS are detected in semen

samples of 25 to 40% of infertile men (4,5). However, a strong body of evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (6).

Spermatozoa, like all cells living under aerobic conditions, constantly face the oxygen (O_2) paradox, *i.e.,* O_2 is required to support life, but its metabolites, such as ROS, can modify cell functions, endanger cell survival, or both (7). Hence, ROS must be continuously inactivated to keep only the small amount necessary to maintain normal cell function. It is not surprising that a battery of different types of antioxidants protect against oxidants (8). An antioxidant is defined as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidative damage to that substrate." The term "oxidizable substrate" includes almost every molecule found in living cells, including proteins, lipids, carbohydrates, and DNA (9).

Spermatozoa are particularly susceptible to the damage induced by excessive ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFA) (10), and their cytoplasm contains low concentrations of scavenging enzymes (7,11–13). In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surround the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection afforded by the seminal plasma, which bathes these cells (14,15). Excessive generation of ROS in the reproductive tract not only attacks 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. There is strong evidence that DNA fragmentation commonly observed in spermatozoa of infertile men is mediated by high levels of ROS.

ROS and Sperm Physiology

Until recently, ROS were exclusively considered toxic to human spermatozoa. The idea that limited amounts of ROS can physiologically regulate some sperm functions was first evoked in a study by Aitken *et al*. (16). They observed that ROS, at low levels, enhanced the ability of human spermatozoa to bind zonae pellucida, an effect that was reversed by the addition of vitamin E. As a general rule, incubating spermatozoa with low concentrations of hydrogen peroxide (H_2O_2) stimulates sperm capacitation, hyperactivation, and the ability of the spermatozoa to undergo acrosome reaction and oocyte fusion (17–21). Reactive oxygen species other than H_2O_2, such as nitric oxide and superoxide anion ($O_2^{\bullet -}$), have also been shown to promote sperm capacitation and acrosome reaction (22,23).

Cellular Origin of ROS in Human Semen

A variety of semen components, including morphologically abnormal spermatozoa, precursor germ cells, and leukocytes, can generate ROS in semen. However, seminal

leukocytes and morphologically abnormal spermatozoa are the main sources of ROS in human ejaculates (24,25).

ROS Production by Spermatozoa

Clear evidence suggests that human spermatozoa produce oxidants (26–28). Levels of ROS produced by pure sperm populations are negatively correlated with quality of sperm in the original semen (29). 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 (Fig. 13.1). Under these circumstances, spermatozoa released during spermiation are thought to be immature and functionally defective (30). Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytostolic enzyme glucose-6-phosphate-dehydrogenase (G_6PD). This enzyme controls the rate of glucose flux through hexose monophosphate shunt, which in turn controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). Spermatozoa use NADPH as a source of electrons to fuel the generation of ROS by an enzyme system known as NADPH-oxidase (31,32).

Spermatozoa may generate ROS in two ways: (i) NADPH-oxidase system at the level of the sperm plasma membrane (26) and (ii) NADH-dependent oxido-reductase (diphorase) at the level of mitochondria (33). The mitochondrial system is the major source of ROS in spermatozoa from infertile men (34) (Fig. 13.1).

The primary ROS generated in human spermatozoa is the $O_2^{\bullet -}$. This one-electron reduction product of oxygen secondarily reacts with itself in a dismutation reaction, which is greatly accelerated by superoxide dismutase (SOD), to generate H_2O_2. In

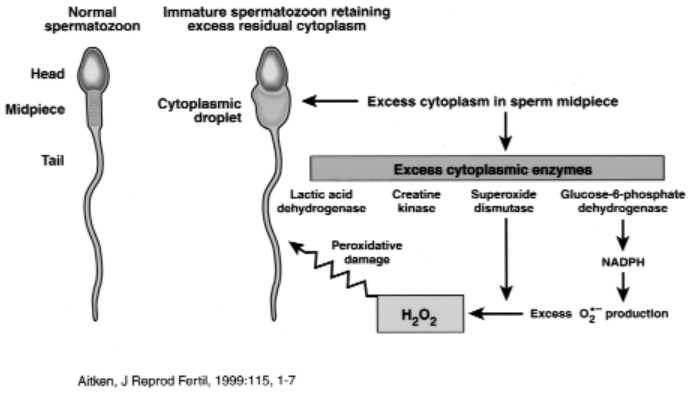

Fig. 13.1. Mechanism of increased production of reactive oxygen species (ROS) by abnormal spermatozoa.

addition to H_2O_2 and $O_2^{\bullet-}$, a variety of secondary cytotoxic radicals and oxidants are generated by human spermatozoa. In the presence of transition metals such as iron and copper, H_2O_2 and $O_2^{\bullet-}$ can interact in a Haber-Weiss reaction to generate the extremely pernicious hydroxyl radical ($OH^{\bullet}$) as in Equation 1.

$$O_2^{\bullet-} + H_2O_2 \rightarrow OH + OH^- + O_2 \quad [1]$$

Alternatively, the hydroxyl radical can be produced from hydrogen peroxide by the Fenton reaction, which requires a reducing agent, such as ascorbate or ferrous ions (Eq. 2) (35).

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \quad [2]$$

The hydroxyl radical, which is thought to be an extremely powerful initiator of the lipid peroxidation (LPO) cascade, can precipitate a loss of sperm function.

ROS Production by Leukocytes

With respect to all nonsperm cells, the majority of the so-called round cells consist of immature germ cells with fewer than 5% leukocytes under normal conditions (36). Peroxidase-positive leukocytes are the major source of ROS in semen (20,37,38). Peroxidase-positive leukocytes include polymorphonuclear (PMN) leukocytes, which represent 50 to 60% of all seminal leukocytes, and macrophages, which represent the remaining 40 to 50% of all seminal leukocytes (39–41). Peroxidase-positive leukocytes in semen are contributed largely by the prostate and the seminal vesicles (42). Sperm damage from ROS produced by leukocytes occurs if seminal leukocyte concentrations are abnormally high, *i.e.,* leukocytospermia (43), if the patient has epididymitis, or if seminal plasma was removed during sperm preparation for assisted reproduction (38). Seminal plasma contains large amounts of ROS scavengers but confers a very variable (10 to 100%) protection against ROS generated by leukocytes (44).

Activated leukocytes can produce 100-fold higher amounts of ROS than nonactivated leukocytes (34). Leukocytes may be activated in response to a variety of stimuli including inflammation and infection (45). Activated leukocytes increase the NADPH production via the hexose monophosphate shunt. The myeloperoxidase system of both PMN leukocytes and macrophages is also activated leading to respiratory burst with production of high levels of ROS (46,47). Such an oxidative burst is an early and effective defense mechanism in cases of infection for killing the microbes (48).

ROS Scavenging Strategies

Interestingly, the seminal plasma is well endowed with an array of antioxidant defense mechanisms to protect spermatozoa against oxidative insult (49). These mechanisms compensate for the deficiency in cytoplasmic enzymes in sperm (50). Seminal plasma

contains enzymatic antioxidants, such as SOD (51), glutathione peroxidase/glutathione reductase (GPX/GRD) system (52), and catalase (53), as well as nonenzymatic antioxidants, such as ascorbate (54), urate (55), vitamin E (56,57), pyruvate (58), glutathione (59), taurine, and hypotaurine (60). Seminal plasma from fertile men has a higher total antioxidant capacity than that of infertile men (61). However, pathological levels of ROS detected in semen from infertile men are more likely due to increased ROS production rather than reduced antioxidant capacity of the seminal plasma (15). Antioxidant defense mechanisms include three levels of protection: prevention, interception, and repair.

Prevention

Prevention of ROS formation is the first line of defense against oxidative damage. An example is the binding of metal ions, iron and copper ions in particular, which prevents them from initiating a chain reaction (8). Chelation of transition metals is a major means of controlling LPO and DNA damage. When transition metals become loosely bound to biological molecules such as oxygen reduction products, they can produce secondary and more reactive oxidants, particularly $OH^{\bullet}$ (9).

Interception

Free radicals have a tendency toward chain reaction, *i.e.,* a compound carrying an unpaired electron will react with another compound to generate an unpaired electron, "radical begets radical." Hence, the basic problem is to intercept a damaging species from further activity, which is the process of deactivation leading to the formation of nonradical end products (8). Vitamin E, a chain-breaking antioxidant, inhibits LPO in membranes by scavenging peroxyl ($RO^{\bullet}$) and alkoxyl ($ROO^{\bullet}$) radicals. The ability of α-tocopherol to maintain a steady-state rate of peroxyl radical reduction in the plasma membrane depends on the recycling of α-tocopherol by external reducing agents, such as ascorbate or thiols (62). In this way, α-tocopherol can function again as a free radical chain-breaking antioxidant, even though its concentration is low (63). A prerequisite for efficient interception is a relatively long half-life of the radical to be intercepted (8). The peroxyl radicals are major reaction partners because their half-life extends into the range of seconds (7 s). In contrast, the hydroxyl radical, with its high reactivity and extremely short half-life (10^{-9} s), cannot be intercepted with reasonable efficiency.

Repair

Protection from the effects of oxidants can also occur by repairing the damage once it has occurred. Unfortunately, spermatozoa are unable to repair the damage induced by ROS because they lack the cytoplasmic enzyme systems that are required to accomplish this repair. This is one of the features that make spermatozoa unique in their susceptibility to oxidative insult (16,51).

The Concept of Oxidative Stress (OS)

Oxidative stress (OS) is the term applied when oxidants outnumber antioxidants (8), when peroxidation products develop (64), and when these phenomena cause pathological effects. Oxidative stress has been implicated in numerous disease states such as cancer, arthritis, connective tissue disorders, aging, toxin exposure, physical injury, infection, inflammation, acquired immunodeficiency syndrome, and male infertility (12,20,26,65).

In the context of human reproduction, a balance is present 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 (66). Excessive ROS production, which is related to abnormalities of the male reproductive tract, can overwhelm all antioxidant defense strategies of spermatozoa and seminal plasma when it exceeds critical levels, causing an OS status (13,67,68).

Mechanisms of ROS Toxicity

Virtually every human ejaculate is contaminated with potential sources of OS, such as peroxidase-positive leukocytes and morphologically abnormal spermatozoa. It follows that some of the sperm cells will incur oxidative damage and a concomitant loss of function in every ejaculate. Thus, the impact of OS on male fertility is a question of degree rather than the presence or absence of the pathology (69). All cellular components, lipids, proteins, nucleic acids, and sugars are potential targets for ROS. The extent of damage caused by ROS depends not only on type and the amount of ROS involved but also on the moment and duration of ROS exposure and on extra-cellular factors such as temperature, oxygen tension, and the composition of the surrounding environment, including ions, proteins, and ROS scavengers.

Lipid Peroxidation

Lipid peroxidation can be broadly defined as "oxidative deterioration of PUFA," *i.e.,* fatty acids that contain more than two carbon-carbon double bonds (70). The LPO cascade occurs in two fundamental stages: initiation and propagation.

Initiation Stage

The hydroxyl radical ($OH^{\bullet}$) is a powerful initiator of LPO (12). Most membrane PUFA 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 that is produced is stabilized by the rearrangement of the double bonds, which forms 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 (47). Conjugated dienes rapidly react with O_2 to form a lipid peroxyl radical ($ROO^{\bullet}$), which abstracts hydrogen atoms

from other lipid molecules to form lipid hydroperoxides (ROOH). Thus, the chain reaction of LPO is continued (70).

Propagation Stage

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 (70). Lipid peroxidation propagation will depend upon the antioxidant strategies employed by spermatozoa. One of the by-products of lipid peroxide decomposition is malonaldehyde (MDA), which has been used as an end product in biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa (12,16). 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 (71,72).

Impairment of Sperm Motility

Increased production of ROS has been correlated with a reduction of sperm motility (14,16,73,74). The link between ROS and reduced motility may be due to a cascade of events that results 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 (7). Another hypothesis is that H_2O_2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes, such as G_6PDH, leading to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione and reduced glutathione. This can cause a decrease in the antioxidant defenses of the spermatozoa, which ultimately leads to the peroxidation of membrane phospholipids (75).

Sperm Nuclear DNA Damage

Sperm DNA is organized in a specific manner to keep the chromatin in the nucleus compact and stable (76). In 1991, Ward and Coffey proposed four levels of organization for DNA packaging in the spermatozoon: (i) chromosomal anchoring, which refers to the attachment of the DNA to the nuclear annulus; (ii) formation of DNA loop domains as the DNA attaches to the newly added nuclear matrix; (iii) replacement of histones by protamines, which condense the DNA into compact doughnuts; and (iv) chromosomal positioning. Chromosomes become organized, with their centromeres located in the center of the nucleus and their telomeres at the nuclear periphery; active genes are localized to the nuclear center and the inactive genes to the periphery (Fig. 13.2).

This DNA organization not only permits the very tightly packaged genetic information to be transformed to the egg but also ensures that the DNA is delivered in a physical and chemical form that allows the developing embryo to access the genetic information (76).

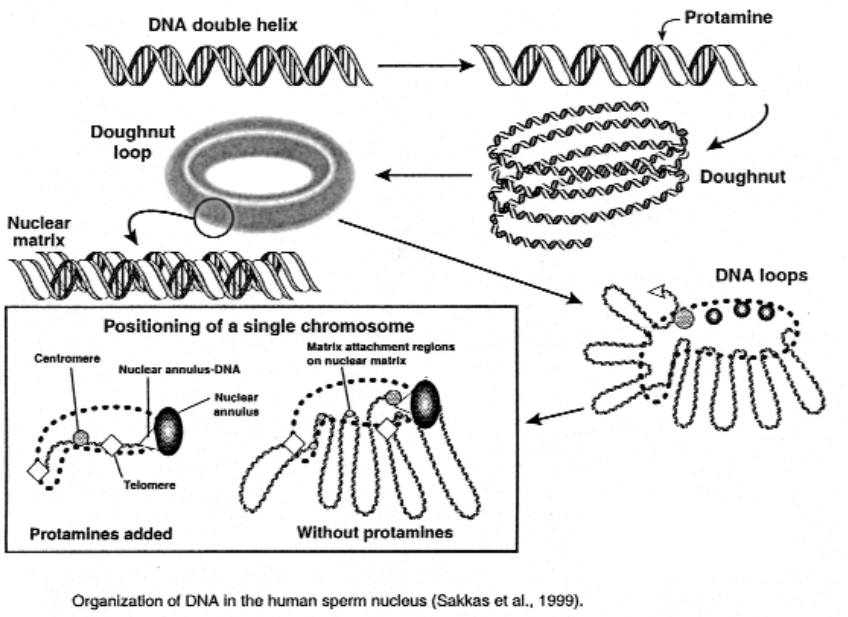

Fig. 13.2. DNA packaging in human spermatozoa.

Origin Of DNA Damage in Spermatozoa

Defective Chromatin Packaging

Endogenous nicks in DNA occur most frequently during the transition from round to elongated spermatids in the testis. In rat and mouse spermatozoa, the nicks occur before protamination is completed (77). Protamination for chromatin packaging might require the formation and ligation of nicks through endogenous nuclease activity. Researchers have proposed that the endogenous nuclease, topoisomerase II (topo II), might play a role in both the creation and ligation of nicks during spermiogenesis. These nicks are thought to relieve stress due to torsion and to aid chromatin rearrangement as histones are replaced by protamines. Therefore, the presence of endogenous nicks in ejaculated spermatozoa indicates incomplete maturation during spermiogenesis (76). This hypothesis is supported by observations that the presence of DNA damage in mature spermatozoa is correlated with poor chromatin packaging due to under-protamination (78,79).

Apoptosis

Spermatogenesis is a dynamic process of germ cell proliferation and differentiation from stem spermatogonia to mature spermatozoa through a complex series of mitot-

ic and meiotic divisions. Apoptosis, also described as programmed cell death, is a physiological phenomenon characterized by cellular morphological and biochemical alterations leading the cell to commit suicide (80). Apoptosis is genetically determined and takes place at specific moments during normal embryonic life to allow definitive forms of tissues to develop and during adult life to discard cells that have an altered function or no function at all (81). In the context of male reproductive function, apoptosis may be responsible for controlling overproduction of male gametes (76,82). Testicular germ cell apoptosis occurs normally and continuously throughout life.

One factor that is thought to play a role in sperm apoptosis is the cell surface protein Fas (83). Fas is a type I membrane protein that belongs to the tumor necrosis factor-nerve growth factor receptor family and mediates apoptosis (84). Binding of Fas-ligand (Fas-L) or agonistic anti-Fas antibody to Fas kills cells by apoptosis (85). In men with abnormal semen parameters, the percentage of Fas-positive spermatozoa can be as high as 50%. Samples with low sperm concentration are more likely to have a high proportion of Fas-positive spermatozoa (76). This evidence suggests that in subfertile men the correct clearance of spermatozoa via apoptosis is not occurring. The presence of spermatozoa that possess apoptotic markers, such as positive Fas and DNA damage, indicates that in men with abnormal semen parameters such as abnormal morphology, abnormal biochemical functions, and nuclear DNA damage, an "abortive apoptosis" has taken place (30,76).

Fas-positive spermatozoa may not be cleared due to dysfunction at one or more levels. First, apoptosis limits any excess in the number of developing germ cells so that the supportive capacity of Sertoli cells is not overloaded. Because Sertoli cells can limit their proliferation by producing Fas-L, the production of spermatozoa may not be enough to trigger apoptosis in cases with hypo-spermatogenesis. In these men, Fas-positive spermatogonia may escape the signal to undergo apoptosis. Second, Fas-positive spermatozoa may also exist because of problems in activating Fas-mediated apoptosis. These problems could be inherent to a particular patient or may be due to lack of synchronization between apoptosis and spermatogenesis. In the latter case, the spermatozoa will go through spermiogenesis and fail to complete apoptosis even though apoptosis has been initiated. This hypothesis may explain why patients with abnormal semen characteristics also possess a high percentage of spermatozoa containing DNA damage and abnormal spermatozoa that display markers of apoptosis (76).

Oxidative Stress-Induced DNA Damage

Two factors protect spermatozoal DNA from oxidative insult: the characteristic tight packaging of the sperm DNA and the antioxidants present in the seminal plasma (86). Exposing the sperm to artificially produced ROS causes DNA damage in the form of modification of all bases, production of base-free sites, deletions, frame shifts, DNA cross-links, and chromosomal rearrangements (87). Oxidative stress is

also associated with high frequencies of single and double DNA strand breaks (86). This information has important clinical implications, particularly in the context of assisted reproductive techniques (ART). Spermatozoa selected for ART most likely originate from an environment experiencing OS, and a high percentage of these sperm may have damaged DNA (88,89). There is a substantial risk that spermatozoa carrying damaged DNA are being used clinically in this form of therapy (90). When intrauterine insemination (IUI) or *in vitro* fertilization (IVF) is used, such damage may not be a cause of concern because the collateral peroxidative damage to the sperm plasma membrane ensures that fertilization cannot occur with a DNA-damaged sperm. However, when intracytoplasmic sperm injection (ICSI) is used, this natural selection barrier is bypassed, and a spermatozoon with damaged DNA is directly injected into the oocyte (6,90).

Whether DNA-damaged spermatozoa used in ICSI can impair the process of fertilization and embryo development is not clear. On one hand, a recent study has indicated that spermatozoa with significantly damaged DNA still retain a residual capacity for fertilization. On the other hand, the percentage of sperm with DNA damage has been negatively correlated to the fertilization rate (91,92). In addition, a recent study has linked sperm DNA damage to increased rates of early embryo death (76). This is also supported by the results of Sanchez and colleagues who, in 1996, reported that miscarriage rates after ICSI were higher than that after conventional IVF.

Sperm preparation techniques involving repeated centrifugation may lead to high ROS production in sperm suspensions processed for ART (74). This may be important because exposing spermatozoa to high levels of ROS may increase the DNA fragmentation rate, which can have adverse consequences if they are used for ICSI (92). In 2000, Zini and colleagues reported that the improvement in sperm motility after Percoll processing is not associated with a similar improvement in sperm DNA integrity. The authors recommended that the current sperm preparation techniques be reexamined with the goal of minimizing sperm DNA damage. This can be accomplished by using more gentle sperm preparation methods such as the swim-up technique, which allows for good sperm recovery with minimal sperm dysfunction (93,94). In a recent study from our center, we found that recovery of sperm with intact nuclear DNA is significantly higher after the swim-up technique than the ISolate gradient technique (unpublished data).

Complications of Sperm Nuclear DNA Damage

Failure of Fertilization

When spermatozoa with DNA damage are selected for ICSI, the initiation or completion of de-condensation may be impeded, thereby preventing fertilization (76). Lopes *et al*. (92) have shown that men with a sperm population containing more than 25% of sperm with DNA damage are more likely to experience a fertilization rate of less

than 20% after ICSI. However, Twigg *et al*. (90) found that the genetically damaged spermatozoa can achieve normal fertilization following ICSI.

Embryo Death

Several studies have indicated that damage to sperm DNA may be linked to an increase in early embryo death (76).

Childhood Cancer

Sperm nuclear DNA damage may have consequences for the health of the offspring, who show a particularly high incidence of childhood cancer (95). In a study from China, paternal smoking was associated with a four-fold overall increased risk of developing a childhood cancer (96). Smoking may induce a state of OS that is associated with free radical-mediated damage to sperm DNA. Furthermore, an independent epidemiological study in the United Kingdom concluded that 14% of all childhood cancers could be attributed directly to paternal smoking (97).

Infertility

Another possible consequence of free radical-mediated DNA damage in the male germ line is infertility in the offspring (6). This possibility relates specifically to forms of male infertility involving deletions on the long arm (q) of the Y chromosome. In this nonrecombining area of the Y chromosome (NRY), three regions have been identified that contain genes of importance to spermatogenesis; these loci have been designated AZF (azoospermia factor) a, b, and c (98). Deletions in each of these areas produce a particular testicular phenotype. Deletions in AZFa produce Sertoli cell only syndrome. Deletions in AZFb are associated with germ cell arrest at the pachytene stage and deletions in AZFc generate arrest at the spermatid stage of development (99). These deletions are not observed in fertile men or in the majority of fathers of affected patients. Therefore, the Y chromosome deletions leading to male infertility must arise *de novo* in the germ line of the patient's fathers (6).

Y chromosome deletions are found in approximately 15% of patients with azoospermia or severe oligozoospermia and in 10% of men with idiopathic infertility (98). Although these are not particularly high frequencies, it should be recognized that more than 90% of the human genome is noncoding and would not produce a phenotypic change on deletion. Moreover, for most of the genome, homologous recombination could provide a theoretical mechanism for repairing double-stranded DNA deletions on autosomes or on the X chromosome. However, since the Y chromosome does not possess a homologue, this repair mechanism cannot be invoked, and deletions on the nonrecombining region of this chromosome will persist. Thus, for Y chromosomal deletions to occur at the frequency observed, there must be an extremely high spontaneous rate of DNA fragmentation in the male germ line, most of which is either undetected or is repaired. However, deletions on the AZF on the NRY cannot be repaired and produce an extremely obvious phenotype (6).

Contributions of the Cleveland Clinic

The role of OS in male infertility has been the main focus of our research in the Cleveland Clinic Foundation during the last decade. Our research team has identified the critical role OS plays in male infertility. The main objective of our research was to transfer this important knowledge from the research bench to clinical practice. This objective was stated in a review article by Sharma and Agarwal (13), which described specific plans and strategies for future research in the area of OS. We designed studies with the aims of (i) understanding the exact mechanisms by which OS develops in semen, (ii) establishing assays for accurate assessment of OS status and running the quality control studies for this purpose, and (iii) identifying the clinical significance of seminal OS assessment in male infertility practice.

Mechanism of Seminal OS

We investigated the cellular origins of ROS in semen to track the source of OS and, accordingly, to create strategies to overcome the problem.

Role of Seminal Leukocytes

Shekarriz *et al*. (43) reported that peroxidase-positive leukocytes are the main source of ROS in semen and found that positive peroxidase staining is an accurate indicator of excessive ROS generation in semen. Recently, Sharma *et al*. (100) observed that seminal leukocytes may cause OS even at concentrations below the WHO cutoff value for leukocytospermia (concentrations greater than 1×10^6 peroxidase positive leukocytes/mL semen). Levels of ROS production by pure sperm suspensions were found to be significantly higher in infertile men with leukocytospermia than in infertile men without leukocytospermia and were strongly correlated with seminal leukocyte concentration (101) (Table 13.1). This new finding led us to postulate that seminal leukocytes play a potential role in enhancing sperm capacity for excessive ROS production either by direct sperm-leukocyte contact or by soluble products released by the leukocytes. This observation has significant implications for the fertility potential of sperm both *in vivo* and *in vitro*. Excessive production of ROS by sperm in the patients with leukocytospermia implies that both the free-radical generating sperm themselves and any normal sperm in the immediate vicinity are susceptible to oxidative damage. Furthermore, once the process of LPO is initiated, the self-propagating nature of this process ensures a progressive spread of the damage throughout the sperm population.

Role of Abnormal Spermatozoa

In addition to ROS production by seminal leukocytes, the production of ROS by human sperm was also the subject of extensive research by our group. Our data indicate that human sperm production of ROS was significantly increased by the repeated cycles of centrifugation involved in the conventional semen processing techniques

TABLE 13.1
Median (25 and 75% Interquartile Value) ROS Levels in Original Cell Suspension (Basal) in Leukocyte-Free Sperm Suspension (Pure Sperm); and ROS-TAC Score in Normal Donors, Nonleukocytospermic Patients, and Leukocytospermic Patients[a]

Variable	Donors ($n = 13$)	Non-Leukocytospermic ($n = 32$)	Leukocytospermic ($n = 16$)	A	B	C
Basal ROS ($\times 10^6$ cpm)	0.4 (0.1, 2.5)	2.7 (0.53, 12)	178 (32, 430)	0.06	0.0001	<0.0001
Pure sperm ROS ($\times 10^6$ cpm)	0.06 (0.01, 0.2)	0.31 (0.09, 1.2)	3.3 (0.5, 7.4)	0.05	0.001	0.002
ROS-TAC Score	54.5 (52, 60)	50.3 (42, 54.8)	27.8 (23.7, 35)	0.01	0.0003	0.0001

[a]A = *P* value of donors *versus* nonleukocytospermic; B = *P* value of donors *versus* leukocytospermic; and C = *P* value of nonleukocytospermic *versus* leukocytospermic. Wilcoxon rank-sum test was used for comparison and statistical significance was assessed at $P < 0.05$ level. ROS = reactive oxygen species; TAC = total antioxidant capacity.

(washing and resuspension) for ART (102). In addition, we have demonstrated that the duration of centrifugation is more important than the force of centrifugation for inducing ROS formation in semen (43). Based on these findings, we recommended the use of more gentle techniques for sperm preparation with shorter centrifugation periods to minimize the risk of OS-induced injury to the sperm.

Our group has also reported that ROS production by human sperm rises as sperm concentration increases and decreases with time (103). In addition, we emphasized the importance of adjusting sperm concentration for ROS measurements when comparing ROS levels between different specimens (104). Results from our most recent studies indicate that ROS production varies significantly in subsets of human spermatozoa at different stages of maturation (28,105). Following ISolate gradient fractionation of ejaculated sperm, ROS production was found to be highest in immature sperm with abnormal head morphology and cytoplasmic retention and lowest in mature sperm and immature germ cells. The relative proportion of ROS-producing immature sperm was directly correlated with nuclear DNA damage values in mature sperm and inversely correlated with the recovery of motile, mature sperm. These interesting findings led to the hypothesis that oxidative damage of mature sperm by ROS-producing immature sperm during their co-migration from seminiferous tubules to the epididymis may be an important cause of male infertility. This suggests that perhaps interventions directed to (i) increase antioxidant levels in immature germ cell membranes during spermatogenesis and (ii) isolate spermatozoa with intact DNA by *in vitro* separation techniques should be of particular benefit to these patients in which a defect in the normal regulation of spermiogenesis and spermiation leads to an abnormal increase in the production of ROS-producing immature sperm.

Assessment of OS Status

Extensive research in the field of male infertility has been conducted to develop adequate indices of OS that would help determine, with accuracy, if OS is a significant contributor in male infertility (106). Levels of OS vary greatly in infertile men (51). Because OS is an imbalance between levels of ROS production and antioxidant protection in semen, it is conceivable that any assessment of OS will rely on the measurement of ROS as well as total antioxidant capacity (TAC) of semen. Recently, a statistical formula described as the ROS-TAC score has been introduced for assessment of OS using principal component analysis (106).

Measurement of ROS

Levels of seminal ROS can be measured by a chemiluminescence assay (107) (Fig. 13.3). Liquefied semen is centrifuged at 300 × *g* for 7 min, and the seminal plasma is separated and stored at –80°C for measurement of TAC. The pellet is washed with phosphate buffered saline (PBS) and resuspended in the same media at a concentration of 20×10^6 sperm/mL. Levels of ROS are measured by a chemiluminescence assay using luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as a probe. Four 100-µL aliquots of the resulting cell suspensions (containing sperm and leukocytes) are used for assessment of basal ROS levels. Eight microliters of horseradish peroxidase (HRP) (12.4 U of HRP Type VI, 310 U/mg; Sigma Chemical Co.) are added to sensitize the assay so that it can measure extracellular hydrogen peroxide. Ten microliters of luminol, prepared as 5-mM stock in dimethyl sulfoxide (DMSO), are added to the mixture. A negative control is prepared by adding 10 µL of 5-mM luminol to 400 µL of PBS. Levels of ROS are assessed by measuring the luminol-dependant chemiluminescence with a luminometer (model:

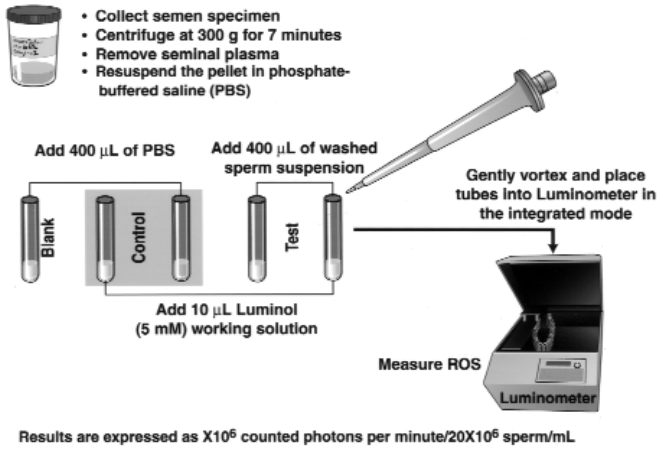

Fig. 13.3. Measurement of reactive oxygen species (ROS) in washed semen by chemiluminescence assay.

LKB 953; Wallac Inc., Gaithersburg, MD) in the integrated mode for 15 min. The results are expressed as $\times 10^4$ counted photons per minute (cpm) per 20×10^6 sperm. Normal ROS levels in washed semen range from 10 to 100×10^4 counted photons per minute (cpm) per 20×10^6 sperm.

Measurement of TAC

Total antioxidant capacity in the seminal plasma can be measured with an enhanced chemiluminescent assay (108). Frozen samples of seminal plasma are thawed at room temperature and immediately assessed for TAC. Seminal plasma is diluted 1:20 with deionized water (dH2O) and filtered through a 0.20-μ filter (Allegiance Healthcare Corporation, McGaw Park, IL). Signal reagent is prepared by adding 30 μL H_2O_2 (8.8 molar/L), 10 μL 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 to give a chemiluminescence output of 3×10^7 cpm. Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, is prepared as a standard solution (25, 50, and 75 μM) for TAC calibration. With the luminometer in the kinetic mode, 100 μL of signal reagent and 100 μL of HRP working solution are added to 700 μL of dH_2O and mixed. The mixture is equilibrated to the desired level of chemiluminescent output (between 2.8 and 3.2×10^7 cpm) for 100 s. One hundred microliters of the prepared seminal plasma is immediately added to the mixture, and the chemiluminescence is measured. Suppression of luminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence are recorded. The same steps are repeated after the Trolox solutions are replaced with 100-μL aliquots of the prepared seminal plasma. The assay is conducted in a dark room because light affects the chemiluminescence. Plotting the three concentrations of Trolox solution versus 10% recovery time results in a linear equation (Fig. 13.4).

TAC Calculation

Seminal TAC levels are calculated using the following equation:

$$y = (Mx \pm C) \times d$$

In this equation, M refers to the slope increase in the value of Trolox equivalent for a one-second increase of the recovery time, whereas C accounts for the daily background variability. The results are multiplied by the dilution (d) factor and expressed as molar Trolox equivalents (106).

ROS-TAC Score: A New Development in the Field of OS

The fact that neither ROS alone nor TAC alone can adequately quantify seminal oxidative stress led us to the logical conclusion that combining these two variables may be a better index for diagnosis of the overall OS affecting spermatozoa. This con-

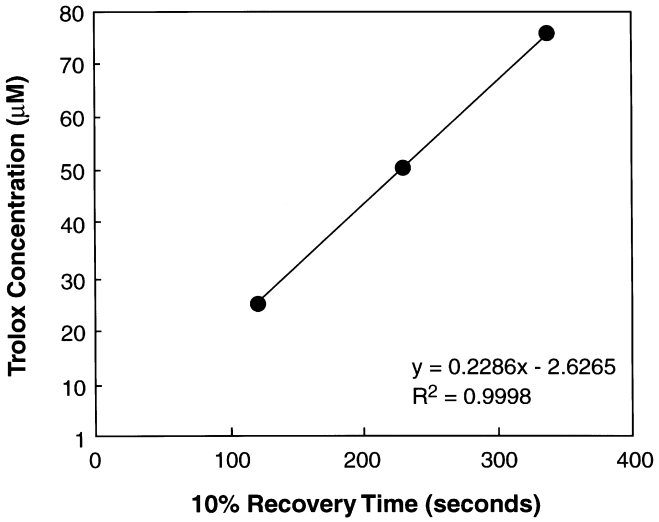

Fig. 13.4. Linear relationship between the concentrations of standard Trolox solution (μM) and 10% recovery time (sec.).

clusion was behind our landmark paper in which we introduced the ROS-TAC score as a new method for accurate assessment of OS status in infertile men (106). The new ROS-TAC score is derived from levels of ROS in washed semen and TAC in seminal plasma (106). The resulting score minimizes the variability present in the individual parameters of OS (ROS alone or TAC alone). In our study, the ROS-TAC score was calculated from a group of normal healthy fertile men who had very low levels of ROS. The composite ROS-TAC score calculated for these men was representative of the fertile population, and any scores significantly below levels in the fertile population were indicative of infertility. We found that individuals with ROS-TAC scores below 30, the lower limits of normal range, are at particular risk for prolonged inability to initiate pregnancies.

Quality Control of OS Indices (ROS and TAC)

It was of utmost importance to standardize the measures that we used as indices for OS, including measurement of ROS in washed semen and TAC in seminal plasma. We have demonstrated that the luminol-dependent chemiluminescence assay for ROS measurement in washed semen is both accurate and reliable when the sperm concentration is greater than 1×10^6/mL and the samples are analyzed within one hour after collection (108). Our results have also indicated that the enhanced chemiluminescence assay is both accurate and reliable for assessment of TAC in seminal plasma (109).

Clinical Significance of Assessment of Seminal OS

It was also of special interest to us to determine levels of seminal OS in different clinical settings (Table 13.2). We found a significant increase in levels of ROS in men with spinal cord injury, which was also associated with poor sperm motility and morphology (5). We also found elevated levels of ROS in infertile men with varicoceles (27). In a recent study, we demonstrated that varicocelectomy resulted in a significant increase in pregnancy and live birth rates for couples who underwent IUI, although standard semen parameters were not improved in all patients (110). We hypothesized that the improvement in pregnancy rates following varicocelectomy may be due to a functional factor not tested during standard semen analysis such as seminal OS or sperm DNA damage. Currently, studies are underway in our center to investigate this hypothesis. Patients with varicocele also had low levels of TAC in their seminal plasma. We speculated that these patients might benefit from antioxidant supplementation (27). A study on rats has indicated that free radical scavengers such as SOD can prevent free radical-mediated testicular damage (111).

Across all clinical diagnoses, the ROS-TAC score was a superior discriminator between fertile and infertile men to either ROS or TAC alone (106). Furthermore, analyses of male patients with a diagnosis of male-factor infertility indicated that those with partners who had subsequent successful pregnancies had an average ROS-TAC score in the normal range compared with significantly lower ROC-TAC scores in those men with partners who did not become pregnant. In addition, we found that the average ROS-TAC score for the fertile vasectomy reversal group was nearly identical to that of the controls (107). Infertile men with male-factor or idiopathic diagnoses had significantly lower ROS-TAC scores than the controls, and men with male-factor diagnoses that eventually were able to initiate a successful pregnancy had significantly higher ROS-TAC scores than those who failed (112). Also, infertile men with chronic prostatitis or prostatodynia have been shown to have lower ROS-TAC score than controls, and this was irrespective of their leukocytospermia status (113). Finally, male partners of couples who achieved pregnancy did not have significantly different ROS-TAC scores than controls. Therefore, the new ROS-TAC score may serve as an important measure in identifying those patients with a clinical diagnosis of male infertility who are likely to initiate a pregnancy over a period of time (45).

ROS in Neat (Raw) Semen: An Accurate and Reliable Test for OS

More recently, our group has introduced an additional test of OS in which ROS levels are measured directly in neat (raw) semen (114). The maximum ROS level observed in neat semen from normal healthy donors with a normal genital examination and normal standard semen parameters was 1.5×10^4 cpm/20 million sperm/mL. The test was subjected to all quality control studies and proved to be an accurate measure for seminal OS status. Using a cutoff value of 1.5×10^4 cpm/20 million sperm/mL, infertile men were reliably classified as either OS-positive ($>1.5 \times 10^4$ cpm/20 million

TABLE 13.2
Mean and Standard Deviation (SD) between ROS, TAC, and ROS-TAC Score in Subgroups of Infertility Patients and Controls[a]

Diagnosis	ROS Log (ROS + 1)	P-value *vs.* controls[b]	TAC (Trolox Equivalent)	P-value *vs.* controls[b]	ROS-TAC score	P-value *vs.* controls[b]
Control ($n = 24$)	1.39 (SD 0.73)		1650.9 (SD 532.2)		50.00 (SD 10.00)	
Varicocele ($n = 55$)	2.10 (SD 1.21)	0.02	1100.1 (SD 410.3)	0.0002	34.87 (SD 13.54)	0.0001
Varicocele with prostatitis ($n = 8$)	3.25 (SD 0.89)	0.0002	1061.4 (SD 425.1)	0.03	22.39 (SD 13.48)	0.0001
Vasectomy reversal (infertile; $n = 23$)	2.65 (SD 1.01)	0.0004	1389.9 (SD 723.9)	0.30	33.22 (SD 15.24)	0.0002
Vasectomy reversal (fertile; $n = 12$)	1.76 (SD 0.86)	0.80	1876.9 (SD 750.8)	0.62	49.35 (SD 12.25)	1.00
Idiopathic infertility ($n = 28$)	2.29 (SD 1.20)	0.01	1052.0 (SD 380.9)	0.0003	32.25 (SD 14.40)	0.0001

[a]TAC = total antioxidant capacity; ROS = reactive oxygen species.
[b]Pairwise P-values from Student's t-test adjusted using Dunnett's method.

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.

We also found that assessing ROS directly in neat semen has diagnostic and prognostic capabilities identical to those obtained from the ROS-TAC score (Table 13.3). Levels of ROS in neat semen were strongly correlated with levels of ROS in washed semen and with ROS-TAC score (Fig. 13.5). A strong positive correlation was seen between ROS levels in neat semen and the extent of sperm chromatin damage (115). However, the difference in the extent of sperm DNA damage between OS-negative and OS-positive patients was not statistically significant, an indication that OS is associated with DNA damage or contributes to it in some way (or both) in some but not all infertility patients.

Strategies to Reduce Seminal OS

Long-term strategies must determine the cause of the enhanced generation of ROS by spermatozoa of infertile men. Reduced levels of OS will be beneficial in ART such as IUI and IVF. An insight into the molecular basis of these defects is vital in order to identify the underlying cause of the etiology of sperm pathologies. Such an understanding will help researchers develop appropriate therapeutic strategies in the treatment for male infertility. Determining the level and origin of ROS production in the ejaculate and precisely evaluating the scavenger system may be useful in treating patients. If the error in spermatogenesis that leads to such atypical activity (excessive ROS production) could be defined, it would provide an important lead in determining the etiology of male infertility, and a sensible basis for the design of effective therapies could be prepared.

The seminal leukocyte population should also be considered potentially detrimental and must be carefully monitored. It is important to minimize the interaction

TABLE 13.3
Median (25^{th} and 75^{th} Percentile) Values of Reactive Oxygen Species (ROS) in Neat Semen, ROS in Washed Semen, Total Antioxidant Capacity (TAC) in Seminal Plasma, and ROS-TAC Score in Donors and Oxidative Stress (OS)-Negative and OS-Positive Patients

Variable	Donors ($n = 9$)	OS-negative ($n = 11$)	OS-positive ($n = 23$)	A[a]	B[a]	C[a]
ROS-neat semen ($\times 10^4$ cpm)	0.3 (0.2, 0.9)	0.3 (0.2, 1)	19 (6, 143)	0.9	0.0001	0.0001
ROS-washed semen ($\times 10^4$ cpm)	10 (4, 17)	79 (13, 177)	1468 (514, 11496)	0.03	0.0003	0.0008
TAC (Trolox Equivalents)	908 (736, 1129)	797 (575, 966)	739 (627, 1047)	0.31	0.24	0.94
ROS-TAC score	53 (51, 55)	49 (47, 52)	36 (32, 44)	0.4	0.001	0.001

[a]A, donors *vs.* OS-negative patients; B, donors *vs.* OS-positive patients; C, OS-negative patients *vs.* OS-positive patients. Results were analyzed by Wilcoxon rank-sum test, $P < 0.05$ was significant.

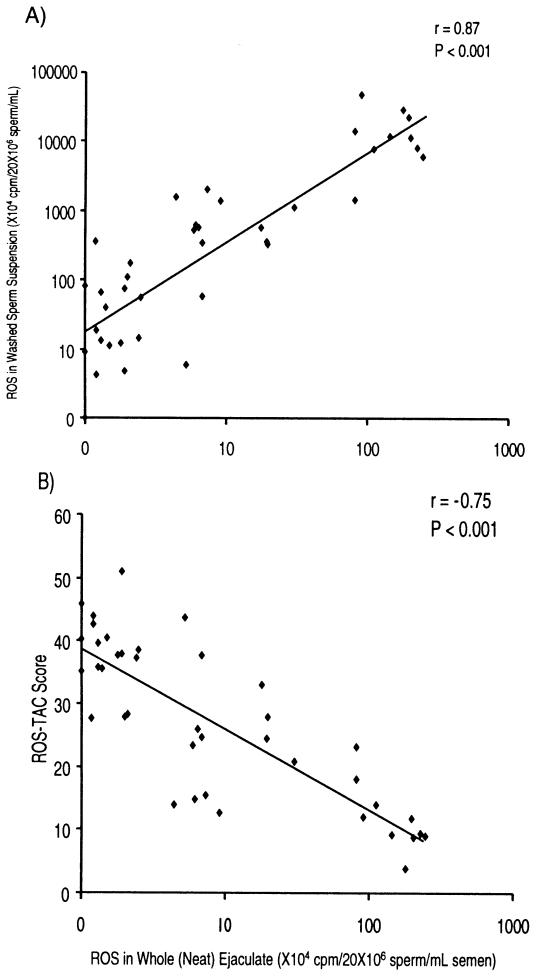

Fig. 13.5. Correlation of reactive oxygen species (ROS) levels in neat semen with (A) levels of reactive oxygen species in washed semen (r = 0.87, $P < 0.001$) and (B) reactive oxygen species – total antioxidant capacity (TAC) score (r = –0.75, $P < 0.001$).

between ROS-producing cells in semen, *e.g.*, PMN leukocytes, and spermatozoa that have a potential to fertilize. Differentiating between spermatozoa and leukocyte sources of ROS is important clinically because this will affect the strategies used to reduce OS on spermatozoa during the course of IVF therapy. It is important for the clinician also to know that sperm preparation techniques used for ART may induce damage to the spermatozoa by removing the seminal plasma with its powerful antioxidants and by inducing ROS generation by spermatozoa.

Future Directions

Future efforts should focus on elucidating why spermatozoa from some patients become over-reactive in the generation of ROS. It is also important to determine the period of sperm differentiation at which this self-destructive activity first appears. Further efforts are also required to identify sperm population at risk of collateral peroxidative damage to the sperm membrane. An interesting area of future research is to investigate the oxidative damage to sperm DNA and its implication on male fertility potential and the outcome of assisted reproductive programs.

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