ROLE OF REACTIVE OXYGEN SPECIES IN MALE INFERTILITY

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A free radical may be defined as any molecule that has one or more unpaired electrons. Highly reactive oxygen radicals such as the superoxide anion ($\cdot O_2^-$), the hydroxyl radical ($\cdot OH$), and the hypochlorite radical ($\cdot OHCl$) comprise what is called the reactive oxygen species (ROS). Oxygen toxicity is an inherent challenge to aerobic life. Although the controlled generation of ROS may have physiologic functions as signaling molecules (second messengers) in many different cell types, their uncontrolled production is considered an important factor in aging, diet, health, and in the etiology of pathologic conditions such as myocardial infarction, cataract, or rheumatoid arthritis.[1] There is a wealth of literature on the damaging effects of ROS on all aerobic cellular systems.[1,2] Recent data indicate that many nonphagocyte cells produce ROS.[3-7] Owing to their high reactivity, ROS produce extensive protein damage and cytoskeletal modifications and inhibit cellular mechanisms.[8-10] To meet this challenge, aerobic organisms are equipped with a powerful battery of mechanisms that protect them from the adverse effects of lipid peroxidation (LPO) and other manifestations of oxygen toxicity.

Defective sperm function frequently causes male infertility, accounting for at least 24% of couples attending infertility clinics.[11,12] This condition covers a wide array of malfunctions that include abnormal flagella movement,[13,14] failure to recognize the zona, and inhibition of sperm–oocyte fusion.[15-17] Human spermatozoa represent a growing list of cell types that exhibit a capacity to generate ROS when incubated under aerobic conditions, such as hydrogen peroxide (H_2O_2), the superoxide anion ($\cdot O_2^-$), the hydroxyl radical ($\cdot OH$), and the hypochlorite radical ($\cdot OHCl$).[18] Superoxide anions ($\cdot O_2^-$) are produced as a primary product that subsequently dismutases to hydrogen peroxide (H_2O_2) under the influence of intracellular superoxide dismutase (SOD).[19-21] In addition to the damage caused by ROS, the nitrogen-derived free radical nitric oxide ($NO\cdot$) also appears as an important mediator of normal sperm function.[22,23] The production of ROS by sperm is a normal physiologic process, but an imbalance between ROS generation and scavenging activity is detrimental to the sperm and is associated with male infertility. Excessive generation of ROS, called positive oxidative stress (OS), is defined as a situation in which there is a shift in the ROS balance toward the pro-oxidants, because of either excess ROS or diminished antioxidants. A shift of pro-oxidants in the semen and vaginal secretions can induce OS on the spermatozoa; concomitantly, a decrease in the antioxidant activities correlates with idiopathic infertility. It appears that the imbalance between ROS production and degradation is likely to be secondary to increased ROS production and not the result of decreased scavenging capacity. Excessive production of ROS may play a role in the etiology of male infertility. A prospective study[24] demonstrated that men with high levels of ROS generation had seven times less chance of effecting a pregnancy in a partner compared to men with low ROS.

It is important to understand the source(s) of ROS production and the impact of excessive production of ROS on the function of the spermatozoa, especially those steps critical for fertilization. This article reviews the impact of ROS on spermatozoal function and male infertility as well as the balance of risks and benefits that characterizes the cellular production of ROS. We discuss the interest generated by these molecules in the medical community, the differences in the antioxidant capacity of the seminal plasma in fertile and infertile men, the significance of antioxidants (ROS scavengers) in reproduction, and new diagnostic and therapeutic strategies in the treatment of the infertile man.

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BENEFICIAL EFFECTS OF REACTIVE OXYGEN SPECIES

Reactive oxygen species are important mediators of normal sperm function, such as signal transduction mechanisms that affect fertility.[25-28] ROS can have beneficial effects on sperm functions depending on the nature and the concentration of the particular ROS involved. These are necessary in regulating the rate of hyperactivation and the ability of the spermatozoa to undergo acrosome reaction.[27,29-31] A sustained and increased amount of $\cdot O_2^-$ is one of the first steps required by the spermatozoa for induction and development of hyperactivation and capacitation.[32] ROS facilitates the acrosome reaction through a stimulatory effect on the phospholipase A2 (PLA_2) activity that is present in the human spermatozoa and that is stimulated both by the calcium and by the formation of lipid peroxides within the plasma membrane.[28,33] This increases membrane fusogenecity by generating conditions that favor enhanced PLA_2 activity. ROS may also help activate tyrosine phosphorylation and may have a physiologic role in mediating the attachment of spermatozoa to oocytes.[34,35]

REACTIVE OXYGEN SPECIES IN FERTILE AND INFERTILE MEN

Under normal physiologic conditions, seminal plasma and normal motile spermatozoa do not produce ROS. It is not detectable in the semen of normal volunteers or azoospermic men.[36] Morphologically abnormal spermatozoa can produce ROS.[36-39] In the infertile man, two ROS generating systems are possibly involved, a hypothetical NADPH-oxidase at the level of sperm membrane[18,19] and the sperm-diphorase (mitochondrial NADH-dependent oxidoreductase).[40] It is conceivable that defects in spermiogenesis in these men, involving the retention of excess residual cytoplasm, might create a situation in which sufficient substrate NADPH would be available to generate the $\cdot O_2^-$. Under the influence of SOD, it could dismutase to H_2O_2.[19-21] The small portion of ROS released outside the spermatozoa suggests that the mitochondrial system is the major source of ROS in spermatozoa from infertile men.[39]

High levels of ROS seen in some infertile patients could be a cause of idiopathic infertility[19] (Table 1). Iwasaki and Gagnon[36] detected ROS levels in 40% of semen samples from infertile patients, whereas no ROS was detected in control and azoospermic subjects. This suggests that certain causes of infertility may be associated with elevated incidence of ROS formation.[44] Recent work[45] has demonstrated that the total antioxidant capacity of the seminal plasma differs in the fertile and infertile man. Seminal plasma from infertile men has lower antioxidant levels than that of fertile men; this is especially true of infertile men with poor sperm motility. Presence of ROS activity in infertile men is also associated with lower levels of chain-breaking antioxidants, especially in the seminal plasma from asthenozoospermic men.[45,46]

REACTIVE OXYGEN SPECIES IN OLIGOZOOSPERMIC PATIENTS

Oligozoospermia (a sperm count of less than 20×10^6)[57] is found in 16% to 41% of infertile couples.[58] The primary difference between oligozoospermic specimens and normal fertile controls is in the relative contribution of the spermatozoa to the ROS generating capacity of the ejaculate. In oligozoospermic patients, the spermatozoa are the predominant source of ROS and generate extremely high levels of ROS compared to those produced by spermatozoa from normal fertile men. Defects in spermatozoa from oligozoospermic patients involve lesions downstream from the calcium influx site where calcium enters to initiate the acrosome reaction.[19] ROS may cause a defect in sperm function through the peroxidation of the unsaturated fatty acids in the plasma membrane.[20,21,44,59,60] Although the normal fertile population responds to these calcium signals,[61] an inverse relationship between the capacity of human spermatozoa to generate ROS and the ability to cause sperm–oocyte fusion in response to calcium ionophore A23187 has been shown.[19,44] About 60% of oligozoospermic samples exhibit an abnormal rate of oocyte penetration (less than 10%), and in 40% of such cases, high levels of ROS have been observed.[44] Possibly this association is causative—the excessive generation of ROS may overwhelm the defense mechanisms of the cell and lead to a loss of sperm function.[24] In these sperm cells, the mechanisms normally controlling the SOD anion generating system that involves the peroxidation of unsaturated fatty acids in the sperm plasma membrane would be ineffective, and as a consequence, the ROS production would rise. Given the high frequency of plasma membrane disruption in the oligozoospermic population, establishing which of these alternative possibilities is correct is clearly a priority, with important therapeutic implications.

ROS IN PATIENTS WITH VARICOCELE AND SPINAL CORD INJURY

ROS has been implicated in reduced fertility in patients with varicocele.[19,41,47,48,62] ROS production in these patients in response to chemoattractants such as phorbol ester (phorbol-12-myristate-13-acetate; PMA), formyl peptide (*N*-formyl-meth-

TABLE I. *Influence of ROS on semen parameters and the etiology of male infertility*

Authors	Subjects	ROS	Semen Parameters	Fertilization
Aitken & Clarkson[19,20] Aitken *et al.*[34] Rao *et al.*[38]	Normal	NA	Normal	+
Mazzilli *et al.*[41]	Infertile	+	Abnormal	NA
Rajasekaran *et al.*[42]	Infertile	+	NA	NA
McKinney *et al.*[43]	Infertile	+	Abnormal	NA
Aitken & Clarkson[19,20] Aitken *et al.*[24] Aitken *et al.*[34,44]	Infertile	+	NA	Reduced oocyte fusion, reduced spontaneous pregnancy
Iwasaki & Gagnon[36] Lewis *et al.*[45] Baker *et al.*[46]	Infertile	+	Low volume, motility, antioxidant levels	NA
Aitken *et al.*[34] Iwasaki & Gagnon[36] Gavella and Lipovac[40] Aitken *et al.*[44] Lenzi *et al.*[47,48] Agarwal *et al.*[49] D'Agata *et al.*[50] Aitken & Clarkson[51]	Infertile	+	Abnormal concentration, motility, morphology, functionally abnormal	NA
Zalata *et al.*[52]	Immunologically infertile	+	NA	NA
Aitken & Clarkson[19] Aitken *et al.*[24] Aitken *et al.*[34] Plante *et al.*[39] Aitken *et al.*[44] Zalata *et al.*[52] Irvine & Aitken[53] Thiele *et al.*[54]	Oligozoospermic	+	Functional defects	Reduced oocyte penetration
Iwasaki & Gagnon[36]	Azoospermic	NA	Normal	NA
Lenzi *et al.*[48] Weese *et al.*[55]	Varicocele	+	Differential chemoattractant response	Reduced fertility
de Lamirande *et al.*[56]	Spinal cord injury	+	Reduced motility, excessive granulocytes	Reduced fertility

KEY: NA = not available.

ionyl-leucyl-phenylalanine, FMLP) complement C5a, nerve growth factor, and A23187 differs from those without varicocele. Fertile men with varicocele demonstrate significantly enhanced chemoattractant-stimulated ROS levels compared to fertile men without varicocele. Similarly, significant differences have been seen in fertile and infertile groups (fertile, no varicocele; fertile varicocele; infertile prevaricocelectomy, and infertile postvaricocelectomy) in their response to stimulated ROS generation, especially toward A23187, FMLP, and complement C5a.[55] ROS stimulated by chemoattractant (complement C5a and FMLP) was elevated in fertile and infertile patients with varicocele, compared to normal fertile patients. However, in infertile men who underwent varicocelectomy, ROS levels after stimulation with C5a and FMLP were comparable to those of the fertile group without varicocele. If ROS production is an active cause of infertility in these men, the fact that many of these men are fertile suggests that the degree of damage inflicted by ROS is not severe enough to result in infertility.[55]

Even though anejaculation can be overcome in 80% to 90% of the patients with spinal cord injury (SCI), more than 90% of these men remain infertile.[63] In our study (unpublished), semen samples from SCI patients showed higher levels of ROS than samples from infertile men. ROS levels in SCI patients have been inversely related to sperm motility and positively related to the presence of polymorphonuclear neutrophils (PMN), the predominant cell type encountered in seminal smears. This could partially explain the low fertility potential in these men even when the problem of anejaculation can be overcome.[56] ROS levels were not influenced by the method of ejaculation (electroejaculation versus vibrator stimulation) or the type of specimen (anterograde versus retrograde).

EFFECTS OF REACTIVE OXYGEN SPECIES ON SPERMATOZOA CHARACTERISTICS

Of the patients attending an infertility clinic, 40% have detectable levels of ROS formation in their semen, and 25% have levels higher than the normal limits.[19,21,36,64] The relation between ROS and sperm motion parameters is controversial. Excessive ROS formation is positively correlated with abnormal sperm concentration, motility, and morphology.[34,36,38,44,47,49-51] ROS formation decreases when motility is greater than 60%, suggesting that in infertile men, a sperm suspension with a high concentration of immotile spermatozoa has a greater probability of producing ROS than a highly motile sperm suspension.[36]

To study the mode of action of ROS on spermatozoa, we can generate ROS artificially under defined conditions, using the xanthine (X) and xanthine oxidase (XO) system.[65,66] This system generates the $\cdot O_2^-$, which dismutases to H_2O_2 without decreasing sperm viability. After ROS addition, the percentage of sperm motility decreases abruptly. In contrast, the flagellar beat frequency drops progressively due to a rapid loss of intracellular adenosine triphosphate (ATP).[67-72] The observed effects on sperm motility (of intact and demembranated-reactivated cells) are probably due to a cascade of events that results in a decrease in axonemal proteins and sperm immobilization.[32] The loss of sperm motility and the capacity for sperm-oocyte fusion have been associated with reduction in membrane fluidity, increase in lipid peroxidation (LPO), and loss of membrane-bound enzyme ATP. The fact that ROS production in semen is inversely correlated with motility[65] underscores the need to devote further study to ROS action on spermatozoa repair mechanisms. Various ROS scavengers, such as catalase, SOD, and dimethyl sulfoxide (DMSO), have been used in an X + XO system to study the damage to sperm motility. Of the scavengers, only catalase conferred full protection to the spermatozoa incubated in the X + XO system, SOD conferred partial protection, and DMSO provided no protection, indicating that H_2O_2 is responsible for most of the damage to spermatozoa.[69] Another hypothesis is that H_2O_2 diffuses across the membranes into the cells and inhibits the activities of some enzymes such as glucose 6 phosphate dehydrogenase (G6PDH), leading to a decrease in the production of NADPH and a concomitant accumulation of GSSG (reduced form of glutathione), GSH (oxidized form of glutathione), and SOD. This causes a decrease in the antioxidant defenses of the spermatozoa which ultimately leads to the peroxidation of the membrane phospholipids by ROS.[73] It changes the membrane fluidity and integrity induced by the accumulation of lipid peroxides and disrupts the ionophore-induced acrosome reaction and sperm motility. Reversible immobilization of the spermatozoa can be explained by the inhibition of energy metabolism by H_2O_2 produced by the X + XO system which is responsible for the inhibition of the antioxidant defense system of the spermatozoa. The production of ROS by this peroxidase-oxidant stops rapidly, however, which suggests that the ROS may be produced by the spermatozoa themselves.[73]

ROS has been hypothesized to play a causative role in the etiology of defective sperm function through the peroxidation of the unsaturated fatty acids in the human sperm plasma membrane.[19,21,59,60] This peroxidation has been suggested as the cause of abnormal acrosome reaction and penetration of the oocyte. Frequently, in patients with defective sperm function, the spermatozoa develop a refractory response to calcium signals such that sperm-oocyte fusion will not occur in the presence of ionophore A23187.[19,61] This refractoriness results from changes in plasma membrane properties that are induced by lipid peroxidation.[20] The mechanism responsible for the impaired sperm-oocyte fusion may involve a reduction in fluidity of the plasma membrane[74] as well as changes in the activities of the key membrane-bound enzymes and ion-channels.[75] To determine whether enhanced ROS generation in cases of defective sperm function is a cause or consequence of lipid peroxidation, studies have been undertaken using A23187 with spermatozoa exposed to ferrous ion promoters and subsequent measurement of malonaldehyde levels.[34] This research indicates that although limited peroxidation of human spermatozoa enhances their capacity to generate ROS—presumably by interfering with cellular mechanisms that normally regulate this activity—such damage is not the primary cause but instead probably a consequence of enhanced ROS production.[34]

LEUKOCYTOSPERMIA AND REACTIVE OXYGEN SPECIES

Acute infections of the genital tract can affect male fertility, but there is a controversial relationship between infection and infertility.[76,77] Some investigators have reported a connection between the presence of bacteria in the semen and reduced fertility potential.[78-81] Reports of decreased numbers of seminal leukocytes and of marked improvement in semen quality[80] and pregnancy rate[81] after antibiotic therapy support the view that microorganisms are one cause of leukocytospermia. However, in general, leukocytospermia and semen microbiology are only weakly correlated,

and there have been no reports of benefit from antibiotic treatment in patients with asymptomatic idiopathic leukocytospermia.

The clinical significance of leukocytic infiltration in human ejaculate is controversial.[82-84] Numerous epidemiologic,[85-88] clinical,[89,90] and experimental[91-93] studies report leukocyte-induced damage to sperm function. Whereas leukocytospermia has been linked with poor semen quality and impaired zona-free hamster oocyte penetration,[24,76,89,94,95] in other cases no correlation was found between seminal leukocyte concentrations and semen analysis.[96-99] However, there is substantial evidence that PMN are a major source of ROS recorded in human sperm suspensions. Luminol-dependent chemiluminescense signals can be detected in unprocessed human semen samples, suggesting that PMN are not only present but activated.[100] Both leukocytes and immature germ cells are unfavorable prognostic factors for fertilization and in vitro fertilization-embryo transfer (IVF-ET) failure.[94,101] Quantifying white blood cells (WBC) is an integral part of semen analysis because they can be indicators and mediators of genital tract infection. The World Health Organization defines leukocytospermia as the presence of greater than 1×10^6 WBC/mL semen.[57] The threshold for clinically relevant leukocytospermia may sometimes be higher than 10^6 WBC/mL.[102] Knowing which WBC populations are present in semen is essential to understand the mechanism of sperm damage by WBC. In general, granulocytes represent 50% to 60% of all WBC in semen. Greater numbers of seminal granulocytes, monocytes or macrophages, and T-lymphocytes have been found in infertile patients than in fertile controls.[85] In terms of numerical dominance, granulocytes are the WBC type most likely to cause sperm damage and are contributed largely by the prostate and the seminal vesicles.[97]

To determine the influence of leukocytes on fertility, we must establish whether the spontaneously infiltrating leukocytes in human ejaculate are capable of generating ROS in such quantities that they can overwhelm the powerful antioxidant factors of human seminal plasma.[37,103] Potent chemoattractants such as FMLP and PMA[104,105] are capable of stimulating the leukocyte system to varying degrees.[106-110] Each of these chemoattractants results in ROS production via discrete pathways, and there are similarities between ROS generating systems of leukocytes and spermatozoa.[55] FMLP can be used as a means of selectively triggering the generation of ROS by leukocytes to monitor the fertilizing capacity of human spermatozoa.[111] In leukocyte-free sperm suspensions, the capacity of the sperm population to generate ROS can be monitored by adding PMA, the most powerful stimulus for oxidant generation by human spermatozoa yet identified.[112] The use of the chemiluminescent procedure to detect the presence of leukocytes in ROS gives excellent correlates of FMLP-induced chemiluminescence with leukocyte numbers.[62] In addition to being quick and convenient, the FMLP test gives a functional assessment of the level of leukocyte contamination and provides information on the capacity of leukocytes to generate ROS.

It is the cellular composition of the ejaculate that is mainly responsible for determining the intensity of ROS generation.[64] Powerful antioxidant systems in seminal plasma are unable to exert the complete removal of ROS, indicating the importance of contaminating granulocytes as a major source of ROS.[36,100] Plante *et al.*[40] found that although the extracellular release of ROS from defective spermatozoa was inadequate to compromise the motility of normal spermatozoa in the immediate vicinity, activated PMN at concentrations greater than 1×10^6/mL had a marked effect on the sperm movement characteristics of washed sperm preparations. This association is causative, because the selective removal of the contaminating leukocytes with anti-CD45-coated magnetic beads led to a significant improvement in sperm function.[100] The impact of contaminating leukocytes on sperm function clearly depends on the number of cells involved, their state of activation, the level of free radical generation, and the point at which they are added to the sperm suspension. Damage to spermatozoa by ROS may be moderated if leukocyte infiltration is confined to the prostate or to the seminal vesicles. Under such circumstances, the spermatozoa would first contact leukocyte-dependent ROS at the moment of ejaculation when they are protected by seminal antioxidants. On the other hand, if the leukocytes originate in the epididymis, ROS could interact with the sperm membrane for a longer period, thus increasing the risk of serious damage. ROS might be the only pathogenic factor acting on otherwise normal spermatozoa, if produced by infiltrating leukocytes.

Polymorphonuclear neutrophils generate ROS in response to a variety of chemical and bacterial stimuli,[113,114] and overwhelm spermatozoa's ability to repair or compensate for damage.[115] Thus, one can hypothesize that in vivo, a relatively short exposure to stimulated PMN could affect sperm function even hours after exposure when PMN are absent or no longer functional. Catalase and DMSO have little or no effect on the measured ROS output from activated PMN as compared with that of SOD, but they preserve sperm motility effectively. This finding as well as the fact that the combination of catalase and DMSO can completely

protect spermatozoa from the effects of PMN strongly suggests that H_2O_2 and the hydroxyl radical are involved in impaired motility. Another study[46] observed the antioxidant effects of thiols such as reduced glutathione, *N*-acetylcysteine, and hypotaurine in protecting human spermatozoa from leukocyte-derived oxidative stress. Combinations of glutathione and hypotaurine have been found to give the best overall results. DMSO, catalase, and vitamins C and E were less effective, suggesting that although H_2O_2 may be an important initiator of LPO,[14] it is the cytotoxic lipids generated during the peroxidative chain reaction that are particularly damaging to the spermatozoa.

SPERM PREPARATION TECHNIQUES AND REACTIVE OXYGEN SPECIES GENERATION

Sperm washing techniques that impair sperm function, such as repeated centrifugation, are associated with significantly higher ROS production than methods that isolate spermatozoa by more efficient techniques, such as swim-up and Percoll procedures.[116] The optimal sperm preparation techniques are those in which the sperm are centrifuged after the motile cells have been selected, as in the swim-up procedure and albumin column and Percoll gradient separation. The swim-up technique is maximally effective when centrifugation is done after swim-up.[117] Both removal of seminal plasma and repeated centrifugation contribute to an increase in ROS formation in conventionally washed (wash and resuspend) spermatozoa.[36] Spermatozoa can be separated from leukocytes while they are still protected by antioxidants in the seminal plasma. This can be easily achieved either with the swim-up separation of spermatozoa from unfractionated semen, or by membrane filter (eg, L4 membrane) that separates morphologically normal sperm from abnormal spermatozoa, undifferentiated cells, and leukocytes[118,119] or by Percoll method.

As a result of increased ROS production, more damage occurs in specimens with normal sperm motility and morphology after washing than in specimens with poor motility and morphology before sperm washing. This difference may occur because already damaged sperm may have lost the capacity to generate ROS.[116] Centrifugation time and speed affect ROS levels. ROS-negative specimens become ROS positive after centrifugation at 200 g or 500 g.[116,120] However, centrifugation time is more important than centrifugation speed.[120] Apparently, damage caused during separation of spermatozoa can generate ROS. This inverse relationship between sperm function and capacity for generating ROS is in agreement with earlier studies of the biochemical basis of defective sperm function.[19,20]

The presence of low-level leukocyte contamination in sperm preparations used for therapeutic IVF has been shown to markedly affect fertilization outcome.[111,121] Such leukocyte-mediated damage is not a reflection of the fertility status of the donor but an iatrogenic artifact created by the sperm preparation procedure that can occur even in a fertile man. This requires the need for constant vigilance in monitoring leukocyte contamination in washed preparations to be used in assisted reproduction procedures.

REACTIVE OXYGEN SPECIES MEASUREMENT

In view of the physiologic and diagnostic significance of ROS generation, developing techniques to detect this activity is important.[3] The methods commonly used for measuring ROS can be categorized into reactions that measure ROS on the cell membrane surface, involving the tetrazolium nitroblue technique for measuring superoxide, involving ferricytochrome C reduction, this lacks adequate sensitivity, requiring sperm concentrations of about 0.8×10^8/mL semen[21]. The latter requirement presents a problem in oligozoospermic men. The second category measures extracellular and intracellular ROS by chemiluminescence method (Table II). Although a number of fluorescence-based techniques are available for measuring H_2O_2 generation,[122,134] lack of sensitivity and cell autofluorescence are the inherent limitations in such methods. ROS can also be measured by electron spin resonance methods, which are sensitive and can identify the type of ROS generated inside the cells. As the capacity for ROS generation by sperm is low compared with leukocytes, the diagnosis of oxidative stress requires sensitive techniques to detect ROS production. The techniques that measure ROS outside or inside the membrane using the luminol-dependant chemiluminescence are the most sensitive.[122] Luminol (5-amino-2,3-dihydro-1,4-pthalazinedione) undergoes intracellular deoxygenation mediated by H_2O_2 and a sperm peroxidase located in the acrosome. The resulting signal can be suppressed with peroxidase inhibitors (phenylhydrazine and sodium azide) and reversed to baseline values by adding an azide-insensitive peroxidase, horseradish peroxidase (HRP).

A refinement of this technique uses a luminol analogue, 7-dimethyl amino-naphthalin-1,2-dicarbonic acid hydrazide (DNDH); using HRP (DNDH/HRP) further increases the sensitivity.[124] In the diagnosis of oxidative stress in human sperm, the DNDH/HRP system has improved sensitivity for a physiologically meaningful range of H_2O_2 concentrations, giving chemi-

TABLE II. ***Currently available tests for detection of reactive oxygen species (direct) or their oxidized products (indirect)***

Assay	Probe	End Product Measured	Extracellular/ Intracellular	References
Direct measurement				
Tetrazolium nitroblue	Ferricytochrome C	$^{\bullet}O_2^-$	Extracellular	21, 40
Chemiluminescence	Luminol	H_2O_2, $^{\bullet}O_2^-$, $^{\bullet}OH$	Both	43, 122
	Luminol + FMLP, PMA	H_2O_2	Extracellular	111, 123
	DNDH/HRP	H_2O_2, $^{\bullet}OH$	Both	124
	Lucigenin	$^{\bullet}O_2^-$, $^{\bullet}OH$	Extracellular	43, 125
	Xanthine-xanthine oxidase system	H_2O_2 SOD-like activity	Extracellular	32, 65, 66, 73, 126, 127
Indirect measurement				
Lipid peroxidation levels	TBA	MDA	Measures oxidized component in the body fluids	21, 34, 37, 128
Antioxidants, micronutrients, vitamins	HPLC	Alpha-tocopherol	Serum and seminal plasma	48, 129
	HPLC	Retinol	Seminal plasma	130
	HPLC	Beta-carotene	Seminal plasma	130
	HPLC	Lycopene	Seminal plasma	130
Ascorbate	HPLC	Ascorbic acid	Seminal plasma	54
Antioxidant enzymes	SOD	$^{\bullet}O_2^-$	Seminal plasma	42, 131
	Catalase	H_2O_2	Seminal plasma	132
	Glutathione peroxidase	ROO$^{\bullet}$	Spermatozoa	21, 73, 133
	Glutathione reductase	H_2O_2	Spermatozoa	
Chemokines	ELISA	Interleukin-6, Interleukin-8	Seminal plasma	42, 83
Antioxidant-pro-oxidant status	Total antioxidant	TEAC	Low chain-breaking levels	45

Key: DNDH/HRP = 7-dimethyl amino-naphthalin-1,2-dicarbonic acid hydrazide/horseradish peroxidase; ELISA = enzyme linked immunosorbent assay, FMLP = N-formyl-methionyl-leucyl-phenylalanine; HPLC = high-performance liquid chromatography; MDA = malonaldehyde; PMA = phorbol-12-myristate 13-acetate, ROO = peroxyl radical; SOD = superoxide dismutase; TBA = thiobarbituric acid; TEAC = trolox equivalent antioxidant capacity.

luminescence signals that are significantly greater than those obtained with luminol and HRP, both in the steady-state situation and after administration of agonists such as PMA and calcium ionophore A23187.

The half-life of ROS is very short,[135] and the effect of leukocytic ROS on sperm function could depend on the length of contact between leukocytes and spermatozoa. Lack of a standardized protocol to assess ROS in humans is partly responsible for the absence of defined ROS levels in normal fertile men. When ROS is measured using luminol as a probe, values greater than 10×10^4 counted photons per minute indicate a ROS-positive specimen[136,137] (Fig. 1). A more specific potential method of measuring ROS measures an oxidized component that can remain in body fluids such as the thiobarbituric acid (TBA)-reacted malonaldehyde (MDA), which is an index of LPO damage.[21] The onset of LPO in susceptible sperm leads to the progressive accumulation of lipid hydroperoxides in the sperm plasma membrane which then decompose to form MDA.

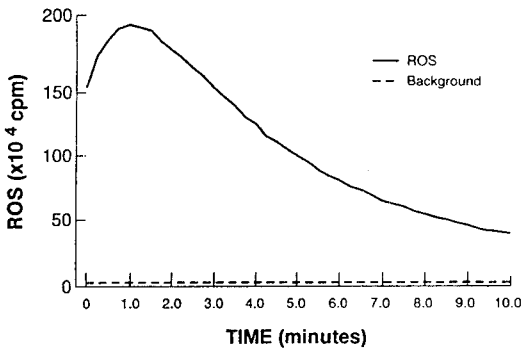

FIGURE 1. *A positive ROS curve in an Endtz-positive specimen. Chemiluminescence peaks 1 to 2 minutes after adding luminol, indicating a positive test. The baseline curve shows the background luminescence.*

ANTIOXIDANT EFFECTS OF SEMINAL PLASMA

Seminal plasma and normal motile spermatozoa do not produce high levels of ROS under normal

conditions.[36] ROS is produced by a variety of semen components including immotile or morphologically abnormal spermatozoa, leukocytes, and morphologically normal but functionally abnormal spermatozoa.[19,36,39] Enzymes that scavenge ROS include SOD, which has been detected in the sperm of several species, including rams,[138] humans,[103,131] rabbits,[131] and mice.[139] The SOD level in spermatozoa is positively correlated with the duration of sperm motility.[21] Also, catalase, which prevents ROS damage,[44,103,131] has been found in both human spermatozoa and seminal plasma.[132] Similarly, a selenium-containing antioxidase enzyme scavenging system, the glutathione peroxidase (GSSG)/reductase (GSH), exists in the sperm of several mammalian species, including human beings.[21,133,140] This system may act directly as an antioxidant and an inhibitor of LPO. A high GSH/GSSG ratio will help spermatozoa to combat oxidative insults.

Normally, a balance is maintained between the amount of ROS produced (pro-oxidants) and that scavenged by a cell (antioxidant). Cellular damage arises when this equilibrium is disturbed, especially when the cellular scavenging systems (SOD, catalase, GSSG, and GSH) cannot eliminate the increase in ROS and correlate with idiopathic infertility.

In studies using cells free of seminal plasma, an association was seen between defective sperm function and the levels of SOD and other ROS produced either spontaneously or after calcium ionophore stimulation.[21,24,44,51] Other substances in semen can also act as ROS scavengers and have SOD-like or catalase-like activity. Proteins such as albumin and small molecules, such as glutathione, pyruvate, taurine and hypo-taurine, and vitamins E and C,[51,66,133,141-144] also protect tissues against oxidative stress. In vitro experiments with vitamin E (one of the major membrane protectants against ROS and LPO) have shown significant protection of the spermatozoa from peroxidative damage and loss of motility. Higher concentrations have resulted in the enhancement of sperm function in a hamster egg penetration assay.[51,65] A recent double-blind, randomized placebo-controlled trial[129] demonstrated that therapy with this powerful antioxidant can be a potential treatment for a well-defined group of infertile men after oral vitamin E administration. Vitamin E is a chain-breaking and not a scavenging antioxidant. It therefore offers protection to membrane components without influencing ROS generation.

Studies have revealed that ROS-scavenging enzyme activity can be induced and that the specificity and extent of induction depend on the cell type and the source of ROS.[66] The increase in catalase-like activity observed in ROS-producing semen samples may be a compensatory mechanism that produces more low molecular weight H_2O_2 scavengers, activates catalase, or both. The extracellular levels of both catalase and glutathione peroxidase are very low and may explain the sensitivity of human spermatozoa to H_2O_2.[34,65,140] This may explain why catalase-like rather than SOD-like activity is increased. Zini *et al.*[64] found that SOD-like activity was similar in seminal plasma (whole or fractionated) and spermatozoa of all samples studied regardless of whether or not ROS was produced. However, the catalase-like activity of whole seminal plasma and of spermatozoa alone was higher (by 37% and 306%, respectively) in ROS-producing samples than in samples that did not produce ROS. Seminal plasma can markedly reduce the amount of ROS that PMN produce, as measured with a chemiluminescence method.[145] Given that seminal plasma bathes ejaculated spermatozoa from the time of exit from the ejaculatory duct until they pass through the cervical canal, it is likely to be a key in interactions between PMN and spermatozoa. SOD and catalase also remove $\cdot O_2^-$ generated by neutrophils (by NADPH-oxidase) and may play an important role in decreasing LPO and protecting the spermatozoa during genitourinary inflammation.

EVIDENCE FOR REACTIVE OXYGEN SPECIES-INDUCED INFERTILITY

The two primary sources of ROS in the male reproductive tract are the spermatozoa and infiltrating leukocytes.[62] Mammalian spermatozoa undergo morphologic transformation, and discard a great majority of their cytoplasm during the final stages of spermiogenesis. Their plasma membranes are rich in polyunsaturated fatty acids and have the ability to generate ROS as a normal process.[59] However, spermatozoa are unable to repair the damage induced by ROS as they are not endowed with cytoplasmic defense enzymes like catalase.[19-21,34,146] As a result, spermatozoa are more susceptible to peroxidative damage or LPO. Lipid peroxidation may be defined as oxidative deterioration of polyunsaturated fatty acids. It is a physiologic phenomenon occurring in all cells that are rich in lipids, especially the polyunsaturated fatty acids. Lipid peroxidation plays a significant role in the etiology of defective sperm function.[35] Spermatozoal generation of ROS has been implicated in the control of normal sperm function[34] and in male infertility associated with a peroxidation-induced loss of plasma membrane function.[19,21,34,37,44,103,132,140] Free radical reactions have two primary characteristics. First, they are produced by a chain reaction mechanism. Second, they consist of a number of competing reactions.

The relative importance of each reaction depends on variables such as the concentration of free radicals and target molecules, and their activities and reactivates. The low lipid solubility of $\cdot O_2^-$ combined with its high reactivity and short half-life limits its capacity to diffuse away from the site of generation. ROS attack biomembranes, thereby injuring or killing the cells at definite sites in the body, and disappear in the process. On the other hand, owing to long half-life and high membrane solubility, H_2O_2 diffuses a considerable distance from its site of generation, causing damage at distant sites. Lipid peroxidation involves the initiation and propagation stage and occurs by the Haber-Weiss reaction and/or the Fenton reaction. Both reactions generate the hydroxyl radical, which then initiates lipoperoxidation (Fig. 2). By itself, neither $\cdot O_2^-$ nor H_2O_2 is energetic enough to initiate LPO directly but, in the presence of catalytic amounts of transition metals, such as iron or copper, they react and form the $\cdot OH$ radical (the Haber-Weiss reaction). In the Fenton reaction, the hydroxyl radical can be directly generated from H_2O_2 if ferrous ions and an alternative reducing agent such as ascorbate are present.[141] The hydroxyl radical is a direct and powerful initiator of the Fenton reaction.

$$\cdot O_2^- \cdot + H_2O_2 \rightarrow \underset{\text{hydroxyl radical}}{\cdot OH} + \underset{\text{hydroxyl ion}}{OH^-} + \cdot O_2 \text{ (Haber-Weiss reaction)},$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \underset{\text{hydroxyl radical}}{\cdot OH} + \underset{\text{hydroxyl ion}}{OH^-} \text{ (Fenton reaction)}.$$

To complete the Fenton reaction, a source of H_2O_2 is required, and the human ejaculate contains all the components (defective spermatozoa and contaminating neutrophils) needed to initiate and propagate this free radical cascade and lipoperoxidation.[21,36,60,62,147,148]

Following the initiation stage of lipid peroxidation cascade, the extent to which the process proceeds will depend on the antioxidant strategies employed by the spermatozoa. Propagation of lipid peroxidation in the sperm plasma membrane will be impeded as a result of the antioxidant mechanism, and lipid peroxides will tend to accumulate. On the other hand, if a transition metal such as iron is added to the sperm suspension, it will result in a sudden acceleration of LPO and loss of sperm function. The peroxidative damage does not appear to result from the initiation stage of the lipid peroxidation chain. Alkyl and peroxyl radicals are generated by the conversion of lipid hydroperoxides effected by the catalytic decomposition of the pre-existing lipid peroxides. In the presence of ferrous ion promoters, these then initiate a chain reaction within the membrane, thereby propagating the damage throughout the cell. Lipid peroxides are extremely cytotoxic to human spermatozoa[37,149] as a result of the decomposition of these peroxides into highly toxic aldehydes that migrate from one site to another in

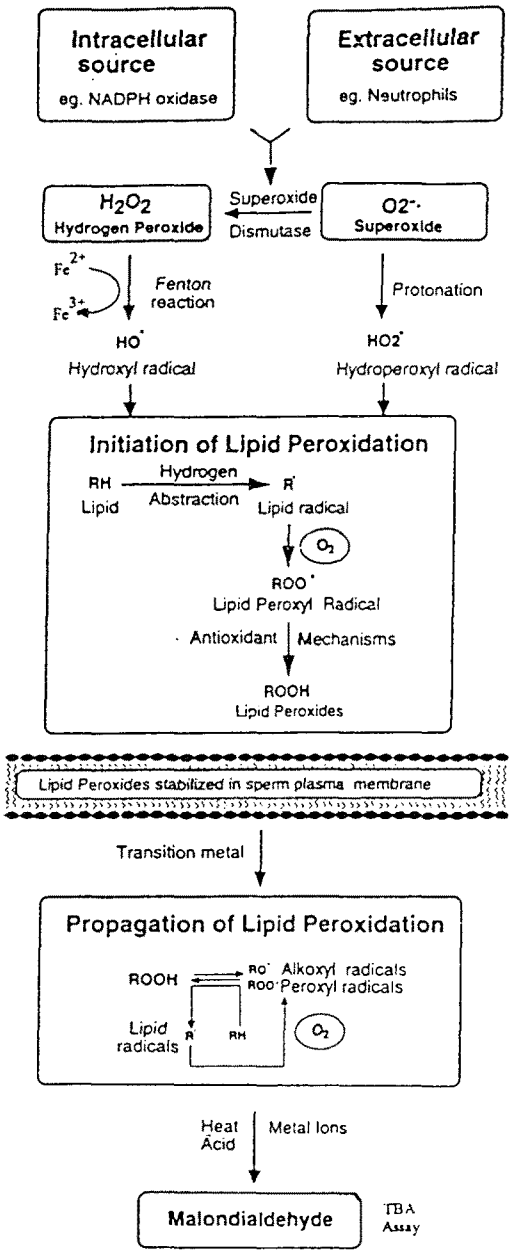

FIGURE 2. *Schematic representation of the major pathways of lipid peroxidation in human spermatozoa (reproduced with permission of ICSU Press from Aitken and Fisher[35]).*

the body (owing to their long life), thus propagating the injuries. Lipid peroxidation often appears to be a late stage in the oxidation-induced cellular injury rather than a mechanism of initiating the injury.[84] This may be due to a loss of membrane fluidity, which is important in sperm function and fusion with the oocyte. An inhibition of the membrane-bound enzymes, especially the adenosinetriphosphatases, is also seen due to accumulation of LPO. Lipid peroxidation also impairs cell membrane ion exchange that is essential for maintaining normal sperm motility.[38] Malonaldehyde is an end product of LPO in the spermatozoa and is induced by ferrous:ascorbate promoter system. This can be measured by the TBA assay and can serve as an important diagnostic tool in LPO measurement. The MDA concentration exhibits excellent inverse relationship with the sperm–oocyte fusion,[34] and a direct relationship with sperm morphology.[150]

STRATEGIES TO REDUCE OXIDATIVE STRESS

Long-term strategies must determine the cause of the enhanced generation of ROS by the spermatozoa of infertile men. Reduced oxidative stress will be beneficial in assisted reproductive techniques such as IVF or donor-assisted intrauterine inseminations (IUI). 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 develop appropriate therapeutic strategies in the treatment for male infertility. Determining the level and origin of ROS production in the ejaculate and precise evaluation of the scavenger system may be useful in treating patients. If the error in spermatogenesis that leads to such atypical activity (excessive ROS generation) could be defined, it would provide an important lead in determining the etiology of male infertility, and a rational basis for the design of effective therapies could then be prepared. Seminal leukocyte population should be considered potentially detrimental and must be monitored using the new generation of biochemical tests based on the release of ROS or seminal concentrations of key cytokines. It is important to minimize the interaction between ROS-producing cells in the semen (PMN) and spermatozoa that have a potential to fertilize. Differentiating between spermatozoa and leukocyte sources of ROS is important clinically, because this has a bearing on the strategies used to reduce oxidative stress on spermatozoa during the course of IVF therapy. Early identification could significantly influence the antioxidant strategies selected for cases in which the diagnostic tests show oxidative stress as the only pathogenic factor, because damage to the sperm membrane may still be contained or reversed. It is important for the clinician to recognize that assisted reproductive techniques for sperm preparation (washing, centrifugation, and Percoll gradients) may induce damage to spermatozoa by removing the scavengers from the seminal plasma or by increasing ROS generation by spermatozoa. ROS detection, in addition to a sperm culture, could provide a marker for some cases of genital tract infection. The damaging effect of ROS originating from leukocytes may be moderated if the site of infiltration is confined to the prostate or the seminal vesicle. Under such circumstances, the first contact between the spermatozoa and leukocyte-derived free radicals would be at the moment of ejaculation when the spermatozoa would be protected by the powerful antioxidant factors in seminal plasma.[37]

SELECTIVE REMOVAL OF CONTAMINATING LEUKOCYTES

The interactions that take place between spermatozoa and activated leukocytes in washed sperm pellets are probably clinically important, for a negative correlation between leukocyte contamination and fertilization rates has been demonstrated in IVF-ET.[112,123] The fact that low levels of leukocyte contamination can impair the fertilizing potential of human spermatozoa has clear implications for the design of IVF protocols that might be used to treat such patients. The negative impact of oxidative stress might be addressed by the development of an antioxidation-supplemented IVF culture medium and/or by removal of contaminating leukocytes. Phorbol stimulation of leukocyte-free suspensions reflects the fact that although spermatozoa have the capacity to generate ROS they do not express this activity spontaneously. It was only after phorbol exposure that the free radical mediated loss of sperm function was observed. These results emphasize the benefits of monitoring ROS for human sperm suspensions prepared for IVF therapy. Whatever the source of ROS, counteracting the availability and the activity of these molecules is important in the development of IVF protocols designed to meet the special problems set by male factor infertility patients. The development of efficient techniques for selectively removing PMN from sperm suspensions or neutralizing their adverse effects could be important for improving fertilization and pregnancy rates.[51,111,149] Separation of spermatozoa on a Percoll gradient results in a fairly high population of sperm; however, a low number of leukocytes remain. Simple biochemical techniques for measuring the degree of leukocytic contamination and

selective removal of these cells might be useful. One such approach that uses paramagnetic beads coated with a monoclonal antibody against the common leukocyte antigen CD45 has proven effective.[24,76,111] FMLP-associated chemiluminescence signals confirming leukocyte contamination are of immediate interest to IVF-ET programs.

ANTIOXIDANT SUPPLEMENTATION

It is difficult to measure the effectiveness of one antioxidant in isolation of another because of a cooperation between various antioxidants. Therefore, measurement of total antioxidant capacity is important. Measuring total chain-breaking antioxidant (CBA) content in seminal plasma of various categories of infertile men is important, as this varies in these men. CBA levels can then be compared to ROS levels in these men. CBAs trap ROS directly and prevent amplification of radical formation and subsequent damage to the sperm. Low levels of CBA may be an indication of sperm stress rather than a primary cause of sperm dysfunction. Decreased CBA will lead to increased free radical damage in semen and reduced capacity to recycle antioxidants in sperm membranes, making the sperm more susceptible to peroxidative damage. It is important to measure individual CBAs to study their level in fertile men and to measure which particular antioxidant is reduced in each infertile group. The most important strategy to reduce oxidative stress is to use antioxidant-supplemented IVF culture media. Damage caused by iron-catalyzed peroxidation can be prevented by including alpha-tocopherol (vitamin E) to the medium.[146] Alpha-tocopherol breaks free radical chain reactions by forming a relatively stable radical tocopheroxyl at a concentration of 10 mM.[34,51,59,151] Oral administration of vitamin E results in a significant improvement of the in vitro function of human spermatozoa,[129] and this may have significant implications for the treatment of these patients using IVF rather than relying on in vivo conception.

H_2O_2 is the most disruptive oxidant species with respect to human spermatozoa. Ascorbic acid also prevents or reduces the oxidation of biological molecules. The addition of catalase into the in vitro culture media can be beneficial in treating some patients. The positive correlation between a high ascorbic acid level and normal sperm morphology suggests that ascorbic acid has a crucial role as an antioxidant.[54] Although ascorbic acid alone cannot scavenge lipophilic radicals in the lipid compartment of the spermatozoa, it can act synergistically with alpha-tocopherol to reduce lipid peroxide radicals by reacting with tocopheroxyl radicals and regenerating active alpha-tocopherol. Sperm preparation for IVF with ascorbic acid could be useful because seminal antioxidants are not available to protect sperm membranes from ROS attack during IVF. The effect of vitamin E supplementation in combination with IVF employing controlled clinical studies will help determine if these putative antioxidants can improve infertility in a subgroup of patients.

Pentoxifylline, a motility stimulator, can also act as a ROS scavenger by reducing the generation of superoxide anion by spermatozoa,[40,152] and may have a clinical role in the treatment of patients susceptible to ROS-induced damage.

GLUTATHIONE THERAPY

Glutathione has a number of physiologic and pharmacologic qualities that act against lipid peroxidation of the cell membrane. Glutathione therapy has been proposed in various pathologic situations in which ROS could be involved in idiopathic infertility. Marked improvement in total sperm motility and morphology occurring during glutathione therapy suggests that glutathione could act indirectly on spermatozoa by improving the metabolic conditions of the epididymal and testicular structures.[153] These results last even after therapy is stopped. Glutathione acts as a free radical scavenger and results in improved semen quality. Baker *et al.*[46] have also demonstrated the effectiveness of glutathione alone or in combination with hypotaurine which is able to react directly with cytotoxic aldehydes produced during LPO and thus protects the thiol groups on the sperm plasma membrane.[154] Reduced glutathione can also neutralize hydroxyl radicals and function in the detoxification of peroxides through its interaction with sperm glutathione peroxidase. It may also facilitate the antioxidant action of alpha-tocopherol in the sperm plasma membrane by participating in the regeneration of this vitamin from tocopheryl radicals. Parenteral administration of glutathione has been shown to improve sperm motility and morphology in infertile men with abnormal semen quality associated with varicoceles or genital tract inflammation.[48]

FUTURE DIRECTIONS

Future efforts should be directed to elucidate why the spermatozoa of some patients become overreactive in the generation of ROS and during what period of differentiation and maturation of the spermatozoa this self-destructive activity first appears. Other areas of research include: (1) development of diagnostic methods and tools for identifying sperm at risk of collateral peroxidative damage to the sperm membrane; (2) identification of the extent to which the various culture media currently used for IVF-ET support the oxidative

process, use of those that do not support oxidative stress, and investigation of whether patients can benefit from the addition of ROS scavengers to the culture medium to minimize the effects of peroxidative damage; (3) identification of individual chain-breaking antioxidants in fertile men in order to study their levels and to determine which particular chain-breaking antioxidant is reduced in each infertile group; and (4) investigation of the involvement of antioxidants in male-factor infertility and the way antioxidants per se affect sperm function in vitro.

SUMMARY

Human spermatozoa exhibit a capacity to generate ROS and initiate peroxidation of the unsaturated fatty acids in the sperm plasma membrane, which plays a key role in the etiology of male infertility. The short half-life and limited diffusion of these molecules is consistent with their physiologic role in key biological events such as acrosome reaction and hyperactivation. The intrinsic reactivity of these metabolites in peroxidative damage induced by ROS, particularly H_2O_2 and the superoxide anion, has been proposed as a major cause of defective sperm function in cases of male infertility. The number of antioxidants known to attack different stages of peroxidative damage is growing, and it will be of interest to compare alpha-tocopherol and ascorbic acid with these for their therapeutic potential in vitro and in vivo. Both spermatozoa and leukocytes generate ROS, although leukocytes produce much higher levels. The clinical significance of leukocyte presence in semen is controversial. Seminal plasma confers some protection against ROS damage because it contains enzymes that scavenge ROS, such as catalase and superoxide dismutase. A variety of defense mechanisms comprising a number of antioxidants can be employed to reduce or overcome oxidative stress caused by excessive ROS. Determination of male infertility etiology is important, as it will help us develop effective therapies to overcome excessive ROS generation. ROS can have both beneficial and detrimental effects on the spermatozoa and the balancing between the amounts of ROS produced and the amounts scavenged at any moment will determine whether a given sperm function will be promoted or jeopardized. Accurate assessment of ROS levels and, subsequently, OS is vital, as this will help clinicians both elucidate the fertility status and identify the subgroups of patients that respond or do not respond to these therapeutic strategies. The overt commercial claims of antioxidant benefits and supplements for fertility purposes must be cautiously looked into, until proper multicentered clinical trials are studied. From the current data it appears that no single adjuvant will be able to enhance the fertilizing capacity of sperm in infertile men, and a combination of the possible strategies that are not toxic at the dosage used would be a feasible approach.

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