Oxidative Stress and Male Infertility: From Research Bench to Clinical Practice

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Extensive research in our center at the Cleveland Clinic indicates that the seminal oxidative stress test has diagnostic and prognostic capabilities beyond those of conventional tests of sperm quality or functions. An oxidative stress test can accurately discriminate between fertile and infertile men and identify patients with a clinical diagnosis of male-factor infertility who are likely to initiate a pregnancy if they are followed over a period of time. In addition, the test can help select subgroups of patients with infertility in which oxidative stress is a significant factor, and who may benefit from antioxidant supplementation. Incorporation of such a test into routine andrology laboratory practice may be of particular importance to the future management of male infertility.

In recent years, the generation of reactive oxygen species (ROS) in the male reproductive tract has become a real concern because of their potential toxic effects at high levels on sperm quality and function. ROS are highly reactive oxidizing agents belonging to the class of free radicals (Aitken, 1994). A free radical is defined as “any atom or molecule that possesses one or more unpaired electrons” (Warren et al, 1987). Recent reports have indicated that high levels of ROS are detected in semen samples of 25% to 40% of infertile men (de Lamirande et al, 1995; Padron et al, 1997).

However, a strong body of evidence suggests that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities (Aitken, 1999).

Spermatozoa, like all cells living in aerobic conditions, constantly face the oxygen (O₂) paradox: O₂ is required to support life, but its metabolites such as ROS can modify cell functions, endanger cell survival, or both (de Lamirande and Gagnon, 1995). Hence, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function. It is not surprising that a battery of different antioxidants is available to protect spermatozoa against oxidants (Sies, 1993).

Seminal oxidative stress (OS) develops as a result of an imbalance between ROS generating and scavenging activities (Sikka et al, 1995; Sharma and Agarwal, 1996; Sikka, 2001). Spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (PUFAs; Alvarez and Storey, 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Jones et al, 1979; Aitken and Fisher, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996). In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by depending on the protection afforded by the seminal plasma (Iwasaki and Gagnon 1992; Zini et al, 1993). Oxidative stress attacks not only the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus (Aitken, 1999).

Even though OS has been established as a major factor in the pathogenesis of male infertility, there is a lack of consensus as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to use an OS test in clinical practice may be the lack of a standard protocol to assess seminal OS. The role of OS in male infertility has been the focus of our research center at the Cleveland Clinic Foundation for the past 10 years. The main objective of our research in this area was to transfer this important knowledge from the research bench to clinical practice. We designed studies with the following aims: 1) to understand the precise mechanism by which OS develops in semen, which we thought could help develop strategies to overcome the problem, 2) to establish assays for accurate and reliable assessment of seminal OS, and 3) to identify the clinical significance of testing seminal OS in the male infertility practice. In this review, we summarize the efforts of our program to explore the role of OS in male infertility. We also include detailed information on the protocols introduced recently by our center for the assessment of seminal OS in a clinical andrology laboratory.
**Reactive Oxygen Species and Sperm Physiology**

Until recently, ROS were exclusively considered toxic to human spermatozoa. The idea that limited amounts of ROS can intervene in a physiological manner in the regulation of some sperm functions was first evoked in a study by Aitken et al (1989). The authors found that low levels of ROS can enhance the ability of human spermatozoa to bind with zona pellucida, an effect that was reversed by adding vitamin E. Other studies have found that incubating spermatozoa with low concentrations of hydrogen peroxide (H$_2$O$_2$) stimulates sperm capacitation, hyperactivation, and the ability of the spermatozoa to undergo the acrosome reaction and oocyte fusion (de Lamirande and Gagnon, 1993; de Lamirande et al, 1993; Griveau et al, 1994; Aitken et al, 1995; Kodoma et al, 1996; Aitken, 1997). ROS other than H$_2$O$_2$, such as nitric oxide and superoxide anion (O$_2$•$^-$), have also been shown to promote sperm capacitation and the acrosome reaction (Griveau et al, 1995a; Zini et al, 1996).

**Mechanism of Antioxidant Protection in Semen**

It is interesting that seminal plasma is well endowed with an array of antioxidant defense mechanisms to protect spermatozoa against OS (Sikka, 1996; Smith et al, 1996; Armstrong et al, 1998). These mechanisms compensate for the deficiency in cytoplasmic enzymes in sperm (Donnelly et al, 1999). Seminal plasma contains a number of enzymatic antioxidants such as superoxide dismutase (SOD; Alvarez et al, 1987), the glutathione peroxidase/glutathione reductase (GPX/GRD) system (Chaudiere et al, 1984), and catalase (Jeulin et al, 1989). In addition, seminal plasma contains a variety of nonenzymatic antioxidants such as ascorbate (Fraga et al, 1991), urate (Thiele et al, 1995), α-tocopherol (Aitken and Clarkson, 1988; Moilanen et al, 1993), pyruvate (de Lamirande and Gagnon, 1992), glutathione (Lenzi et al, 1994), taurine, and hypotaurine (Alvarez and Storey, 1983).

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

**Prevention**—Prevention of ROS formation is the first line of defense against oxidative insult. An example is the binding of metal ions, iron, and copper ions in particular, which prevents them from initiating a chain reaction (Sies, 1993). Chelation of transition metals is a major means of controlling sperm lipid peroxidation (LPO) and DNA damage. When transition metals become loosely bound to oxygen reduction products, they can produce secondary and more reactive oxidants, particularly the hydroxyl radical (OH•) (Halliwell, 1990).

**Interception**—Free radicals have a tendency toward chain reaction (ie, 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 break this chain reaction by the formation of nonradical end products (Sies, 1993). Alpha-tocopherol (vitamin E), a chain-breaking antioxidant, inhibits LPO in membranes by scavenging peroxyl (RO•) and alkoxyl (ROO•) 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 (Wefers and Sies, 1988). In this way, α-tocopherol is able to function again as a free radical chain-breaking antioxidant, even though its concentration is low (Buettner, 1993). For efficient interception, the radical to be intercepted must have a relatively long half-life (Sies, 1993). The peroxyl radicals are major reaction partners because their half-life extends into the range of seconds (7 seconds). In contrast, the hydroxyl radical, with its high reactivity and extremely short half-life (10$^{-9}$ seconds), cannot be intercepted with reasonable efficiency (Sies et al, 1992).

**Repair**—In some situations, the damage caused by oxidants may be repaired. Unfortunately, spermatozoa are unable to repair the damage induced by OS 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 (Alvarez et al, 1987; Aitken et al, 1989).

**Pathogenesis of Seminal Oxidative Stress**

The term oxidative stress is applied when oxidants outnumber antioxidants (Sies, 1993), peroxidation products develop (Spitteler, 1993), and when these phenomena cause pathological effects (Janssen et al, 1993). Oxidative stress has been implicated in numerous disease states such as cancer, connective tissue disorders, aging, infection, inflammation, acquired immunodeficiency syndrome, and male infertility (Clark et al, 1986; Aitken et al, 1992, 1995).

In the context of human reproduction, a balance normally exists between ROS generation and scavenging in the male reproductive tract. As a result, only a minimal amount of ROS remains, which is needed to regulate normal sperm functions such as sperm capacitation, acrosome reaction, and sperm-oocyte fusion (Gagnon et al, 1991; de Lamirande and Gagnon, 1994; Griveau and Le Lannou, 1997). Excessive ROS production that exceeds critical levels can overwhelm all antioxidant defense strategies of spermatozoa and seminal plasma causing OS...
Lipid Peroxidation of Sperm Plasma Membrane—Lipid peroxidation is broadly defined as “oxidative deterioration of PUFA” (i.e., fatty acids that contain more than two carbon-carbon double bonds; Halliwell, 1984). The LPO cascade occurs in two fundamental stages: initiation and propagation. The hydroxyl radical (OH•) is a powerful initiator of LPO (Aitken and Fisher, 1994). Most membrane PUFAs have unconjugated double bonds that are separated by methylene groups. The presence of a double bond adjacent to a methylene group makes the methylene C-H bonds weaker and, therefore, hydrogen is more susceptible to abstraction. Once this abstraction has occurred, the radical produced is stabilized by the rearrangement of the double bonds, which 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 (Blake et al., 1987). Conjugated dienes rapidly react with O2 to form a lipid peroxy radical (ROO•), which abstracts hydrogen atoms from other lipid molecules to form lipid hydroperoxides (ROOH). Lipid hydroperoxides are stable under physiological conditions until they contact transition metals such as iron or copper salts. These metals or their complexes cause lipid hydroperoxides to generate alkoxyl and peroxy radicals, which then continue the chain reaction within the membrane and propagate the damage throughout the cell (Halliwell, 1984). Propagation of LPO depends on the antioxidant strategies employed by spermatozoa. One of the by-products of lipid peroxide decomposition is malondialdehyde. This by-product has been used in biochemical assays to monitor the degree of peroxidative damage in spermatozoa (Aitken et al., 1989; Aitken and Fisher, 1994). The results of such an assay exhibit an excellent correlation with the degree to which sperm function is impaired in terms of motility and the capacity for sperm-oocyte fusion (Aitken et al., 1993; Sidhu et al., 1998).

Impairment of Sperm Motility—The increased formation of ROS has been correlated with a reduction of sperm motility (Aitken et al., 1989; Iwasaki and Gagnon, 1992; Lenzi et al., 1993; Agarwal et al., 1994a; Armstrong et al., 1999). The link between ROS and reduced motility may be due to a cascade of events that result in a decrease in axonemal protein phosphorylation and sperm immobilization, both of which are associated with a reduction in membrane fluidity that is necessary for sperm-oocyte fusion (de Lamirande and Gagnon, 1995). Another hypothesis is that H2O2 can diffuse across the membranes into the cells and inhibit the activity of some enzymes such as glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). This in turn is used as a source of electrons by spermatozoa to fuel the generation of ROS by an enzyme system known as NADPH oxidase (Aitken et al., 1997). Inhibition of G6PD leads to a decrease in the availability of NADPH and a concomitant accumulation of oxidized glutathione and reduced glutathione. This can reduce the antioxidant defenses of the spermatozoa and increase peroxidation of membrane phospholipids (Grievou et al., 1995a).

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

Strong evidence suggests that high levels of ROS mediate the DNA fragmentation commonly observed in spermatozoa of infertile men (Kodama et al., 1997; Sun et al., 1997). 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 (Kodama et al., 1997; Lopes et al., 1998). There is a substantial risk that spermatozoa carrying damaged DNA are being used clinically in this form of therapy (Twigg et al., 1998b). When intrauterine insemination or in vitro fertilization 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 may be directly injected into the oocyte (Twigg et al., 1998b; Aitken, 1999).

Sources of Excessive Reactive Oxygen Species Production in Semen
Morphologically abnormal spermatozoa and seminal leukocytes have been established as the main sources of high
ROS production in human ejaculates (Aitken and West, 1990; Kessopolou et al, 1992). Virtually every human ejaculate is contaminated with potential sources of ROS (Aitken, 1995). It follows that some 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 pathology.

Clear evidence suggests that human spermatozoa produce oxidants (Aitken et al, 1992; Hendin et al, 1999; Gil-Guzman et al, 2001). Spermatozoa may generate ROS in two ways: 1) the NADPH oxidase system at the level of the sperm plasma membrane (Aitken et al, 1992), and 2) the NADH-dependent oxidoreductase (diphorase) at the level of mitochondria (Gavella and Lipovac, 1992). The mitochondrial system is the major source of ROS in spermatozoa in infertile men (Plante et al, 1994).

Effect of Sperm Morphology on ROS Production—Gomez et al (1998) have indicated that levels of ROS production by pure sperm populations were negatively correlated with the quality of sperm in the original semen. The link between poor semen quality and increased ROS generation lies in the presence of excess residual cytoplasm (cytoplasmic droplet). When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective, and spermatozoa are released from the germinal epithelium carrying surplus residual cytoplasm. Under these circumstances, the spermatozoa that are released during spermiation are believed to be immature and functionally defective (Huszar et al, 1997). Retention of residual cytoplasm by spermatozoa is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme G6PD (Figure 1) (Aitken, 1999).

Recent studies by Ollero et al (2001) and Gil-Guzman et al (2001) have shown that levels of ROS production in semen were negatively correlated with the percentage of normal sperm forms as determined by World Health Organization (WHO, 1999) classification and by Kruger’s strict criteria (Kruger et al, 1987). The correlation of seminal ROS levels with morphologically abnormal sperm was also evident when morphology slides were scored using a sperm deformity index (SDI) \( (r = .31; P = .01) \) (unpublished data). The SDI was introduced by Aziz et al (1996) as a novel expression of sperm morphological abnormalities that were found to be highly correlated with fertilization in vitro. This new method for sperm morphology assessment uses a multiple entry scoring technique in which an abnormal sperm is classified more than once if more than one deformity is observed. SDI is calculated by dividing the total number of deformities observed by the number of sperm randomly selected and evaluated, irrespective of their morphological normality. In our study, SDI was significantly higher in a group of infertile men who had high levels of seminal ROS than in a group of infertile men with low ROS and in a group of fertile sperm donors (Table 1). The link between seminal ROS and high SDI may be causal and related to the greater capacity of morphologically abnormal spermatozoa to produce ROS.

Effect of Sperm Preparation Techniques on ROS Production—Studies from our center have indicated that human spermatozoa significantly increase levels of ROS production in response to repeated cycles of centrifuga-

![Figure 1](image_url). Mechanism of increased production of ROS by abnormal spermatozoa (spermatozoa with cytoplasmic retention).
Sperm deformity index
Normal morphology by Kruger (%)
Normal morphology by WHO
Sperm motility (%)
ROS-producing immature sperm during their comigration
the hypothesis that oxidative damage of mature sperm by
of motile, mature sperm. These interesting findings led to
mature sperm and inversely correlated with the recovery
production was found to be highest in immature sperm
Santa Ana, Calif) fractionation of ejaculated sperm, ROS
stages of maturation (Ollero et al, 2001; Gil-Guzman et
significantly varies in subsets of human spermatozoa at different
results from
significantly higher after the swim-up technique than after the
sperm with intact nuclear DNA was found to be signifi-
be reevaluated with the goal of minimizing sperm DNA
improvement in sperm DNA integrity. The authors rec-
lected from a nonselected group of infertility patients (n
For the purpose of this study, semen samples were col-
according to the temperature at which semen is incubated.
and 2) isolate spermatozoa with intact DNA by in vitro
in immature germ cell membranes during spermatogenesis,
and 2) isolate spermatozoa with intact DNA by in vitro
separation techniques should be of particular benefit to
these patients in whom a defect in the normal regulation
products of spermiogenesis leads to an abnormal increase in the
production of ROS-producing immature sperm.
Effect of Temperature on ROS Production—Prelimi-
nary data from a recent unpublished study in our center indicate that levels of ROS production in semen may vary according to the temperature at which semen is incubated. For the purpose of this study, semen samples were collected from a nonselected group of infertility patients (n = 12) and from a group of healthy sperm donors (n = 12) after 2 to 3 days of sexual abstinence. We examined levels of ROS in washed sperm suspensions immediately after liquefaction (basal ROS) and compared the results with ROS levels in aliquots of the same samples after 1-hour incubation at 3 different temperatures (4°C, 25°C, and 37°C). Our results indicate that ROS levels are signifi-
cantly lower in aliquots of semen incubated at 37°C for 1 hour compared to aliquots of the same samples incubated at 4°C and 25°C (Table 2). Sperm motion kinetics (curvilinear velocity, straight-line velocity, average path velocity, and amplitude of lateral head displacement) as determined by a computer-assisted semen analyzer (CASA) were significantly higher in samples incubated at 37°C than at 4°C (P = < .001, < .001, < .001, and < .001, respectively) although not significantly different from samples incubated at 25°C (P = .69, .51, .95, and .79, respectively). Therefore, it may be speculated that 37°C could be the optimum temperature at which semen can be collected and processed for different diagnostic and therapeutic purposes. Handling semen samples at

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fertile Donors (n = 8)</th>
<th>Infertile Men with Low ROS (≤1 × 10^6 cpm; n = 14)</th>
<th>Infertile Men with High ROS (&gt;1 × 10^6 cpm; n = 25)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seminal ROS (× 10^6 cpm)</td>
<td>0.1 (0.1, 0.5)</td>
<td>0.3 (0.1, 0.5)</td>
<td>5 (3.60)</td>
<td>0.51</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Seminal leukocytes (× 10^9/mL)</td>
<td>0.2 (0.1, 0.2)</td>
<td>0.1 (0.0, 0.2)</td>
<td>1.2 (0.3, 2.4)</td>
<td>0.61</td>
<td>0.005</td>
<td>0.007</td>
</tr>
<tr>
<td>Sperm concentration (× 10^6/mL)</td>
<td>68 (42, 90)</td>
<td>56 (38, 72)</td>
<td>20 (12, 26)</td>
<td>0.08</td>
<td>0.004</td>
<td>0.07</td>
</tr>
<tr>
<td>Sperm motility (%)</td>
<td>73 (67, 76)</td>
<td>59 (39, 63)</td>
<td>38 (33, 52)</td>
<td>0.002</td>
<td>&lt; 0.001</td>
<td>0.07</td>
</tr>
<tr>
<td>Normal morphology by WHO (1999) (%)</td>
<td>33 (31, 40)</td>
<td>23 (17, 32)</td>
<td>16 (12, 23)</td>
<td>0.02</td>
<td>&lt; 0.001</td>
<td>0.07</td>
</tr>
<tr>
<td>Normal morphology by Kruger (%)</td>
<td>12 (8, 14)</td>
<td>7 (5, 11)</td>
<td>6 (3, 8)</td>
<td>0.12</td>
<td>0.008</td>
<td>0.14</td>
</tr>
<tr>
<td>Sperm deformity index</td>
<td>1.6 (1.5, 1.7)</td>
<td>1.6 (1.5, 1.9)</td>
<td>2 (1.7, 2.2)</td>
<td>0.86</td>
<td>0.009</td>
<td>0.003</td>
</tr>
</tbody>
</table>

ROS = reactive oxygen species measured in washed sperm suspensions (after simple wash and resuspension in PBS) by chemiluminescence assay. Values are median and interquartile range (25th and 75th percentiles). A = P value of donors versus infertile men with low ROS; B = P value of donors versus infertile men with high ROS; and C = P value of infertile men with low ROS versus infertile men with high ROS. Wilcoxon rank-sum test was used for comparison, and statistical significance was considered at P < .05.

tion involved in the conventional sperm preparation tech-
niques used for ART (Agarwal et al, 1994a). The duration of centrifugation was found to be more important than the force of centrifugation for inducing ROS formation by human spermatozoa (Shekarriz et al, 1995b). These data are significant because exposing spermatozoa to high lev-
evels of ROS may cause DNA fragmentation, which can have adverse consequences if they are used for ART, par-
ticularly ICSI (Lopes et al, 1998). This may be true par-
ticularly in light of a recent report by Zini and coworkers (2000) who found that improvement in sperm motility
after Percoll processing was not associated with a similar improvement in sperm DNA integrity. The authors rec-
commended that the current sperm preparation techniques be reevaluated with the goal of minimizing sperm DNA
damage. In a recent study in our center, the recovery of sperm with intact nuclear DNA was found to be signifi-
cantly higher after the swim-up technique than after the ISolate gradient technique (unpublished data).

Our group has also reported that ROS production by human sperm increases with sperm concentration and de-
creases with time (Shekarriz et al, 1995b, c). Results from
our recent studies indicated that ROS production signifi-
cantly varies in subsets of human spermatozoa at different
stages of maturation (Ollero et al, 2001; Gil-Guzman et
al, 2001). Following ISolate gradient (Irvine Scientific, Santa Ana, Calif) 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 comigration from seminiferous tubules to the epididymis may be an
important cause of male infertility. If this is the case, per-
haps interventions directed to 1) increase antioxidant levels in immature germ cell membranes during spermatogenesis,
and 2) isolate spermatozoa with intact DNA by in vitro
separation techniques should be of particular benefit to
these patients in whom a defect in the normal regulation
of spermiogenesis leads to an abnormal increase in the
production of ROS-producing immature sperm.

Table 1. Comparison of semen parameters and sperm deformity index (SDI) in fertile donors, infertile men with low ROS, and infertile men with high ROS
37°C may help avoid deleterious effects of ROS on human spermatozoa.

**Reactive Oxygen Species Production by Seminal Leukocytes**—With respect to all nonsperm cells, the majority of the so-called “round cells” consist of immature germ cells with less than 5% leukocytes under normal conditions (Eggert-Kruse et al., 1992). Peroxidase-positive leukocytes were found to be the major source of high ROS production in semen (Tomlinson et al., 1993; Aitken et al., 1995; Rajasekaran et al., 1995; Shekarriz et al., 1995a; Ochsendorf, 1999). Peroxidase-positive leukocytes include polymorphonuclear (PMN) leukocytes, which represent 50% to 60% of all seminal leukocytes and macrophages, which represent another 20% to 30% (Wolff and Anderson, 1988; Fedder et al., 1993; Thomas et al., 1997). Peroxidase-positive leukocytes in semen are contributed largely by the prostate and the seminal vesicles (Wolff, 1995).

Activated leukocytes are capable of producing 100-fold higher amounts of ROS than nonactivated leukocytes (Plante et al., 1994). Leukocytes may be activated in response to a variety of stimuli including inflammation and infection (Pasqualotto et al., 2000a). Activated leukocytes increase NADPH production via the hexose monophosphate shunt. The myeloperoxidase system of both PMN leukocytes and macrophages is also activated, which leads to respiratory burst and production of high levels of ROS (Blake et al., 1987). Such an oxidative burst is an early and effective defense mechanism in cases of infection for killing the microbes (Saran et al., 1999).

Sperm damage from ROS that is produced by leukocytes occurs if seminal leukocyte concentrations are abnormally high (ie, leukocytospermia; Shekarriz et al., 1995a), if the patient has epididymitis, or if seminal plasma was removed during sperm preparation for assisted reproduction (Ochsendorf, 1999). However, Sharma et al (2001) observed that seminal leukocytes may cause OS even at concentrations below the WHO cutoff (ie, $<1 \times 10^6$ peroxidase positive leukocytes/mL semen) value for leukocytospermia. This may be due to the fact that seminal plasma contains large amounts of ROS scavengers but confers a variable (10% to 100%) protection against ROS generated by leukocytes (Kovalski et al., 1992).

Recent yet unpublished data from our center indicated that levels of ROS production by pure sperm suspensions from infertile men with a laboratory diagnosis of leukocytospermia were significantly higher than those in fertile men without leukocytospermia (Table 3). This observation led to the postulation that seminal leukocytes play a role in enhancing sperm capacity for excessive ROS production either by direct sperm-leukocyte contact or by soluble products released by the leukocytes. Studies are

### Table 2. Comparison of ROS levels in donors and in a nonselected group of infertility patients at different temperatures (values are median and interquartile range)

<table>
<thead>
<tr>
<th>Variable</th>
<th>ROS After 1-Hour Incubation at 4°C ($\times 10^6$ cpm)</th>
<th>ROS After 1-Hour Incubation at 25°C ($\times 10^6$ cpm)</th>
<th>ROS After 1-Hour Incubation at 37°C ($\times 10^6$ cpm)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donors (n = 12)</td>
<td>1.0 (0.2, 2.6)</td>
<td>1.4 (0.1, 3.1)</td>
<td>0.8 (0, 7.2)</td>
<td>0.60</td>
<td>0.84</td>
<td>0.002</td>
<td>0.75</td>
<td>0.008</td>
<td>0.004</td>
</tr>
<tr>
<td>Infertility patients (n = 12)</td>
<td>2.2 (0.3, 67.2)</td>
<td>2.2 (0.4, 61.2)</td>
<td>2.2 (0.3, 58.5)</td>
<td>0.60</td>
<td>0.18</td>
<td>0.001</td>
<td>0.40</td>
<td>0.004</td>
<td>0.04</td>
</tr>
</tbody>
</table>

ROS = reactive oxygen species measured in washed sperm suspensions (after simple wash and resuspension in PBS) by chemiluminescence assay. A = $P$ value of basal ROS vs. ROS after 1-hour incubation at 4°C. B = $P$ value of basal ROS vs. ROS after 1-hour incubation at 25°C. C = $P$ value of basal ROS vs. ROS after 1-hour incubation at 37°C. D = $P$ value of ROS after 1-hour incubation at 4°C vs. ROS after 1-hour incubation at 25°C. E = $P$ value of ROS after 1-hour incubation at 4°C vs. ROS after 1-hour incubation at 37°C. F = $P$ value of ROS after 1-hour incubation at 25°C vs. ROS after 1-hour incubation at 37°C. Wilcoxon rank-sum test was used for comparison, and $P$ value $<.05$ was significant.

### Table 3. Basal ROS levels in original cell suspensions (containing sperm and leukocytes) and in pure sperm suspensions (leukocyte-free sperm suspensions after complete removal of leukocytes using anti-CD45 coated paramagnetic beads) in 3 study groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Donors (n = 13)</th>
<th>Nonleukocytospermic (n = 32)</th>
<th>Leukocytospermic (n = 16)</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal ROS ($\times 10^6$ cpm)</td>
<td>0.4 (0.1, 2.5)</td>
<td>2.7 (0.53, 12)</td>
<td>178 (32, 430)</td>
<td>0.06</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pure sperm ROS ($\times 10^6$ cpm)</td>
<td>0.06 (0.01, 0.2)</td>
<td>0.31 (0.09, 1.2)</td>
<td>3.3 (0.5, 7.4)</td>
<td>0.05</td>
<td>0.001</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Values are median (25th, 75th percentiles). ROS = reactive oxygen species measured by chemiluminescence assay. 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 $P < .05$ level.
Currently underway to investigate this hypothesis. However, this new finding may have significant implications for the fertility potential of sperm both in vivo and in vitro. Excessive production of ROS by sperm in patients with leukocytospermia implies that both the free-radical generating sperm themselves and any normal sperm in the immediate vicinity will be 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.

Assessment of Seminal Oxidative Stress in the Andrology Laboratory

Extensive research in the field of male infertility has been conducted to develop adequate indices of OS that would help determine, with accuracy, whether OS is a significant contributor to male infertility (Sharma et al, 1999). Levels of OS vary greatly in infertile men (Alvarez et al, 1987). Because OS is an imbalance between levels of ROS production and antioxidant protection in semen, it is conceivable that assessment of OS will rely on the measurement of ROS as well as TAC of semen.

Protocol for Measurement of ROS by Chemiluminescence Assay—Levels of ROS can be measured in washed sperm suspensions using a chemiluminescence assay (Kobayashi et al, 2001; Figure 2). With this protocol, liquefied semen is centrifuged at 300 × g for 7 minutes, 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 washing media at a concentration of 20 × 10^6 sperm/mL. Four-hundred-microliter aliquots of the resulting cell suspensions (containing sperm and leukocytes) are used to assess basal ROS levels. Eight microliters of horseradish peroxidase (HRP) (12.4 units of HRP Type VI, 310 U/mg; Sigma Chemical Company, St Louis, Mo) are added to the cell suspension. The HRP sensitizes the assay so that it measures the extracellular H_2O_2. Ten microliters of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma), prepared as 5 mM stock in dimethyl sulfoxide (DMSO), are added to the mixture and serve as a probe.

Luminol is an extremely sensitive oxidizable substrate that has the capacity to react with a variety of ROS at neutral pH. The reaction of luminol with ROS results in production of a light signal that is then converted to electrical signal (photon) by a luminometer. 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 the lumi-
nometer (model LKB 953; Wallac Inc, Gaithersburg, MD) in the integrated mode for 15 minutes. The results are expressed as \(10^6\) counted photons per minute (cpm) per \(20 \times 10^6\) sperm. Normal ROS levels in washed sperm suspensions range from 0.10 to \(1.0 \times 10^6\) cpm per \(20 \times 10^6\) sperm.

**Protocol for Measurement of Total Antioxidant Capacity in Semen by Enhanced Chemiluminescence Assay**—
Total antioxidant capacity in the seminal plasma can be measured using an enhanced chemiluminescence assay (Kolettis et al, 1999). Frozen samples of seminal plasma are thawed at room temperature and immediately assessed for TAC. Seminal plasma is diluted 1:20 with deionized water (dH2O) and filtered through a 0.20-µm filter (Allegiance Healthcare Corporation, McGaw Park, Ill). Signal reagent is prepared by adding 30 µL of H2O2 (8.8 molar/L), 10 µL of para-iodophenol stock solution (41.72 µM), and 110 µL of luminol stock solution (3.1 mM) to 10 mL of Tris buffer (0.1 M pH 8.0). Horseradish peroxidase working solution is prepared from the HRP stock solution by making a dilution of 1:1 of dH2O. Light emission occurs when the chemiluminescent substrate luminol is oxidized by H2O2 in a reaction catalyzed by HRP. HRP catalyzes the reaction between a hydrogen acceptor (oxidant) and a hydrogen donor. Under normal circumstances, this reaction produces low-intensity light emission that may decay rapidly. The characteristics of the reaction can be altered substantially by the addition of para-iodophenol as an enhancer that gives a more intense, prolonged, and stable light emission. The continuous light output depends on the constant production of free radical intermediates derived from para-iodophenol (enhancer), luminol (substrate), and H2O2 (oxidizer) in the presence of HRP.

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 dH2O and mixed. The mixture is equilibrated to the desired level of chemiluminescence output (between 2.8 and 3.2 \(10^7\) cpm) for 100 seconds. One hundred microliters of the standard Trolox solution is immediately added to the mixture, and the chemiluminescence is measured. Suppression of luminescence and the time from the addition of standard Trolox solution to 10% recovery of the initial chemiluminescence are recorded. Plotting the three concentrations of Trolox solution versus 10% recovery time results in a linear equation (Figure 3).

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. 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 1-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 (Sharma et al, 1999).

**Quality Control of Oxidative Stress 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 sperm suspensions and TAC in seminal plasma. We have demonstrated that the luminol-dependent chemiluminescence assay for ROS measurement in washed sperm suspensions is both accurate and reliable when the sperm concentration is greater than \(1 \times 10^9\)mL and when the samples are analyzed within 1 hour after collection (Kobayashi et al, 2000). Similarly, assessment of TAC in seminal plasma using the enhanced chemiluminescence assay was found to be both accurate and reliable, with very low intraassay, interassay, and interobserver variations (\(P \geq 0.8\)) (unpublished data). The intraassay reliability was 91% [coefficient of variation (CV) = 5%], the interobserver reliability was 89% (CV = 13%), and interassay reliability was 92% (CV = 13%).

**ROS-TAC Score: An Accurate Method for Assessment of Seminal Oxidative Stress**—The fact that neither ROS alone nor TAC alone can adequately quantify seminal OS led to the logical conclusion that combining these two variables may be a better index for the diagnosis of overall OS affecting spermatozoa. This conclusion led to the introduction of the ROS-TAC score as a new method for assessment of OS status in infertile men (Sharma et al, 1999). The new ROS-TAC score is a statistical formula.
derived from levels of ROS in washed sperm suspensions and TAC in seminal plasma using principal component analysis. The resulting score minimizes the variability in the individual parameters of OS (ROS alone or TAC alone). The ROS-TAC score is an accurate measure of seminal OS and low ROS-TAC scores indicate high seminal OS (Sharma et al., 1999). A cutoff value of 30 was determined as the lower limit of normal range for the ROS-TAC score, and individuals with scores below this cutoff value were found to be at particular risk for prolonged inability to initiate pregnancies.

Assessment of ROS in Whole (Unwashed) Ejaculates: A Reliable Alternative for ROS-TAC Score—Our group has recently introduced an additional test of OS in which ROS levels are directly measured in the whole (unwashed) ejaculate following liquefaction (unpublished data). Semen samples in our study were collected from 34 infertile men and from 9 normal, healthy donors. Donors were selected on the basis of normal genital examination and normal standard semen parameters according to WHO (1999) guidelines. All semen specimens were produced by masturbation after the patients had abstained from sex for a period of 48 to 72 hours. Eight microliters of HRP were added to 400 μL- aliquots of liquefied semen. Levels of ROS were measured by the chemiluminescence assay using luminol (10 μL) as the probe with the luminometer set in the integrated mode for 15 minutes. The results were expressed as \( \times 10^4 \) cpm per 20 × 10⁶ sperm. In addition, levels of ROS in the washed sperm suspensions and TAC in seminal plasma were measured in aliquots from the same samples in order to calculate the ROS-TAC scores.

Our results indicated that levels of ROS (median [25th and 75th percentiles]) in the whole ejaculate of the normal donors (\( \times 10^4 \) cmap/20 million sperm/mL) were significantly lower than in infertile men (0.3 [0.2, 0.9] vs. 5.8 [1.0, 81.0] \( \times 10^4 \) cmap/20 million sperm/mL; \( P = .004 \)). The maximum level of ROS in the whole ejaculate of normal donors was 1.5 \( \times 10^4 \) cmap/20 million sperm/mL. Sixty-eight percent (23 of 34) of infertile men had ROS levels >1.5 \( \times 10^4 \) cmap/20 million sperm/mL and were classified as OS-positive. The remaining 32% (11 of 34) of infertile men had ROS levels ≤1.5 \( \times 10^4 \) cmap/20 million sperm/mL and were accordingly classified as OS-negative.

Leukocytospermia (concentrations greater than 1 \( \times 10^6 \) leukocytes/mL of semen) was found in 9% (1 of 11) of OS-negative samples and in 39% (9 of 23) of OS-positive samples. It is interesting that ROS-TAC scores were significantly higher (ie, lower OS, [49 {47, 52}] in the group classified as OS-negative based on ROS levels in whole [unwashed] ejaculates than the OS-positive group [36 {32, 44}], \( P = .001 \)). The OS-negative group scores were not significantly different from the normal controls (51, 55, \( P = .4 \)). In addition, levels of ROS in whole (unwashed) ejaculate were correlated positively with levels of ROS in washed sperm suspensions and negatively with ROS-TAC scores (Figure 4).

ROS levels in whole ejaculates were correlated negatively with sperm concentration (\( r = -.52, P = .0003 \)), motility (\( r = -.41, P = .006 \)) and morphology by the WHO method (\( r = -.34, P = .02 \)), and positively with seminal leukocyte concentrations (\( r = .65, P < .0001 \)). Furthermore, quality control studies of assessment of ROS in whole ejaculates indicated low variability (in-
tra assay [CV = 17, with 99% reliability] and inter assay [CV = 18, with 99% reliability]. Therefore, this test can be used as an alternative to ROS-TAC score for accurate and reliable assessment of seminal OS in the andrology laboratory. The strong positive correlation between levels of ROS in whole ejaculates and seminal leukocyte concentrations suggests that treatment of OS-positive patients should include plans for effective control of excessive leukocyte infiltration, antioxidant supplementation, or both.

Nitroblue Tetrazolium Test for Identification of ROS-Producing Cells in Semen—It is important to determine the source of ROS in a given semen sample because the clinical implications of infiltrating leukocytes are quite different from those of pathological conditions in which spermatozoa themselves are the source of ROS (Aitken, 1994). The luminol-dependent chemiluminescence assay that was described above helps assess levels of seminal OS by measuring the total amount of ROS in semen using a luminometer. This assay, however, does not provide information on the differential contribution of spermatozoa and leukocytes to ROS production in semen or on the state of activation of individual cells. Nitroblue tetrazolium (NBT) is a yellow, water-soluble dye that can be reduced by accepting electrons in the presence of free oxygen radicals to form a blue-black water-insoluble compound known as formazan (Baehner et al, 1967). The cytoplasmic NADH, which is produced by oxidation of glucose through the hexose monophosphate shunt, serves as an electron donor. The oxidase systems available in the cytoplasm help transfer electrons from NADH to NBT and reduce NBT into formazan (Baehner et al, 1967).

Thus, the NBT reaction indirectly reflects the ROS generating activity in the cytoplasm of cells and can help determine the cellular origin of ROS in a suspension containing a variety of cells such as semen. Earlier studies have shown that NBT reduction and formazan deposition in blood neutrophils are related to their phagocytic activity (Park et al, 1968; Segal and Levi, 1973). In the same studies, the state of activation of neutrophils was determined by scoring the blue-black formazan granules deposited in the cytoplasm. In a recent prospective study (unpublished data), we assessed ROS generating activity in semen from a group of infertile men using the NBT reduction test and also by the chemiluminescence assay. Nitroblue tetrazolium (0.1%) was prepared by adding 10 mg of NBT powder to 100 mL of PBS (pH 7.2) and stirring at room temperature for 1 hour. The NBT solution was filtered using a 0.2-micron filter. Equal volumes of NBT solution (0.1%) and unwashed semen were incubated for 30 minutes at 37°C. After another 30 minutes of incubation at room temperature, the mixture was centrifuged at 250 x g for 5 minutes. Smears were prepared from the pellet, air-dried, and stained with Wright stain. A total of 100 spermatozoa and 100 neutrophils were scored using 100× magnification. Leukocytes were scored as follows: cells filled with formazan (+++), intermediate density (+), scattered or few formazan granules (+), and no detectable formazan (−). Spermatozoa were scored as follows: formazan occupying ≤50% of cytoplasm (+) and >50% of cytoplasm (++).

The most important finding from our study was a significant increase in the percentage of NBT-positive leukocytes in semen from leukocytospermic samples (70%) as compared with nonleukocytospermic (14.5%) and donor samples (7%) (P < .0001). Furthermore, the results of the NBT test were inversely correlated with ROS-TAC scores. These results indicate that the NBT reduction test can help assess the differential contribution of leukocytes and defective spermatozoa to the amount of ROS generation in semen. The NBT test is easily performed and can be used as a routine andrology laboratory procedure to assess seminal OS without the need for expensive equipment such as the luminometer.

Clinical Significance of Assessment of Seminal Oxidative Stress

Currently, no single laboratory test can accurately and precisely assess a man’s total fertility (Evenson et al, 1999). The entire purpose of performing a diagnostic test in the evaluation of the male partner of a suspected infertile couple is to learn whether or not his fertility is impaired (Matson, 1997). Therefore, any test must then have a threshold above and below which it will provide discrimination and predictive capabilities with little overlap between fertile and infertile men. Determining which tests are valid in the evaluation of male infertility is difficult for a number of reasons. First, the term “male infertility” does not constitute a defined clinical syndrome but rather a collection of disparate conditions with a variety of causes and prognoses (Aitken et al, 1995). In addition, various clinical diagnoses are unable to determine the underlying cause of sperm dysfunction and pathophysiology of infertility. Relatively few men will have conditions causing absolute sterility such as azoospermia. Rather, the majority of infertile men have abnormal semen quality of varying severity and poorly understood etiology. Until the causes of male infertility are better understood, it is unlikely that any given descriptive test of sperm quality or sperm function will predict with absolute certainty that a man will be fertile or infertile in a given time period (Van Voorhis and Sparks, 1999).

The second reason that testing a man’s fertility is dif-
difficult to do is that confounding factors are always present. Tests of semen are confounded by intraindividual variability in the quality of semen samples. Semen quality can vary a great deal in the same individual due to factors such as days of abstinence from ejaculation, febrile illness, stress, and even problems with collection of the sample. Another important factor is the female partner's relative fertility, which can vary tremendously (Van Voorhis and Sparks, 1999).

With these facts in mind, the ultimate goal of our extensive research in the area of OS was to reach a consensus as to the clinical significance of seminal OS testing in an infertility clinic. The first step toward reaching such a consensus was to standardize the protocols for assessing seminal OS indices (ROS and TAC). Next, we developed methods for accurately assessing seminal OS by calculating the ROS-TAC scores or measuring ROS levels in unwashed semen. These important initial steps helped our group to determine levels of seminal OS in different clinical settings (Table 4). We found that levels of ROS were significantly higher in men with spinal cord injury, which were associated with poor sperm motility and morphology (Padron et al, 1997). We also found elevated levels of ROS in infertile men with varicoceles (Hendin et al, 1999). In a recent study, varicocelectomy was associated with a significant increase in pregnancy and live birth rates for couples who underwent intrauterine insemination, although standard semen parameters were not improved in all patients (Daitch et al, 2001). This observation led to our speculation that an improvement in pregnancy rates following varicocelectomy may be due to a functional factor not tested during standard semen analysis such as seminal OS. Patients with varicocele also had low levels of TAC in their seminal plasma and, therefore, may benefit from antioxidant supplementation (Hendin et al, 1999). An earlier study on rats has indicated that free radical scavengers such as SOD can prevent free radical-mediated testicular damage (Agarwal et al, 1997).

Across all clinical diagnoses, the ROS-TAC score was a superior discriminator between fertile and infertile men than either ROS or TAC alone (Sharma et al, 1999). The average ROS-TAC scores for fertile men who underwent vasectomy reversal was nearly identical to those of normal fertile donors (Kolettis et al, 1999). Infertile men with an idiopathic or male-factor diagnosis had significantly lower ROS-TAC scores compared to normal controls (Pasqualotto et al, 2000a; 2001). In addition, patients with a male-factor diagnosis who eventually initiated a successful pregnancy had significantly higher ROS-TAC scores than those who did not (Pasqualotto et al, 2001). Our results also indicated that infertile men with a diagnosis of chronic prostatitis or prostatodynia have significantly lower ROS-TAC scores than controls, irrespective of their leukocytespermia status (Pasqualotto et al, 2000a).

In a recent study (unpublished data), cigarette smoking in infertile men who had a normal genital examination was significantly correlated with higher levels of seminal OS, as evidenced by lower ROS-TAC scores (Table 5). Lower ROS-TAC scores in infertile smokers may be due to the significant increase in seminal ROS levels and decrease in TAC levels. The link between cigarette smoking and high seminal ROS may be at least in part related to the significant increase in leukocyte concentrations observed in semen of infertile smokers in the same study. An earlier study also reported an association between cigarette smoking in infertile men and greater leukocyte infiltration into semen (Close et al, 1990). The precise mechanism or mechanisms of greater seminal leukocyte infiltration into semen of infertile smokers is not clear. One potential explanation is that smoking metabolites may induce an inflammatory reaction in the male genital tract with a subsequent release of chemical mediators of

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>ROS</th>
<th>P value vs. Controls*</th>
<th>TAC (Trolox equivalent)</th>
<th>P value vs. Controls*</th>
<th>ROS-TAC Score</th>
<th>P value vs. Controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 24)</td>
<td>1.39 ± 0.73</td>
<td>0.0002</td>
<td>1650.93 ± 532.22</td>
<td>0.0002</td>
<td>50.00 ± 10.00</td>
<td>0.0001</td>
</tr>
<tr>
<td>Varicocele (n = 55)</td>
<td>2.10 ± 1.21</td>
<td>0.02</td>
<td>1100.11 ± 410.30</td>
<td>0.03</td>
<td>34.87 ± 13.54</td>
<td>0.0001</td>
</tr>
<tr>
<td>Varicocele with prostatitis (n = 8)</td>
<td>3.25 ± 0.89</td>
<td>0.0002</td>
<td>1061.42 ± 425.11</td>
<td>0.03</td>
<td>22.39 ± 13.48</td>
<td>0.0001</td>
</tr>
<tr>
<td>Vasectomy reversal (infertile; n = 23)</td>
<td>2.65 ± 1.01</td>
<td>0.0004</td>
<td>1389.89 ± 723.92</td>
<td>0.30</td>
<td>33.22 ± 15.24</td>
<td>0.0002</td>
</tr>
<tr>
<td>Vasectomy reversal (fertile; n = 12)</td>
<td>1.76 ± 0.86</td>
<td>0.80</td>
<td>1876.93 ± 750.82</td>
<td>0.62</td>
<td>49.35 ± 12.25</td>
<td>1.00</td>
</tr>
<tr>
<td>Idiopathic infertility (n = 28)</td>
<td>2.29 ± 1.20</td>
<td>0.01</td>
<td>1051.98 ± 380.88</td>
<td>0.0003</td>
<td>32.25 ± 14.40</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Pairwise P values from the Student t-test adjusted using the Dunnett method. ROS = reactive oxygen species in washed sperm suspensions (after simple wash and resuspension in PBS) measured by chemiluminescence assay. TAC = total antioxidant capacity in seminal plasma measured by enhanced chemiluminescence assay. ROS-TAC score is a statistical formula calculated using principal component analysis.

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**Table 4. Mean and standard deviation of levels of ROS, TAC, and ROS-TAC score in subgroups of infertile men and controls**
inflammation. Inflammatory mediators such as interleukin (IL)-6 and IL-8 can recruit and activate leukocytes (Comhaire et al, 1999). In turn, activated leukocytes can generate high levels of ROS in semen, which may overwhelm the antioxidant strategies, resulting in OS (Aitken et al, 1999). Another theory is that toxic metabolites of cigarette smoke may impair spermatogenesis, and leukocytes infiltrate the male reproductive tract to eliminate defective spermatozoa by phagocytosis (Tomlinson et al, 1992). Blood and seminal plasma cotinine levels in fertile (n = 107) and infertile (n = 103) men who smoke were correlated with the number of cigarettes smoked per day (Wong et al, 2000). A statistically significant correlation was found between cotinine concentrations in seminal plasma and the percentage of abnormal sperm morphology. Chia et al (1994) examined the relationship between cigarette smoking, and blood and seminal plasma concentrations of cadmium, and sperm quality in 184 men who were undergoing initial screening for infertility. They found that levels of cadmium in blood and seminal plasma were negatively correlated with semen volume and sperm concentrations.

An additional factor that may explain why semen from smokers had higher levels of ROS may be that cigarette smoke itself contains high levels of ROS such as superoxide anion, H₂O₂, and hydroxyl radicals (Stone and Bermedes, 1986; Church and Pryor, 1990). The finding of higher levels of seminal OS in association with cigarette smoking is of significance and may have important implications in the fertilizing potential of infertile men.

**Summary**

Production of very low amounts of ROS in semen appears to play a physiological role in regulating normal sperm functions, whereas high levels of ROS endanger sperm function and viability. Oxidative stress due to excessive production of ROS, impaired antioxidant defense mechanisms, or both precipitates a range of pathologies that are currently believed to negatively affect the male reproductive function. Oxidative stress-induced damage to sperm may be mediated by lipid peroxidation of the sperm plasma membrane, reduction of sperm motility, and damage to the DNA in the sperm nucleus. Despite the established role of OS in the pathogenesis of male infertility, there is a lack of consensus as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to utilize the OS test in clinical practice is related to the lack of a standard protocol for assessment of seminal OS.
Introduction of ROS-TAC score as an accurate measure of seminal OS by our program has helped in our effort to translate the important information gained on OS from the research bench to the clinical practice of andrology. In addition, results of our recent studies indicate the availability of alternate protocols for assessment of seminal OS by measuring ROS directly in the whole (unwashed) ejaculate or assessing the differential contribution of spermatozoa and leukocytes to ROS production in semen using the NBT reduction test.

Our results indicate that the diagnostic and prognostic capabilities of the seminal OS test are beyond those of conventional tests of sperm quality and function. The OS test can accurately discriminate between fertile and infertile men and identify patients with a clinical diagnosis of male-factor infertility that are likely to initiate a pregnancy when followed over a period of time. In addition, the test can help select subgroups of infertile men in whom OS is a significant factor and who might benefit from antioxidant supplementation. We strongly believe that incorporating such a test into the routine andrology workup is an important step for the future of the male infertility practice.

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