

SHOULD SEMINAL OXIDATIVE STRESS MEASUREMENT BE OFFERED ROUTINELY TO MEN PRESENTING FOR INFERTILITY EVALUATION?

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ABSTRACT

Objective: To determine if seminal oxidative stress measurement should be offered routinely to men presenting for infertility evaluation.

Methods: We performed an extensive review of the English-language literature by searching MEDLINE for studies published between 1980 and 2007.

Results: Research conducted during the last decade has provided growing support for the concept that excessive production of reactive oxygen species (ROS) is related to abnormal semen parameters and sperm damage. Routine semen analysis remains the backbone of clinical evaluation in male infertility, but determining the levels and sources of excessive ROS generation in semen is currently not included in the routine evaluation of subfertile men. However, the diagnostic and prognostic capabilities of seminal oxidative stress measurement exceed the capabilities of conventional sperm quality tests. An oxidative stress test may accurately discriminate between fertile and infertile men and identify those 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, such a test can help select subgroups of patients with infertility in which oxidative stress is an important factor and those who may benefit from antioxidant supplementation. Although consensus is still required about the type and dosage of

antioxidants to be used, rationale and evidence exist supporting their use in infertile men with elevated oxidative stress.

Conclusion: Consensus is growing about the clinical utility of seminal oxidative stress testing in infertility clinics, but standardization of protocols to measure ROS is crucial before introducing these tests into routine clinical practice. (**Endocr Pract.** 2008;14:484-491)

Abbreviation:

ROS = reactive oxygen species

INTRODUCTION

Free radicals are a group of highly reactive chemical molecules with 1 or more unpaired electrons that can oxidatively modify biomolecules that they encounter. Consequently, free radicals react almost immediately with any substance in their surrounding area, beginning a chain reaction that finally leads to disruption of the cells (1). Reactive oxygen species (ROS) comprise a wide group of molecules that includes a collection of radicals and non-radical oxygen derivatives. In addition, there is another class of free radicals that are nitrogen derived, called reactive nitrogen species (2). These reactive species are readily transformed into reactive nonradical species by enzymatic or nonenzymatic chemical reactions that in turn can give rise to new radicals.

In healthy men, a delicate balance exists between ROS production and the antioxidant scavenging system in the male reproductive tract (3). Low physiologic levels of ROS are essential in the regulation of sperm functions including sperm capacitation, acrosome reaction, and sperm-oocyte fusion (4,5). Oxidative stress is the condition that exists once oxidants outnumber antioxidants (6,7). It is a frequent situation caused by biologic systems in aerobic conditions such that antioxidants cannot scavenge the free radicals. This leads to an excess of ROS, which damages cells, tissues, and organs (8,9).

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IMPORTANCE OF ASSESSING SEMINAL OXIDATIVE STRESS

Elevated seminal ROS levels have potentially toxic effects on both sperm quality and function (10-12). Excessive ROS production in semen has been correlated with poor sperm motility, defective acrosome reaction, and impaired fertility (13). The degree of ROS-related damage occurring in sperm cells depends on the nature, amount, and duration of their exposure to excessive free radicals. Studies also suggest that the integrity of the DNA sperm nucleus is jeopardized by ROS attacks that result in base modifications, DNA strand breaks, and chromatin cross-linking (12,14).

The large quantities of polyunsaturated fatty acids present in the sperm plasma membrane and low concentrations of scavenging enzymes in their cytoplasm afford spermatozoa only limited defense mechanisms against oxidative attack on their DNA (4,11). These mechanisms also include the packaging arrangement of DNA and seminal plasma presence. As a result, spermatozoa are unable to repair damage provoked by excessive ROS (15,16). Pathologic levels of ROS detected in the semen of infertile men are more likely caused by increased ROS production than by reduced antioxidant capacity of the seminal plasma (17).

In vivo, such damage may not be a cause for concern because collective peroxidative damage to the sperm membrane ensures that oxidative stress-exposed spermatozoa are unable to participate in the fertilization process. These safeguards, however, are circumvented during intracytoplasmic sperm injection, and therefore, spermatozoa with DNA fragmentation may produce unfavorable results. Reactive oxygen species also may initiate a chain reaction that ultimately leads to apoptosis. Recently, ROS levels were positively associated with apoptosis in mature spermatozoa (18,19).

OXIDATIVE STRESS POTENTIAL IN INFERTILITY SUBGROUPS

Idiopathic Infertility

Infertility affects approximately 15% of all couples attempting to conceive, and male factor infertility is the sole or contributing cause in nearly half of these cases. The term idiopathic infertility is applied to the large population of men with apparently normal semen parameters who experience problems impregnating their partners (20). Men with idiopathic infertility commonly have significantly higher seminal ROS levels and a lower antioxidant potential than healthy fertile controls (9,21). High ROS levels have been detected in 25% to 40% of infertile male semen samples (22,23).

Varicocele

Varicocele is implicated as a factor in 35% to 50% of men with primary infertility and in up to 81% of men with secondary infertility (24). The association between seminal ROS levels and varicocele has been well documented over the past few years (25-27). Mitropoulos and colleagues found high levels of nitric oxide synthase, xanthine oxidase, nitric oxide, peroxynitrite, and S-nitrosothiols in dilated varicocele spermatic veins compared with peripheral blood samples (25). According to a new meta-analysis from our center, patients with varicocele had significantly higher ROS and lipid peroxidation and decreased antioxidant concentrations than were found in healthy sperm donors (28). Another recent study reported a positive correlation between seminal ROS levels and varicocele grade (29). In a study of 25 infertile men by Koksai et al, those with grade III varicocele had significantly higher levels of malondialdehyde than did those with grade I or II varicocele. These findings indicate that higher grades of varicocele are associated with increasing oxidative stress levels (30). Patients with varicocele also present with low seminal plasma antioxidant capacity levels and increased 8-hydroxy-2'-deoxyguanosine levels, indicating a deficient antioxidant defense system and increased oxidative DNA damage, respectively (26,31).

Recently, Mostafa et al demonstrated that varicocelectomy reduces seminal plasma ROS levels and increases concentrations of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, and vitamin C in seminal plasma from infertile men (32). Cervellione et al showed that peroxidative plasma levels were significantly reduced 1 year after varicocele repair in adolescents (33). Shiraishi et al showed that levels of the oxidative stress marker testicular 4-hydroxy-2-nonenal modified proteins were significantly higher in patients who responded to varicocele repair compared with those who did not demonstrate any change in sperm motility postoperatively (34). The authors suggest that varicocelectomy reduces oxidative stress in the testis, and that the proteins identified in the study can predict postoperative improvement in spermatogenesis.

Genitourinary Infection

The World Health Organization defines leukocytospermia as leukocyte counts greater or equal to $1 \times 10^6/\text{mL}$ of semen (35). The incidence of leukocytospermia among infertile male patients is approximately 15% (36). Although the exact importance of leukocytes in semen is controversial, many studies have found that leukocytospermia is correlated with diminished sperm motility and fertilization capacity (37-39).

Recent studies have shown that even leukocyte counts below $1 \times 10^6/\text{mL}$ were associated with significant production of seminal ROS and decreased sperm DNA integ-

rity (40,41). Peroxidase-positive leukocytes are the most important source of ROS in semen and include polymorphonuclear leukocytes, which represent 50% to 60% of all seminal leukocytes, and macrophages, which represent 20% to 30% (42). The capacity of leukocytes to produce ROS depends on their activation, which may occur in response to a variety of stimuli such as inflammation or infection (43). After stimuli, nicotinamide adenine dinucleotide phosphate production is elevated, and the activated myeloperoxidase system of leukocytes results in a respiratory burst with the subsequent liberation of a high quantity of ROS (44).

Many reports have shown that seminal leukocyte ROS production induces spermatozoal damage during sperm preparation for assisted reproduction (9,16,40). Men with genitourinary accessory gland infection exhibit both leukocytospermia and elevated ROS levels. In these men, sperm function defects may be due to abnormal lipid peroxidation stimulated by the high ROS levels secondary to infection (45). A correlation between prostatitis and male infertility has been reported previously, but the mechanism responsible is still poorly understood (43). Prostatitis is associated with the existence of granulocytes in prostatic fluid. However, even without leukocytospermia, increased seminal oxidative stress has been reported in men with chronic prostatitis and prostatodynia (43).

Spinal Cord Injury

Spinal cord injury typically occurs in young men. Many of these men desire to father children, but they usually have poor seminal quality. This most likely is due to sperm damage caused by genital tract stasis resulting from a lack of peristalsis (46). Padron and colleagues reported that seminal ROS levels were significantly elevated in men with spinal cord injury and were associated with poor sperm motility and morphology (23). In a 1995 study, de Lamirande et al reported that seminal samples and Percoll-washed spermatozoa from men with spinal cord injuries produced ROS at much higher frequency and levels than did equivalent preparations from infertile men or healthy volunteers. They also found an inverse correlation between motility and ROS production in Percoll-washed spermatozoa from men with spinal cord injuries. Hence, they observed that high ROS levels may play an important role in the low sperm motility and infertility observed in these men (47).

Environmental Factors and Lifestyle Behaviors

Many reports have suggested that seminal quality decreases with advancing age. However, a decline in semen quality over time independent of increasing age also has been documented in fertile men (48). Environmental pollution; occupational exposure to industrial agents; radiation; heavy metals; and lifestyle risk factors such as smoking, caffeine, and alcohol intake may play a role in

this trend (49,50). Whatever the exposure route, environmental factors clearly can affect the development and function of the male reproductive tract through hormonal regulation of spermatogenesis and/or direct gonadotoxicity. A recent prospective study found smoking to be associated with a 48% increase in seminal leukocyte concentrations. Additionally, the infertile men who smoked cigarettes had higher levels of seminal oxidative stress than the infertile nonsmokers (51). Not surprisingly, the authors suggested that physicians should advise infertile men to quit smoking. Oxidative stress may also play an important role in the poor sperm quality caused by exposure to xenobiotics such as pesticides, herbicides, cosmetics, preservatives, cleaning materials, municipal and private waste, pharmaceuticals, and industrial byproducts (52).

DETERMINATION OF SEMINAL OXIDANTS

The apparent strong correlation between high oxidative stress levels and reduced fertility makes measurement of ROS a useful tool in the evaluation of infertile men (7). Currently, clinical practice as to the inclusion of ROS measurement is variable, primarily because of the lack of standardization of ROS analytic methods, equipment, and range of normal levels of ROS in semen (41). The evidence defining high ROS levels as a cause or an effect of abnormal semen parameters and sperm damage is still insufficient on both sides of the question (53). However, a recent study reported high levels of ROS as an independent marker of male factor infertility in leukocytospermic samples after adjustment for semen characteristics (53). This finding suggests that ROS may play an important role in the etiology of male factor infertility and encourages the use of ROS measurement as a diagnostic tool in clinical practice, particularly in cases of idiopathic infertility.

Numerous assays for ROS measurement have been introduced (Table 1). The *chemiluminescence assay*, the

Table 1
Methods of Determining Oxidative Stress

Method
Direct
Chemiluminescence
Nitro blue tetrazolium reduction
Cytochrome-c reduction
Flow cytometry
Electron spin resonance spectroscopy
Xylenol orange-based assay
Indirect
Thiobarbituric acid-reactive substances
Isoprostane
DNA damage
Total antioxidant capacity

most commonly used technique (6,54), quantifies both intracellular and extracellular ROS. Depending on the probe used (luminol [5-amino-2,3-dihydro-1,4-phthalazine-dione; also, 3-aminophthalic hydrazide] and lucigenin [*N,N'*-dimethyl-9,9'-biacridinium dinitrate]), this method can differentiate between spermatozoic production of superoxide and hydrogen peroxide. The most important confounding factor is the presence of leukocytes, and the assay's main limitation is the need for fresh semen samples with a high sperm count ($>1 \times 10^6/\text{mL}$) (54). The chemiluminescence assay does not provide information on the differential contribution of spermatozoa and leukocytes to ROS production in semen. Determining the exact source of ROS is crucial because the clinical implications of infiltrating leukocytes are quite different from those of pathologic conditions in which spermatozoa themselves are the source of ROS (40). Other factors that can interfere with chemiluminescence measurement include the concentration of reactants, sample volume, reagent injection, temperature control, instrument sensitivity, and background luminescence. Of the various types of luminometers commercially available to measure the light intensity resulting from the chemiluminescence reaction, single/double tube luminometers are relatively inexpensive, sensitive, and suitable for most clinical laboratories (54).

Nitro blue tetrazolium reduction is a readily available, easily performed, inexpensive, and highly sensitive test for assessing the differential contribution of spermatozoa and leukocytes to ROS production in semen (55). *Cytochrome c reduction* is the criterion standard for detecting extracellularly released superoxide in *in vitro* assays and activated leukocytes. Cytochrome c does have restricted intracellular access that limits its ability to measure superoxide formation within intact cells as opposed to cellular or tissue extracts (56). *Flow cytometry* measures the fluorescent intensity of compounds oxidized by ROS. Fluorescent probes like 2,7-dichlorodihydrofluorescein diacetate, dihydroethidine, and dihydrorhodamine 123 can be oxidized by ROS generated within the cell, making them highly fluorescent. The fluorescence can be quantified, which reflects the rate and quantity of the ROS produced (57). This assay can be used to identify sperm populations that may be dysfunctional because of intracellular ROS with the advantage of measuring intracellular ROS exclusively in the viable portion of the sperm population. ROS measured by methods other than flow cytometry might be present extracellularly only or in a small proportion of primarily nonviable sperm (58). *Electron spin resonance spectroscopy* uses the magnetic properties of unpaired electrons to detect free radicals. It is the most direct and least ambiguous method for detecting free radicals of interest without artifacts from added chemicals (59), and efforts are underway to develop this technique for the *in vivo* evaluation of radical generation and redox status. Recently, a new colorimetric automated test based on the *xylenol orange assay* has been proposed.

Proponents claim it to be a rapid, easy, stable, reliable, sensitive, inexpensive, and fully automated assay that merits further investigation (60).

Seminal oxidative stress also can be assessed indirectly by measuring ROS-induced lipid peroxidation levels by either the *thiobarbituric acid-reactive substances* or *isoprostane* methods. While the thiobarbituric acid-reactive substances method is the most widely employed assay for screening and monitoring lipid peroxidation, it lacks specificity (61). Measurement of isoprostane is a reliable and attractive marker of measuring oxidative stress *in vivo* (62). Another useful indirect marker of seminal oxidative stress is *sperm DNA damage*. Some of the currently available tests that are used to evaluate the integrity of sperm DNA include terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling, sperm chromatin structure assay, comet assay, *in situ* nick translation, and DNA breakage detection fluorescent *in situ* hybridization assay. The level of sperm DNA fragmentation (DNA fragmentation index measured by the sperm chromatin structure assay) appears to be related to *in vitro* fertilization pregnancy rates and embryo quality (63). The odds of achieving pregnancy with intrauterine insemination is 16 times higher in patients with a DNA fragmentation index less than 27% compared with patients with a DNA fragmentation index greater than 27% (64). Miscarriage rates are highest with intracytoplasmic sperm injection in patients with a DNA fragmentation index greater than 30% (63,65). These findings suggest that DNA fragmentation index status determined by the sperm chromatin structure assay has the potential to become a useful tool in infertility evaluation.

Because seminal oxidative stress is an imbalance between ROS production and antioxidant protection, it can be measured by assessing the *total antioxidant capacity* by colorimetric assay (66), a simple, rapid, relatively inexpensive, and reliable methodology. Neither measurement of ROS levels nor total antioxidant capacity alone can quantify oxidative stress precisely, but it is possible to combine these 2 parameters into 1 index that minimizes the variability of the individual parameter scores (7). The *ROS-total antioxidant capacity score* was found to be better than ROS or total antioxidant capacity alone in discriminating between fertile and infertile men. The average ROS-total antioxidant capacity score for fertile, healthy men was significantly higher than that of infertile patients. The probability of successful pregnancy was less than 10% when values of ROS-total antioxidant capacity were below 30, and the probability increased as the ROS-total antioxidant capacity score increased.

Assessment of ROS levels in neat semen has proven to be more accurate and reliable for evaluating oxidative stress status than assessments made in washed specimens (67). Direct ROS measurements made in neat semen have diagnostic and prognostic capabilities identical to those of

the ROS–total antioxidant capacity score. This technique accurately represents an individual's true in vivo oxidative stress status and overcomes the drawbacks of earlier methods that involve processing semen, a step that may generate ROS by itself. The ROS level for healthy donors with normal standard semen parameters is 1.5×10^4 cpm/20 million sperm/mL. Using this value as a cutoff, infertile men can be classified as either oxidative stress-positive ($>1.5 \times 10^4$ cpm/20 million sperm/mL) or oxidative stress-negative ($\leq 1.5 \times 10^4$ cpm/20 million sperm/mL), regardless of their clinical diagnosis or standard semen analysis results (67).

ROLE OF ANTIOXIDANTS IN MANAGEMENT OF MALE INFERTILITY

Because of the major role of oxidative stress in the pathogenesis of male infertility, treatment strategies to reduce seminal oxidative stress levels would enhance natural conception as well as assisted reproductive technologies. Spermatozoa produce low levels of ROS that must be inactivated continuously to maintain ROS at the level needed for normal physiologic cell functions (17,68).

Antioxidants are the most important defense against free radical–induced oxidative stress. Both the enzymatic and nonenzymatic antioxidants contained naturally in the seminal plasma can minimize free radical–induced damage. Three natural enzyme systems are known to protect spermatozoa against oxygen toxicity: catalase, glutathione peroxidase, and superoxide dismutase (69). In addition to the enzymatic defenses, other compounds present in human semen such as albumin, α -tocopherol, β -carotene, lycopene, urate, and ascorbic acid play an important role in protecting the spermatozoa (70). Nonenzymatic antioxidants can be classified as preventative or scavengers. Metal chelators and metal-binding proteins are preventative antioxidants that block the formation of new ROS (68). Vitamins E and C, β -carotene, glutathione and enzymes, and other antioxidant dietary supplements act as scavengers to remove ROS already generated by cellular oxidation (15).

A variety of clinical trials have reported the beneficial effects of antioxidants in selected cases of male infertility (71–77), whereas others have failed to report similar benefits (78,79). Pregnancy, the most relevant outcome parameter of fertility, was reported in only a few published studies. A randomized double-blind placebo-controlled trial demonstrated significant improvement in sperm motility ($P < .001$) in 60% of asthenozoospermic patients with 300 mg twice a day of vitamin E compared with 11% in the placebo arm. The pregnancy rate was 21% (11/53) in the control group whereas no pregnancies were reported in the placebo group (71). A dosage of 600 mg/d of oral vitamin E has been shown to improve sperm function as assessed by zona binding test in a randomized crossover study (78). Comhaire et al demonstrated improvement in pregnancy

rate with combination therapy of vitamin E and acetylcysteine or vitamin A, E, and essential fatty acids in infertile men (72). In a more recent double-blind randomized controlled trial by the same group, treatment with a strong antioxidant, Astaxanthin, 16 mg/d for 3 months, increased total (54.5% vs 10.5%) and per cycle (23.1% vs 3.6%) pregnancy rates compared with the placebo group (73). When infertile men with high production of ROS were treated with 600 mg glutathione intramuscularly, significant increase in sperm motility ($P < .003$) and sperm forward progression ($P < .001$) was seen within 30 days (74). In another double-blind placebo-controlled crossover trial involving 100 infertile patients, 8 pregnancies were achieved in the observation period of oral levocarnitine therapy (75). Likewise, Vicari and Calogero demonstrated that carnitines (levocarnitine, 1 g twice a day, and acetylcarnitine, 0.5 g twice a day) for 3 months improved sperm forward progression and viability in abacterial nonleukocytospermic prostatico-vesiculo-epididymitis and led to spontaneous pregnancy in 12% (4/34) of the patients (76). However, Rolf et al found no change in the sperm parameters and pregnancy rates after high-dose oral treatment with vitamin C and E for 56 days in infertile men (79).

Assisted reproductive techniques also have shown significantly higher success rates with in vitro supplementation of antioxidants and metal chelators (5). In a prospective randomized double-blind trial involving 60 couples with severe male factor infertility, 1 capsule a day of Menevit antioxidant for 3 months before the partner's in vitro fertilization cycle improved the viable pregnancy rate compared with the placebo group (38.5% vs 16% of transferred embryos) (77).

Some of the problems in assessing the efficacy of antioxidants are the ability of spontaneous improvement in semen quality and pregnancy being dependent on female fertility status in addition to the semen quality. The lack of consensus regarding the use of antioxidants in infertile men is because of insufficient studies and heterogeneous methods used in the studies conducted. Patient selection must also be considered in the forthcoming studies since oxidative stress may not be the only cause of male infertility. Effectiveness of antioxidant therapy depends on the cause of infertility (5). Therapeutics directed against each specific etiology of elevated ROS should be attempted first. Once the primary cause of infertility has been treated or no specific etiology can be identified (idiopathic infertility), patients may be advised to take optimal antioxidant supplementation doses.

FUTURE TRENDS

The discovery of biomarkers is generating interest worldwide as recognition of their role in many reproductive disorders expands. Identification of key biomarkers

has the potential for important diagnostic and therapeutic interventions. This realization has led to the development of a new scientific discipline—metabolomics.

Metabolomics is the systematic study of the inventory of metabolites as small molecule biomarkers representing the functional phenotype in a cell, tissue, or organism. Recent research suggests that biomarkers of oxidative stress (–CH, –NH, –SH, C=C, and –OH) can be quantified in semen using this technology platform based on various forms of analytic, biochemical, and spectral analyses. Different levels of oxidative stress biomarkers are uniquely associated with normal semen plasma vs different forms of male factor infertility (80). In the future, metabolomic semen profiling using near-infrared spectroscopy and proprietary chemometrics and bioinformatics may prove to be a rapid, noninvasive, and cost-effective technique for detecting semen abnormalities related to ROS damage and oxidative stress.

CONCLUSION

Research conducted over the last decade has bolstered support for the concept that excessive production of ROS is related to abnormal semen parameters and sperm damage. Routine semen analysis remains the backbone of clinical evaluation in male infertility, but determining the levels and sources of excessive ROS generation in semen currently is not included in the routine evaluation of subfertile men. The evaluation of seminal ROS levels in infertile men may help develop new therapeutic strategies and improve pregnancy rates through sexual intercourse as well as through assisted reproductive techniques. An oxidative stress test may accurately discriminate between fertile and infertile men and identify those 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, such a test can help select subgroups of patients with infertility in which oxidative stress is an important factor and those who may benefit from antioxidant supplementation. Although consensus is still required about the type and dosage of antioxidants to be used, rationale and evidence exist supporting their use in infertile men with elevated oxidative stress. As diagnostic and prognostic capabilities of seminal oxidative stress measurement exceed the capabilities of conventional sperm quality tests, consensus regarding the clinical utility of seminal oxidative stress testing in infertility clinics is growing. Standardization of protocols to measure ROS is crucial before introducing these tests into routine clinical practice.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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