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Oxidative Stress in Normospermic Men Undergoing Infertility Evaluation

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ABSTRACT: The purpose of this study was to determine whether normospermic infertile men have high seminal oxidative stress, using 3 measures of oxidative stress: reactive oxygen species (ROS), total antioxidant capacity (TAC), and a composite ROS-TAC score. Forty-three normospermic men without leukocytospermia and 19 healthy donors who came to our infertility clinic were included. Patients were categorized into 3 groups: group I, varicocele and no female factor (n = 16); group II, positive female factor (n = 16); and group III, idiopathic infertility (n = 11). In addition, 52 treated male factor patients and 19 donors were included as reference groups. We measured seminal ROS, TAC, and the ROS-TAC score in the patient groups and the controls. Normospermic infertile patients as a group had higher ROS levels (mean log [ROS + 1] 1.76 ± 0.13) compared with controls (1.39 ± 0.16; P = .03). Patients in the idiopathic subgroup had significantly higher ROS levels (2.29 ± 0.25; P = .004) than controls. Normospermic infertile patients as a group not only had reduced TAC levels (970.18 ± 73.95 Trolox equivalents), but each subgroup also had significantly lower TAC than controls (1650.93 ± 95.87; P < .003). The ROS-TAC scores in all normospermic infertile patients as a group (35.7 ± 1.8) as well as in each subgroup was significantly reduced compared with the ROS-TAC levels in the controls (50.0 ± 2.1; P < .005). We conclude that oxidative stress is associated with male factor infertility. The presence of oxidative stress in infertile normospermic men may explain previously unexplained cases of infertility otherwise attributed to female factors.

Key words: Antioxidants, male infertility, reactive oxygen species, spermatozoa.
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Free radicals are important in both the normal function and the pathophysiology of human spermatozoa (Jones et al, 1979; Aitken, 1999). Human spermatozoa rely on reduction-oxidation processes for normal functions such as hyperactivation, capacitation, and acrosome reaction (Sharma and Agarwal, 1996; Aitken, 1997). However, spermatozoa are also vulnerable to peroxidative damage from oxygen free radicals because the spermatozoal membrane is rich in polyunsaturated fatty acids needed to maintain membrane fluidity for membrane fusion during fertilization (Alvarez et al, 1987; Aitken and Clarkson, 1989; Sharma and Agarwal, 1996; Aitken, 1997). High levels of reactive oxygen species (ROS) can increase sperm membrane permeability, cause morphological abnormalities, and impair fertility (Alvarez et al, 1987; Aitken et al, 1992; de Lamirande and Gagnon, 1993; de Lamirande and Gagnon, 1995; Alkan et al, 1997). Studies have shown that 40% to 88% of nonselected infertile men have high levels of ROS (Lewis et al, 1995).

Most ROS affecting human spermatozoa come from 2 sources, defective spermatozoa and seminal leukocytes, commonly found in ejaculate from patients with accessory gland infections (Aitken et al, 1992,; Shekarriz et al, 1995). Sperm from humans and most other mammals possess a number of defensive mechanisms to prevent damage from ROS (de Lamirande and Gagnon, 1995; Lewis et al, 1995; Sharma and Agarwal, 1996; Smith et al, 1996; Lewis et al, 1997). Antioxidant enzymes are abundant in seminal plasma (Smith et al, 1996; Lewis et al, 1997; Geva et al, 1998). Additional antioxidant enzymes found in sperm cytoplasm, such as superoxide dismutase, glutathione peroxidase/reductase, and catalase, are not very effective against extracellular free radicals (Smith et al, 1996; Lewis et al, 1997; Geva et al, 1998; Zini et al, 1998).

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Excessive oxidative stress may be associated with serious defects in semen characteristics, such as severe oligospermia and asthenospermia (Aitken et al, 1992; Iwasaki and Gagnon, 1992; Huszar and Vigue, 1994; Lewis et al, 1995; Sharma and Agarwal, 1996; Griveau and de Lannou, 1997; Aitken, 1999). However, many men seeking infertility treatment have normal semen characteristics according to the World Health Organization (WHO) standards (WHO, 1999). We have speculated that infertility in these men may be explained by oxidative stress.

The purpose of this study was to assess oxidative stress in normospermic men without leukocytospermia who were seeking treatment for infertility. We measured ROS levels and total antioxidant capacity (TAC) in semen samples and computed a composite ROS-TAC score. To determine which of the 3 measures of oxidative stress was the most useful, we also used receiver-operating characteristics (ROC) curves to compare their ability to distinguish the healthy controls from the infertile patients.

Materials and Methods

Subjects

The study was approved by the Institutional Review Board of the Cleveland Clinic Foundation. Semen specimens were obtained from 299 consecutive patients attending the male infertility clinic for infertility evaluation from 1998 to 1999. All patients were evaluated with a complete medical history, physical examination, and semen analysis. Men were excluded if they had less than 1 year of infertility.

Forty-six of the patients were considered normospermic (WHO, 1999). Three of these patients were excluded because of leukocytospermia, defined as a white blood cell count of 1×10^6/mL or higher. Men were evaluated for abnormalities related to male infertility such as undescended testis, varicocele, atrophic testis, or chronic prostatitis, which were considered male factors. Their partners were evaluated for abnormalities related to infertility, such as tubal occlusion, endometriosis, or ovulatory dysfunction, which were considered female factors. Forty-three normospermic patients were divided into three subgroups: group I-varicocele and no female factor (n = 16), group II-positive female factor (n = 16), group III-idiopathic infertility (n = 11). In addition, semen specimens from 19 healthy normal men were used as a control group and the role of oxidative stress in fertility was assessed by comparisons with 52 men of known fertility status following treatment for male factor diagnoses. The group of men with known fertility status (n = 52) included 13 fertile and 39 infertile patients from our previous study (Sharma et al, 1999) with male factor diagnoses of varicocele and vasectomy reversal. These men had an average follow-up period of 17.4 ± 12.3 months (median, 12.6 months). Out of these 52, 13 (25%; 12 vasectomy reversal and 1 varicocele) patients eventually initiated normal pregnancies.

Semen Analysis

Semen samples were obtained from patients and controls by masturbation after at least 48 to 72 hours of abstinence. Samples were collected into sterile containers for immediate analysis. Following liquefaction at 37°C for 30 minutes, semen samples were analyzed for sperm concentration, percentage motility, and morphology according to WHO criteria. Computer-assisted semen analysis (CASA) was performed on all specimens with use of a Motion Analysis VP 50 semen analyzer (Motion Analysis Corporation, Santa Rosa, Calif). For each measurement, a 5-µL aliquot was loaded on a counting chamber (MicroCell; Conception Technologies, La Jolla, Calif). Four to 8 representative fields containing 200 or more spermatozoa were examined. Samples were analyzed for concentration, percent motility, and complex motion characteristics. To ensure the accuracy of the CASA results, a manual assessment was done each time along with CASA analysis.

White Blood Cell Count

White blood cells (WBCs) in semen specimens were stained with a myeloperoxidase test (Shekarriz et al, 1995). A 20-µL volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar,

Corp Cambridge, Mass): 20 µL of phosphate-buffered saline (PBS; pH 7.0) and 40 µL of benzidine solution was added. The mixture was vortexed and allowed to sit for 5 minutes. Five microliters were placed on a Makler Chamber (Sefi Medical, Haifa, Israel) and examined for cells that had stained dark brown, indicating that they contained peroxidase (Shekarriz et al, 1995). Leukocytospermia was defined as at least 1 *x* 10^6 WBCs/mL. In our study, we excluded 3 patients who had leukocytospermia.

Reactive Oxygen Species

Aliquots of liquefied semen were centrifuged at 300 x g for 10 minutes. Seminal plasma was aliquoted and frozen at -20°C for later measurement of total antioxidant levels. The sperm pellet was washed twice with PBS, pH 7.4, and resuspended in the same media at a concentration of 20 X 10^6 sperm/mL. ROS production was measured by the chemiluminescence assay method using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Company, St. Louis, Mo) as the probe. Ten microliters of 5 mM luminol prepared in dimethyl sulfoxide (Sigma) was added to 400 µL of the washed sperm suspension. ROS levels were determined by measuring chemiluminescence with a luminometer (LKB 953; Wallac Inc, Gaithersburg, Md) in the integrated mode for 15 minutes, and results were expressed as 10° counted photons per minute (cpm) per 20 x10^6 sperm (Hendin et al, 1999). Reliability (the ratio of interassay variability to total variability) for measuring ROS was 93.8% in studies within our laboratory. Analysis of variance (ANOVA) procedures used in our study partition components of variability into interassay and infra-assay variability in planned experiments of multiple measures of the same sample. The sum of interassay variability and infra-assay variability provides the measure of total variability, which is used to estimate reliability.

Total Antioxidant Measurement

Total antioxidant activity was measured in seminal plasma using the enhanced chemiluminescence assay (Kolettis et al, 1999). Aliquots of the seminal plasma stored at -20°C were thawed at room temperature and immediately assessed for their antioxidant capacity as follows. Seminal plasma was diluted 1:10 with deionized water dH_2O and filtered through a 0.20-micron Millipore filter (Allegiance Healthcare Corporation, McGaw Park, Ill). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckinghamshire, United Kingdom). Twenty microliters of horseradish peroxidase (HRP)-linked immunoglobulin (Amersham Life Science) was added to 4.98 mL dH_2O. This was further diluted 1:1 to give a working solution with the desired luminescence output (3 X 10^7 cpm). Trolox (6hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble tocopherol analogue, was added as the standard at concentrations of between 50 and 150 µM. With the luminometer set in the kinetic mode, 100 µL of signal reagent and 100 µL of HRP were added to 700 µL of dH_2O and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between 2 and 3 X 10^7 cpm) for 100 seconds. One hundred microliters of the prepared seminal plasma was added to the signal reagent and HRP and the chemiluminescence were measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence was recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

ROS-TAC Score

The ROS and TAC values from controls were used to create a scale of these 2 variables that uses the control values as reference points (Sharma et al, 1999). The log of (ROS + 1) was used in calculations so that both values were normalized to the same distribution. First, both log (ROS + 1) and TAC were standardized to z-scores (mean = 0, standard deviation [SD] = 1) so that both would have the same variability. These standardized scores were calculated by subtracting the mean values of the controls from each individuals observed value and dividing by the SD of the control- population. For instance, among the controls, the mean log (ROS + 1) is 1.3885, and their SD is 0.7271, these values are applied below:

For log (ROS + 1): standardized ROS + [log (ROS + 1) - 1.3885] / 0.7271

For TAC: standardized TAC = (TAC - 1650.93) / 532.22

These 2 standardized variables were then analyzed with principal component analysis, which provides linear combinations (or weighted sums) that account for the most variability among correlated variables. The first principal component provided the following linear equation:

Principal component = (-0.707 x standardized ROS) + (0.707 x standardized TAC)

To ensure that the distribution of the standardized ROS-TAC score would have a mean of 50 and an SD of 10 in normal controls, the ROS-TAC score was transformed as:

ROS-TAC score = 50 + (principal component x 10.629)

For example, if a donor specimen gives an ROS value of 10.3 and a TAC of 2499.0, initially the ROS would be converted to log (10.3 + 1), or 1.05. Next, the values would be standardized as follows:

Standardized ROS = [1.05 - 1.3885] / 0.7271 = (-0.3385 / 0.7271) = -0.46

Standardized TAC = (2499.00 - 1650.93) / 532.22 = (848.07 / 532.22) = 1.59

This will then be converted to the ROS-TAC score as follows:

ROS-TAC score = 50 + [(-0.707 x -0.460) + (0.707 x 1.590)] x 10.629

ROS-TAC score = 50 + [(0.33) + (1.12)] x 10.629 = 50 + 15.42 = 65.42

By comparison, a patient with a high ROS value of 92,-36.36 cpm or log (ROS + 1) value of 4.97 and low TAC of 1106.85 Trolox equivalents would have an ROS-TAC score of 5.33.

Statistical Methods

The semen characteristics, ROS, and TAC levels were compared among the 3 groups of infertile normospermic men and the control group with ANOVA. If significant differences were indicated ($P < .05$, two-tailed tests), pairwise Dunnett's tests were used to compare each group of infertile men with the control group. The sample was sufficient to detect 13-point differences (the differences previously observed between infertile and fertile patients) between patient groups and donors with at least 90% power (two-tailed tests; $P < .05$). Percentage of patients with detectable WBCs and the amount of cells were compared among groups with Fisher's exact tests and Kruskal-Wallis tests, respectively. Spearman's rank-correlation was used to assess the relationship between WBCs and the ROS-TAC score. ROCS, which illustrate sensitivity and specificity over the entire range of values, were used to compare the ability of ROS. TAC, and the ROS-TAC score to discriminate between patients and donors. The percentage area under the curve indicates the estimated percentage that would be correctly classified as either fertile or infertile if used as a diagnostic tool.

In addition to comparisons with controls, the normospermic patients' ROS-TAC scores were compared with 2 other male factor patient groups. These were 13 fertile and 39 infertile male factor patients from 2 groups of patients with varicocele and vasectomy reversal from our earlier study (Sharma et al, 1999). These male factor patients did not differ in semen characteristics, however, their ROS-TAC scores differed significantly. The estimated probability of infertility (with 95% confidence intervals [CIs]) in normospermic males was calculated using the logistic regression estimates. These were based on the known fertility status of patients and used to provide a point of comparison for ROS-TAC levels.

All summary statistics are presented as mean ± standard error of the mean (SEM). The SAS statistical software package (SAS Institute Inc, version 6.12, Cary, NC) was used for statistical calculations.

Results

Comparisons of semen characteristics indicated no significant differences in sperm concentration (P = .52), WHO morphology (P = .28), and Kruger's morphology (P = .63); among the patient groups. Significant differences in motility were observed among the groups (P = .04) and patients with varicocele had higher motility (66.1% ± 3.8%) than the controls (52.0% ± 3.0%; P = .006). However, none of the patient groups had reduced motility compared with the controls. Although all patients with leukocytospermia were excluded from the study, 6 of the 43 (14%) patients had either a WBC count of 0.4 x 10^6/mL (n = 3) or 0.8 x 10^6/mL (n = 3). There were no differences among the 3 groups in their white cell counts (Table 1), and there was no correlation between WBCs and the ROS-TAC score (r = -.16; P = .32).

Table 1. Percentage of normospermic subfertile men with detectable white blood cell levels and mean levels among the 3 groups *

Groups	Percentage with Detectable WBCs	Mean WBCs (x 106/mL)
Normospermic infertile group	14.0% (95°/a Cl, 5.3%--27.9%)	0.08 ± 0.03
Varicocele only	18.8% (95% Cl, 0.2%-41.3%)	0.13 ± 0.07
Female factor only	12.5% (95% Cl, 4.1%-45.6%)	0.08 ± 0.06
Idiopathic	9.1°/a (95% Cl, 1.6%-38.3%)	0.04 ± 0.04
P value †	0.87	0.69

* Values are mean ± SE. All P values indicate significance of differences comparing three diagnoses. WBC indicates white blood cell.
† P < .05 was considered significant.

Despite the fact that semen parameters were not significantly altered among the patients, evidence of increased oxidative stress was seen in these patient groups. When patients were considered as a single group, mean ROS level was significantly higher compared with the controls (Table 2). However, among the subgroups, the only patient subgroup that was significantly different from controls was the idiopathic infertility group. ROC curves were used to compare *ROS,* TAC, and the ROS-TAC score by quantifying their sensitivity and specificity over their values. The resulting area under the curve represents the rates of the scores, which accurately classifies patients and donors. When an ROC curve was calculated to determine the ability of the ROS level to distinguish donors from infertile men, the area under the curve was 63.2% (95% CI, 50.0% to 77.3%; Figure 1). Also, total antioxidant capacity was higher in controls than patients, both when considered as an overall group and when divided into subgroups (Table 2). The area under the ROC curve for TAC measurements was 86.0% (95% CI, 76.9% to 95.1%; Figure 1).

In addition, ROS-TAC scores were significantly higher in controls than patients, both when patients were considered as a group and when they were divided into subgroups (Table 2). The area under the ROC curve for ROS-TAC scores was 82.4% (95% CI, 71.7% to 93.2%; Figure 1).

Figure 2 illustrates the distribution of the ROS-TAC score among the known control and the 3 groups of normospermic infertile men. In addition, a control group and treated male factor patients with known fertility outcome (fertile and infertile) from a previous study that validated the ROS-TAC score (Kolettis et al, 1999) were also compared. ROS-TAC scores in the 3 groups with unknown fertility outcome were significantly lower than both donors and fertile-treated male factor patients. The normospermic patient groups were comparable to infertile male factor patients. The ROS-TAC score is scaled to have a mean of 50 and an SD of 10 among the control group. Of the 43 normospermic men, 15 (35%) had ROS-TAC scores within 1 SD of the control mean, 16 (37%) were between 1 and 2 SDs below the mean, and the remaining 12 (28%) were 2 SDs below the mean or more (Table 3). To illustrate the potential effect of these low ROS-TAC scores on fertility, logistic regression indicated that men with lower ROS-TAC scores had higher probability of infertility during follow-up. Based exclusively on ROS-TAC scores of other infertile men with male factor diagnoses, we estimated that approximately 78.9% (95% CI, 62.6%-89.2%) of normospermic infertile males would fail to conceive children during a year of follow-up.

Because female-factor patients may have confounding diagnoses that affect their fertility rates, if only idiopathic and varicocele are considered, their 1-year fertility rate is estimated at 20.6% (95% CI, 10.6%37.1%). Obviously, many factors play important roles in male infertility, however, using this group of male factor patients, we can demonstrate that normospermic infertile men have oxidative stress levels that may be of clinical significance.

Table 2. *ROS and TAC levels and the ROS-TAC score in normospermic subfertile men and controls* *

Groups	Log (ROS + 1) (Count Per Minute)	TAC (Trolox Equivalent)	ROS-TAC Score
Controls	1.39 ± 0.16	1650.93 ± 95.87	50.0 ± 2.1
Normospermic infertile group	1.76 ± 0.14 (P = .004)	970.19 ± 73.95 (P < .001)	35.7 ± 1.8 (P < .001)
Varicocele only	1.46 ± 0.22 (P = .81)	888.5 ± 127.19 (P < .001)	39.0 ± 3.1 (P = .005)
Female factor only	1.62 ± 0.21 (P = .41)	951.87 ± 121.2 (P < .001)	36.8 ± 3.1 (P < .001)
Idiopathic	2.29 ± 0.25 (P = .004)	1103.80 ± 145.03 (P = .002)	30.7 ± 3.3 (P < .001)

* Values are mean ± SE. All P values indicate significance of difference when compared with control value. *P < .05* was considered significant. ROS indicates reactive oxygen species; TAC, total antioxidant capacity.

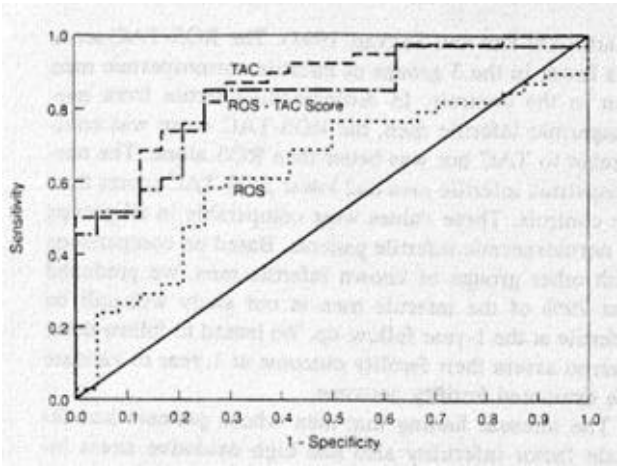

Figure 1. ROC curves show that levels of ROS, and particularly TAC and the ROS-TAC score, significantly distinguished controls from normospermic patients in our sample. By calculating sensitivity and specificity over all values, the area under the curves represent the proportion of accurately classified patients and donors.

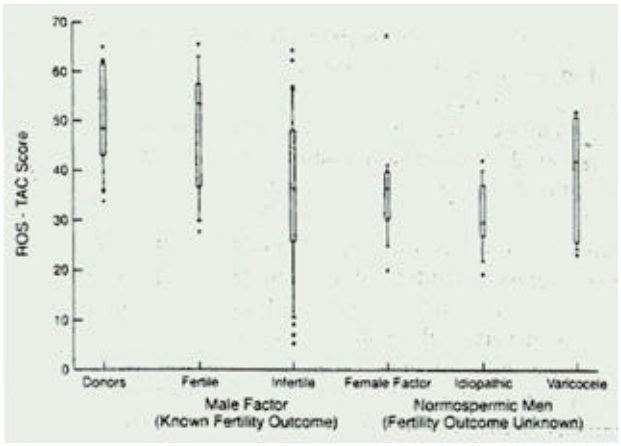

Figure 2. Box-plots illustrating ROS-TAC score values across 6 groups of males: controls, fertile male factor patients, infertile male factor patients, and 3 groups of normospermic patients (female factor only, idiopathic, and varicocele). The box represents the 25th and 75th percentiles, and the line crossing through the box is the median.

Discussion

Normal sperm counts and other variables measured in routine semen analysis do not ensure fertility. Other factors not measured .in routine semen analysis, such as cytokines and ROS, have been related to infertility (Iwasaki and Gagnon, 1992; Huszar and Vigue, 1994; Sharma and Agarwal, 1996). ROS, which can be generated by sperm, polymorphonuclear neutrophils (PMNs), and macrophages, impair sperm function (Alvarez et al, 1987; Aitken and Clarkson, 1989; Aitken et al, 1992; de Lamirande and Gagnon, 1993, 1995; Lewis et al, 1995; Shekarriz et al, 1995; Sharma and Agarwal, 1996; Aitken, 1997, 1999; Alkan et al, 1997). In our study, we excluded patients with high seminal WBC concentrations to rule out known causes of male infertility. This measure ensured that the production of ROS in the specimen was primarily due to the spermatozoa.

Higher levels of ROS are found in samples from men with oligospermia, asthenospermia, and teratospermia (Aitken et al, 1992; Lewis et al, 1995; Sharma and Agarwal, 1996; Griveau and de Lannou, 1997). Also, sperm membrane lipid peroxidation has been reported in samples from 12% of fertile normospermic men (Huszar and Vigue, 1994).

Some physicians will refer the male partner of a couple seeking treatment for infertility to an andrologist

only when semen analysis finds abnormal sperm count, motility, or morphology. However, some normospermic men still fail to conceive despite aggressive treatment of the female, because the male partner has abnormalities in the fertilizing ability of the spermatozoa that are not manifested in a simple semen analysis.

Table *3. Relationship between ROS-TAC score,* diagnosis, and predicted chances* of *1-year infertility after 1-year follow-up in 34 normospermic infertile men, on the basis* of *comparisons with male factor patients* of *known fertility outcome*

ROS-TAC Score	No. Subjects	Mean ± SE	Predicted Chance of Fertility †
All diagnoses, by ROS-TAC Score			
<30	12	25.2 ± 0.9	10.8% (95% Cl, 3.7%--28.1 %)
30 to 40	16	35.6 ± 0.7	18.5% (95% Cl, 9.2%-33.9%)
>40	15	47.2 ± 1.9	31.9% (95°/a Cl, 18.2%-48.6%)
Total	43	35.7 ± 1.8	21.1% (95% Cl, 10.8%-37.4%)
By Clinical Diagnoses			
Female factor	16	36.8 ± 3.1	21.8% (95% Cl, 11.2%-37.9%)
Idiopathic	11	30.7 ± 3.3	17.4% (95% Cl, 8.3%-34.1%)

* ROS-TAC = reactive oxygen species-total antioxidant capacity. Healthy donor's mean ROS-TAC score = 50.
† Predicted fertility rates are based on logistic regression estimates comparing fertile and infertile male factor patients, and based only on ROS-TAC scores.

As oxidative stress plays an important role in male infertility (Jones et al, 1979; Aitken and Clarkson, 1987; Alvarez et al, 1987; Sharma and Agarwal, 1996; Twigg et al, 1998; Lopes et al, 1998; Aitken, 1999), we hypothesized that an imbalance between ROS generation and antioxidant ability to scavenge ROS could be one of these hidden factors in the face of apparent normal sperm characteristics. Our results show that 3 types of infertile men (those with varicocele with partners without female factors, those with no male factor but with partners with female factors, and those with idiopathic infertility) have relatively high ROS levels in their semen. In our earlier work, we demonstrated that men with clinical varicocele have elevated levels of ROS and that this may be responsible for their infertility as a result of oxidative stress (Hendin et al, 1999). Current studies in our laboratory are assessing if varicocelectomy in these groups of patients results in a reduction of their seminal oxidative stress and its relationship with their fertility. Despite the small sample size of our idiopathic infertility group, our results were compatible with data from a previous report demonstrating the presence of oxidative stress in men with idiopathic infertility (Alkan et al, 1997).

The fact that oxidative -stress plays an important role in male infertility is well documented (Alvarez et al, 1987; Sharma and Agarwal, 1996; Lopes et al, 1998; Twigg et al, 1998; Aitken, 1999). One of the possible mechanisms contributing in the pathophysiology of male infertility in the face of apparent normal semen characteristics could be an imbalance between the generation of ROS and the ability to scavenge ROS by antioxidants. Our results show that men who have varicoceles with no female factor, pure female factor, or idiopathic infertility have increased ROS levels in their semen.

Oxidative stress is the result of an imbalance between the production of ROS and the ability to scavenge by antioxidants (Geva et al, 1996, 1998; Sharma and Agarwal, 1996; Griveau and de Lannou, 1997). It is difficult to evaluate the effectiveness of any antioxidant in isolation because there appears to be a cooperation between various enzymatic or nonenzymatic antioxidants (de Lamirande and Gagnon, 1993; Lenzi et al, 1993; Lewis et al, 1995, 1997; Geva et al, 1996, 1998; Suleiman et al, 1996). Therefore, we measured total nonenzymatic antioxidant capacity (Martin-Du Pan and Sakkas, 1998). However, the seminal TAC levels of infertile normospermic patients are unknown.

We found that infertile normospermic men, irrespective of the diagnosis, had lower TAC levels than controls. We also measured ROS and a composite ROS-TAC score, which incorporates both measures of oxidative stress and thus may better represent any imbalance between them (Martin-Du Pan and Sakkas, 1998). The ROS-TAC score was lower in the 3 groups of infertile normospermic men than in the controls. In distinguishing fertile from normospermic infertile men, the ROS-TAC score was comparable to TAC but was better than ROS alone. The normospermic infertile men had lower ROS-TAC scores than the controls. These values were

comparable in all groups of normospermic infertile patients. Based on comparisons with other groups of known infertile men, we predicted that 79% of the infertile men in our study will still be infertile at the 1 year follow-up. We intend to follow these men to assess their fertility outcome at 1 year to validate the estimated fertility outcome.

The unusual finding that men whose partners had female factor infertility also had high oxidative stress indicates that the true cause of infertility in these men is not the female factor alone, but oxidative stress that was not diagnosed earlier. Normospermic men seeking infertility evaluation should be examined for oxidative stress. Men with normal semen analysis and high oxidative stress may benefit from antioxidants.

Empirical trials of oral vitamin E or intramuscular glutathione among infertile men have shown promising results, including improved semen characteristics and high rates of fertilization in vitro (Lenzi et al, 1993; Geva et al, 1996; Suleiman et al, 1996; Ford and Whittington, 1998; Martin-Du Pan and Sakkas, 1998; Tarin et al, 1998). Furthermore, ascorbic acid (vitamin C) protects human spermatozoa against endogenous oxidative DNA damage (Fraga et al, 1991). Antioxidants are likely to benefit only men whose infertility is caused by oxidative stress. Further studies could evaluate antioxidant therapy for normospermic infertile men.

The equations for calculation of the ROS-TAC score may appear cumbersome, however, it basically involves including observed ROS and TAC values into an equation that includes several constants. These constants can be included in a spreadsheet or database for automatic calculation of the score. For quality control purposes, it is necessary to measure ROS and TAC among a group of donors and statistically test whether they differ from "50" with a 1-sample t test. If differences exist, it is important to use the mean and SDs of the new sample in the ROS-TAC equations to maintain the properties of the mean of 50 and SD of 10. Also, quality control should be performed regularly to assess intraobserver and interobserver variability to evaluate levels of reliability.

Infertility in men with normal semen characteristics may be the result of seminal oxidative stress. Total antioxidant capacity and ROS-TAC scores can better distinguish fertile from infertile men than ROS alone, and thus are probably better measures of oxidative stress. One of the limitations of our study was the fact that we did not study the actual pregnancy rates. In this pilot study, the observation that normospermic men demonstrate increased levels of oxidative stress indicates that it may be important that men seeking fertility treatment, irrespective of normal semen analysis, be tested for ROS, and TAC as an additional measure to infertility evaluation. This may be beneficial to the patient because if the infertility is largely related to oxidative stress, antioxidant therapy may be helpful. However, further research using randomized controlled trials is necessary to verify if additional antioxidant supplementation will actually help these men.

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