

## Semen quality and oxidative stress scores in fertile and infertile patients with varicocele

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**Objective:** To compare semen quality and levels of seminal oxidative stress among three groups: infertile men with varicocele, fertile men with varicocele, and healthy semen donors (controls) without varicocele.

**Design:** Prospective study.

**Setting:** Academic medical centers.

**Intervention(s):** None.

**Patient(s):** Semen specimens were obtained from 21 infertile patients with varicocele, 15 fertile men with varicocele, and 17 healthy fertile men with normal semen characteristics.

**Main Outcome Measure(s):** Principal component analysis was applied to nine semen characteristics to provide a standardized semen quality score. Reactive oxygen species (ROS) production and total antioxidant capacity (TAC) were measured by chemiluminescence assays to create an ROS-TAC score.

**Result(s):** The mean semen quality scores of the infertile patients with varicocele were lower than those of the control subjects but similar to those of the fertile men with varicocele. Compared with the healthy subjects, the infertile men with varicocele had higher ROS levels but lower TAC levels. They also had significantly lower ROS-TAC scores compared with control subjects, but the scores were not significantly different than those seen in fertile men with varicocele.

**Conclusion(s):** These findings not only provide us with valuable information regarding semen quality but also can serve as a warning that the fertility potential in fertile varicocele patients can decline due to oxidative stress. (Fertil Steril® 2007; ■: ■-■. ©2007 by American Society for Reproductive Medicine.)

**Key Words:** Semen, sperm, oxidative stress, antioxidants, reactive oxygen species, varicocele

Semen analysis typically produces a wide variety and number of semen characteristics that are correlated, indicating that underlying measures of semen quality may be used to reduce the number of variables evaluated (1–3). In fact, the clinical value of traditional semen parameters in the diagnosis of male infertility is the subject of considerable debate (4–7). After the introduction of computer-assisted semen analysis (CASA), the number of sperm characteristics examined increased to the extent that each semen evaluation quantifies many different semen characteristics (3, 8, 9). Because many of these characteristics are interrelated, an overall semen score can be developed by appropriate statistical model, as previously described (3).

Varicocele is characterized by abnormal tortuosity and dilatation of the veins of the pampiniform plexus within the

spermatic cord and is considered an important cause of male infertility (10–12). The exact mechanism by which an incidental varicocele becomes pathologic remains unclear. In addition, a functional factor not measured in routine semen analysis may affect pregnancy rates in the female partners of patients with varicocele.

However, the exact etiology of male infertility in patients with varicocele is still unknown (13, 14). The controlled generation of reactive oxygen species (ROS) in spermatozoa is associated with normal physiologic functions (15–17). Uncontrolled and excessive production of ROS, produced by both seminal leukocytes and abnormal sperm, however, appears to be one of the major factors leading to an infertile status (18). Excessive ROS production causes oxidative stress (OS), resulting in decreased sperm motility and viability and increased midpiece sperm defects impairing sperm capacitation and acrosome reaction (17–19). Human spermatozoa are rich in polyunsaturated fatty acids and are therefore susceptible to ROS attack. An imbalance between ROS production and the total antioxidant capacity (TAC) in seminal

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fluid leads to OS and is correlated with male infertility (1). A composite ROS-TAC score may be more strongly correlated with infertility than ROS or TAC alone (1). Infertility may occur if spermatozoal impairment caused by OS crosses a critical line. The purpose of the present study was to compare semen quality and seminal oxidative stress levels (ROS-TAC score) in three groups of men: infertile men with varicocele, fertile men with varicocele, and normal semen donors (control subjects) without varicocele. The ROS-TAC score might indicate the severity of spermatozoal impairment and the need for early varicocele repair.

## PATIENTS AND METHODS

### Subjects

The study was approved by the Institutional Review Board of the Cleveland Clinic Foundation, and all of the patients granted their informed consent. Semen specimens were obtained from 21 infertile patients with varicocele and 15 fertile men with varicocele attending our Urological Institute between 1998 and 1999. We evaluated only one sample for each patient. Three patients had a bilateral varicocele, and the remaining patients had left varicocele. The minimum duration of infertility required was defined as a failure to establish a pregnancy within 1 year of unprotected intercourse. Seventeen healthy fertile men with normal semen characteristics according to the World Health Organization (WHO) guidelines were recruited to serve as control subjects.

All patients were evaluated with a complete medical history, physical examination, and semen analyses. Patients with azoospermia or leukocytospermia were excluded.

### Semen Analysis

Semen samples were obtained by masturbation after at least 48 hours of abstinence. Samples were collected into sterile containers and allowed to liquefy at 37°C for 30 minutes and analyzed for sperm concentration, percentage motility, and morphology according to WHO criteria.

### White Blood Cells

The presence of granulocytes in semen specimens was assessed by a myeloperoxidase test (1). A 20- $\mu$ L volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar, Cambridge, MA); 20  $\mu$ L phosphate-buffered saline (PBS; pH 7.0) and 40  $\mu$ L benzidine solution were added. The mixture was vortexed and allowed to sit for 5 minutes. Five microliters of the specimen was placed on a Makler chamber (Sefi Medical, Haifa, Israel) and examined for cells that stained dark brown, which indicated that they were positive for neutrophils. Leukocytospermia was defined as the presence of at least  $1.0 \times 10^6$  white blood cells (WBC)/mL. We excluded patients who had  $\geq 1.0 \times 10^6$  WBC/mL.

### Reactive Oxygen Species

Aliquots of liquefied semen were centrifuged at 300g for 7 minutes. Seminal plasma was aliquoted and frozen at

$-20^\circ\text{C}$  for later measurement of total antioxidant levels. The sperm pellet was washed twice with PBS, pH 7.4, and re-suspended in the same medium at a concentration of  $20 \times 10^6$  sperm/mL. Production of ROS was measured by the chemiluminescence assay method using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical, St. Louis, MO) as the probe. Ten microliters of 5 mmol/L luminol prepared in dimethyl sulfoxide (Sigma Chemical) were added to 400  $\mu$ L of the washed sperm suspension. Levels of ROS were determined by measuring chemiluminescence with an Autolumat LB 953 luminometer (Berthold Technologies, Bad-Wildbad, Germany) in the integrated mode for 15 minutes. The results were expressed as  $10^4$  counted photons per minute (cpm) per  $20 \times 10^6$  sperm.

### Total Antioxidant Capacity

Total antioxidant capacity was measured in seminal plasma using the enhanced chemiluminescence assay. Aliquots of the seminal plasma stored at  $-20^\circ\text{C}$  were thawed at room temperature and immediately assessed for their antioxidant capacity as follows. Seminal plasma was diluted 1:10 with deionized water ( $\text{dH}_2\text{O}$ ) and filtered through a 0.20  $\mu$  Millipore filter (Allegiance Healthcare, McGaw Park, IL). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckinghamshire, England). Twenty microliters of horseradish peroxidase-linked immunoglobulin (HRP-linked Ig; Amersham Life Science) were added to 4.98 mL  $\text{dH}_2\text{O}$ . This was further diluted 1:1 to give a working solution with the desired luminescence output ( $3 \times 10^7$  cpm). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was added as the standard at concentrations between 50 and 150  $\mu\text{mol/L}$ . With the luminometer set in the kinetic mode, 100  $\mu$ L signal reagent and 100  $\mu$ L horseradish peroxidase-linked immunoglobulin were added to 700  $\mu$ L  $\text{dH}_2\text{O}$  and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between  $2 \times 10^7$  and  $3 \times 10^7$  cpm) for 100 seconds. One hundred microliters of the prepared seminal plasma were added to the signal reagent and HRP, and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence were recorded. Antioxidant capacity was expressed as molar trolox equivalents.

### ROS-TAC Score

The ROS and TAC values from the control subjects were used to create a scale of these two variables that uses the control values as reference points. The log of (ROS +1) was used in calculations so that both values were normalized to the same distribution. First, both TAC and log (ROS +1) were standardized to z-scores so that both would have the same variability. These standardized scores were calculated by subtracting the mean values for the control subjects from

the mean value for the patients and dividing by standard deviation of the control population:

$$\text{Standardized ROS} = [\log(\text{ROS} + 1) - 1.3885]/0.7271$$

$$\text{Standardized TAC} = (\text{TAC} - 1650.93)/532.22$$

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

$$\begin{aligned} \text{Principal component} = & (-0.707 \times \text{Standardized ROS}) \\ & + (0.707 \times \text{Standardized TAC}) \end{aligned}$$

To ensure that the distribution of the ROS-TAC score would have a mean of 50 and standard deviation of 10 in control subjects, the ROS/TAC score was transformed as:

$$\text{ROS-TAC score} = 50 + (\text{Principal component} \times 10.629)$$

An ROS-TAC score was formulated using principal components to predict fertility potential in these men.

### Semen Quality Score

The following nine semen characteristics were included to compute semen quality scores: concentration, motility, curvilinear velocity (VCL), straight line velocity (VSL), average path velocity (VAP), lateral head displacement (ALH), linearity, and morphology (both by WHO and Kruger's strict criteria). Log transformation (base 10 logarithms after adding a constant of 1 to each semen characteristic) was done to reduce the effect of high outliers and to scale the variables. Principal component analysis was applied to the covariance matrix of the nine log-transformed semen parameters. This produced nine new components, each with "eigenvectors" (which were the weights assigned to the original variables). Only those components that accounted for at least 10% of the overall variability of the nine semen parameters were used. The resultant principal component scores were converted to semen scores with a mean of 100 and a standard deviation of 10 by using the donor group.

Previous reports from our laboratory have demonstrated that the ROS-TAC score is superior to ROS or TAC alone in predicting fertility during follow-up of patients with male-factor infertility. Therefore, we compared our patients with varicocele with fertile ( $n = 13$ ) and infertile ( $n = 39$ ) men with male-factor diagnoses. The probability of remaining infertile 1 year after the infertility diagnosis was calculated based on logistic regression estimates of the known fertile and infertile men, as previously described (3). The

probabilities of infertility in these patients were examined solely on the logistic regression estimates. These, in turn, were based on the known fertility status of the patients and were used to examine the potential clinical relevance of the observed ROS-TAC levels. Statistical significance was assessed with two-tailed tests, and  $P$  was considered to be statistically significant when  $<.05$ . Summary statistics are presented as mean  $\pm$  standard error. Statistical tests were performed using SAS version 6.12 (SAS Institute, Cary, NC).

### RESULTS

The mean ages of the study participants were  $34.6 \pm 3.5$  years for the infertile varicocele patients,  $32.4 \pm 2.5$  years for the fertile varicocele patients, and  $34.1 \pm 2.8$  years for the control subjects (infertile vs. fertile  $P=.045$ ; infertile vs. control  $P=.09$ ; fertile vs. control  $P=.043$ ). Compared with the control subjects, the infertile patients had significantly lower sperm concentration, motility, and morphology (Table 1). When these three variables were compared between the infertile men with varicocele and fertile men with varicocele, no statistically significant differences were detected.

Infertile patients with varicocele had lower semen quality scores ( $81.7 \pm 10.5$ ) than the control subjects ( $98.9 \pm 10.3$ ;  $P=.002$ ), but the scores were not significantly different from those of the fertile men with varicocele ( $86.1 \pm 13.6$ ;  $P=.52$ ).

Infertile men with varicocele had higher ROS levels ( $2.1 \pm 0.25$ ) than the healthy control subjects ( $1.3 \pm 0.3$ ;  $P=.02$ ) but lower TAC levels ( $1,186.0 \pm 96.9$ ) than the control subjects ( $1,443.0 \pm 105.0$ ;  $P=.049$ ). In addition, the infertile varicocele patients had significantly lower ROS-TAC scores than the control subjects ( $41.7 \pm 13.1$  vs.  $51.3 \pm 9.9$ , respectively;  $P=.03$ ), but their scores were not statistically significantly different from those of the fertile men with varicocele ( $41.7 \pm 13.1$  vs.  $37.6 \pm 11.0$ , respectively;  $P=.349$ ). An estimated 74% of men with infertility will remain infertile during 1-year follow-up based on logistic regression analysis.

### DISCUSSION

All living aerobic cells are normally exposed to some ROS. But if ROS levels rise, OS occurs, which results in oxygen and oxygen-derived oxidants and ultimately cellular damage (6, 14). Oxidative stress has been shown to be a major cause of male infertility. Indeed, a large proportion of infertile men have elevated levels of seminal ROS (1, 14). Several forms of sperm DNA damage are caused by ROS, e.g., chromatin cross-linking, chromosome deletion, DNA strand breaks, and base oxidation (18, 20–23).

The impact of these factors on male infertility, their clinical significance, and management options have always been a subject of controversy (24). In general, ROS production is highest in immature spermatozoa from men with abnormal semen values (14). However, immature spermatozoa with cytoplasmic retention are not the only abnormal male germ

**TABLE 1**

**Comparison of semen characteristics, measures of oxidative stress, and semen scores between infertile patients with varicocele with normal healthy men (control subjects) and fertile patients with varicocele.**

Variable	Infertile varicocele patients (n = 21)	Control subjects (n = 17)	Fertile patients with varicocele (n = 15)	P1	P2
Concentration ( $\times 10^6$ /mL)	37.0 $\pm$ 5.9	64.9 $\pm$ 9.1	42.3 $\pm$ 10.2	.003	.43
Motility (%)	35.6 $\pm$ 3.5	55.5 $\pm$ 4.9	48.6 $\pm$ 5.0	.002	.21
WHO morphology (%)	30.6 $\pm$ 2.8	39.8 $\pm$ 2.5	32.0 $\pm$ 3.2	.007	.25
Semen score	81.7 $\pm$ 10.5	98.9 $\pm$ 10.3	86.1 $\pm$ 13.6	.002	.52
Log (ROS + 1)	2.1 $\pm$ 0.25	1.3 $\pm$ 0.3	1.99 $\pm$ 0.26	.02	.16
TAC	1,186.0 $\pm$ 96.9	1,443.0 $\pm$ 105.0	939.0 $\pm$ 107.0	.049	.13
ROS-TAC score	41.7 $\pm$ 13.1	51.3 $\pm$ 9.9	37.6 $\pm$ 11.0	.03	.3492

*Note:* All values expressed as mean  $\pm$  SE;  $P < .05$  was considered to be significant.  $P1 = P$  between infertile varicocele patients and control subjects;  $P2 = P$  between infertile varicocele patients and fertile varicocele patients. ROS = reactive oxygen species; TAC = total antioxidant capacity; WHO = World Health Organization.

*Pasqualotto. Semen quality and ROS-TAC scores in varicocele men. Fertil Steril 2007.*

cells that are associated with high levels of DNA damage and ROS production (21, 23). Spermatozoa with abnormal head morphology, midpiece defects, and tail defects also have the same characteristics (14). Production of ROS positively correlates with the sperm deformity index, which is calculated by dividing the total number of deformities observed by the number of sperm evaluated (23). Even though varicocele is accepted as the “most common cause of correctable infertility,” the exact pathogenesis is poorly understood (25).

It is believed that heat, oxidative stress, and alterations in hemodynamics are some of the mechanisms for infertility in varicocele patients. Reactive oxygen species may be an important factor, because elevated levels have been detected in infertile patients with varicocele along with reduced levels of both seminal and blood plasma antioxidants (16, 17). In the present study, levels of ROS were higher in the infertile patients with varicocele than in the normal semen donors. Studies have shown that ROS production is higher in fertile men with varicocele than in men without varicocele (26).

A major cause of sperm dysfunction in humans is caused by OS. There is an inverse relationship between total nonenzymatic antioxidants in seminal plasma and lipid peroxidation (27). It has been reported that infertile men have lower levels of antioxidants than fertile men (27).

Because OS is the result of an imbalance between ROS levels and TAC of the seminal plasma, it is important to assess both variables when evaluating the OS status of a given sample. The chemiluminescence assay is one of the most commonly used methods to detect free radicals (28). The assay is accurate and reliable when the sperm concentration is  $>1 \times 10^6$ /mL and the samples are analyzed within the first hour after specimen collection. In the present study, infertile men with varicocele had lower TAC levels than healthy se-

men donors. Even though no differences were detected in ROS and TAC levels between infertile men with varicocele and fertile men with varicocele, the present study demonstrates that the presence of varicocele can increase seminal OS levels among men with varicocele.

Our previous studies have proven that the ROS-TAC score is a reliable indicator of OS in male infertility (1). Many IVF laboratories have attempted to create new reliable assays to evaluate OS and determine the role that antioxidants play in male infertility. Such efforts have great potential in therapeutic practice and have resulted in the establishment of the ROS-TAC score (1). The concept of overall expression of OS status and its assessment by a ROS-TAC score may help improve results of assisted reproductive technologies via antioxidant therapy and may benefit infertile men in whom OS plays a significant role. In the present study, the ROS-TAC score was lower in infertile men with varicocele than in healthy control subjects.

Numerous studies have reported the predictive value of semen parameters in the determination of IVF and pregnancy outcome (29, 30). These include concentration of motile spermatozoa and quantitative and computerized measurements of spermatozoa motility and morphology. After the introduction of CASA, the number of semen characteristics examined has increased to the extent that each semen evaluation quantifies nine semen characteristics (8, 9). Although these characteristics are unique measures of semen quality, they are not independent of one another, e.g., patients with low motility tend to have low concentration and vice versa.

Perhaps the most widely utilized semen characteristic is sperm count. Men with less than  $20 \times 10^6$  spermatozoa/mL are typically deemed subfertile, and men with counts less than  $5 \times 10^6$  spermatozoa/mL are often considered to be

infertile (3). Semen samples containing less than 14% normal forms by Tygerberg's strict criteria are reported as subfertile, and those containing less than 5% normal forms are considered to be severely impaired, causing some centers to recommend donor insemination for couples attempting pregnancy (30). However, like its predecessors, strict sperm morphology is not absolutely accurate in predicting fertility. In recent years, deficiencies in using these measures of semen parameters have been reported. The predictive value of spermatozoa concentration, for example, has been criticized because of the natural day-to-day variations that occur in spermatozoa concentration (3).

Therefore, the semen score can provide important information on the semen quality and the likelihood of establishing a pregnancy (3). Also, semen scores provide more meaningful information than individual semen characteristics. In the present study, the mean semen quality score was higher in normal semen donors than in infertile men with varicocele. Interestingly, even though semen quality was higher in fertile men with varicocele than in infertile men with varicocele, the differences did not reach statistical significance. In addition, logistic regression analysis estimated that 74% of men with infertility will remain infertile during the 1-year follow-up.

Measuring the ROS-TAC score gives us the benefit of measuring two reliable parameters: ROS and the total antioxidant levels. In the present study, there was a large number of fertile varicocele patients with seminal OS, which indicates that the extent of damage caused by ROS was not critical enough to result in infertility. This finding not only provided us with valuable information regarding semen quality but also can serve as a warning that the fertility potential in fertile varicocele patients can take a turn due to the existence of seminal OS.

Therefore, we conclude that infertile patients with varicocele have lower semen quality scores and markers of oxidative stress (ROS, TAC, and ROS-TAC score) than healthy men. Seventy-four percent of infertile men with varicocele tend to remain infertile within 1 year of the diagnosis. Both semen and ROS-TAC scores provide important information about semen quality and fertilizing potential. This information may be useful in the medical management of infertile patients with idiopathic etiologies. Although there was not much of a difference in the ROS-TAC score between infertile varicocele patients and fertile varicocele patients, it may be important to note that fertile patients with varicocele may be at risk of becoming infertile. This can be reinforced by the fact that the ROS-TAC score has proven to be a reliable tool in predicting the risk of OS in male infertility. This will be useful for clinicians who can suggest early varicocele repair for their patients and prevent further sperm damage by OS.

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