

The reactive oxygen species—total antioxidant capacity score is a new measure of oxidative stress to predict male infertility*

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The imbalance between reactive oxygen species (ROS) production and total antioxidant capacity (TAC) in seminal fluid indicates oxidative stress and is correlated with male infertility. A composite ROS–TAC score may be more strongly correlated with infertility than ROS or TAC alone. We measured ROS, TAC, and ROS–TAC scores in semen from 127 patients and 24 healthy controls. Of the patients, 56 had varicocele, eight had varicocele with prostatitis, 35 had vasectomy reversals, and 28 had idiopathic infertility. ROS levels were higher among infertile men, especially those with varicocele with prostatitis (mean \pm SE, 3.25 ± 0.89) and vasectomy reversals (2.65 ± 1.01). All infertile groups had significantly lower ROS–TAC scores than control. ROS–TAC score identified 80% of patients and was significantly better than ROS at identifying varicocele and idiopathic infertility. The 13 patients whose partners later achieved pregnancies had a mean ROS–TAC score of 47.7 ± 13.2 , similar to controls but significantly higher than the 39 patients who remained infertile (35.8 ± 15.0 ; $P < 0.01$). ROS–TAC score is a novel measure of oxidative stress and is superior to ROS or TAC alone in discriminating between fertile and infertile men. Infertile men with male factor or idiopathic diagnoses had significantly lower ROS–TAC scores than controls, and men with male factor diagnoses that eventually were able to initiate a successful pregnancy had significantly higher ROS–TAC scores than those who failed.

Key words: infertility/oxidative stress/reactive oxygen species/ROS–TAC score/spermatozoa

Introduction

Male infertility accounts for 40% of infertility problems (Fleming *et al.*, 1995). Spermatozoa are highly susceptible to

damage induced by reactive oxygen species (ROS). This is due to the high content of polyunsaturated fatty acids within the plasma membranes and a low concentration of scavenging enzymes within the cytoplasm (Jones *et al.*, 1979; Ochsendorf and Fuchs, 1993; Aitken and Fishel, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996). Elevated ROS levels are detected in 25–40% of the semen of infertile men and in up to 96% of the semen of patients with spinal cord injury (Iwasaki and Gagnon, 1992; de Lamirande *et al.*, 1995; Padron *et al.*, 1997).

There is growing evidence that oxidative stress significantly impairs sperm function, and plays a major role in the aetiology of defective sperm function. This may lead to the onset of male infertility via mechanisms involving the induction of peroxidative damage to the plasma membrane (Jones *et al.*, 1979; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992; Aitken and Fischel, 1994; Sharma and Agarwal, 1996; Griveau and Le Lannou, 1997). Both spermatozoa and seminal plasma possess antioxidant systems capable of counteracting the harmful effects of ROS. Studies have demonstrated that infertile men are more likely than fertile ones to have depressed total antioxidant capacity (TAC) and lower levels of individual antioxidants (Smith *et al.*, 1996; Lewis *et al.*, 1995, 1997). The production of abnormal levels of ROS is thought to be involved in many aspects of male infertility, where spermatozoa are rendered dysfunctional by lipid peroxidation and altered membrane function, together with impaired metabolism, morphology, motility, and fertility (Cummins *et al.*, 1994). Men whose spermatozoa produce excessive ROS are less likely to impregnate their wives through natural intercourse. The incidence of spontaneous pregnancy is negatively correlated with the generation of ROS in approximately 50% of oligozoospermic patients who exhibit increased ROS production (Aitken *et al.*, 1991).

Various clinical diagnoses are unable to determine the underlying cause of sperm dysfunction and pathophysiology of infertility. Since oxidative stress has been demonstrated to play a key role in male infertility, it is imperative to identify measures that would help predict, with accuracy, if oxidative stress is a significant contributor of infertility in any given clinical setting (Aitken *et al.*, 1991; Lewis *et al.*, 1995). Given that oxidative stress is associated with infertility, we postulated that it might be possible to validate the infertility status based on the initial clinical diagnoses. After measuring sperm ROS and seminal TAC we derived a composite ROS–TAC score, a novel indicator of oxidative stress. The aim of this prospective study was to investigate the significance of ROS and TAC in different subsets of clinical diagnoses of male infertility. We also examined if the ROS–TAC score could better identify

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infertile patients than either variable alone. Finally, we investigated whether the ROS–TAC score could identify the probability of patients who were likely to initiate a pregnancy.

Materials and methods

The Institutional Review Board approved the study. Patients attending our infertility clinic ($n = 127$) provided semen samples. Based on their clinical diagnosis, patients were classified into four groups: group I = varicocele ($n = 56$), group II = varicocele with prostatitis ($n = 8$), group III = vasectomy reversal ($n = 35$; infertile 23, fertile 12), and group IV = idiopathic infertility ($n = 28$). Patients in the varicocele with prostatitis group were defined as those who had leukocytospermia and inflammation. Within the varicocele and vasectomy reversal subgroups, 52 patients with at least 6 months follow up were evaluated for subsequent pregnancy outcome. These pregnancies were achieved naturally without assisted reproduction. Based on this subgroup, patients with pregnancies during follow-up were compared to those without, using logistic regression. Estimated probability of successful pregnancies was calculated over a range of ROS–TAC scores. In addition, semen specimens from normal healthy volunteers ($n = 24$) who had initiated a pregnancy in the past two years were examined according to the World Health Organization criteria (WHO, 1993) and served as controls. The specimens were examined for the presence of neutrophils by myeloperoxidase or the Endtz test (Shekarriz *et al.*, 1995) as a part of the analysis. Of the 127 patients, 116 (91.3%) tested negative by the Endtz test and 11 (8.7%) were positive. The distribution of Endtz positive specimen was: varicocele (3/56, 5.3%), varicocele with prostatitis (4/8, 50%), vasectomy reversal (2/35, 5.7%), and idiopathic infertility (2/28, 7.1%).

Reactive oxygen species measurement

Aliquots of liquefied semen were centrifuged at 300 *g* for 7 min. Seminal plasma was aliquoted and frozen at -20°C for later measurement of total antioxidant levels. The sperm pellet was washed with phosphate-buffered saline (PBS, pH 7.4) and resuspended in the same medium at a concentration of 2×10^6 sperm/ml. Levels of ROS were determined by chemiluminescence assay using luminol (5-amino-2,3 dihydro-1,4 phthalazinedione; Sigma Chemical Co., St. Louis, MO, USA) as the probe (Shekarriz *et al.*, 1995). Measurements were made using a Berthold luminometer (Autolumat LB 953, Wallac Inc., Gaithersburg, MD, USA). Five-millimolar luminol prepared in dimethyl sulphoxide (Sigma Chemical Co.) was added to 400 μl of the washed sperm suspension. Levels of ROS were determined by measuring chemiluminescence for 15 min and results were expressed as $\times 10^4$ counted photons per minute (cpm).

Total antioxidant capacity measurement

Total antioxidant capacity was measured in the seminal plasma using an enhanced chemiluminescence assay (Kolettis *et al.*, 1999). Aliquots of the seminal plasma were thawed at room temperature and assessed for their antioxidant capacity. Seminal plasma was diluted 1:10 with deionized water and filtered through a 20 μm Millipore filter (Allegiance Health Care Corporation, McGaw Park, IL, USA). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckingham, UK). A constant source of ROS was produced by horseradish peroxidase-linked immunoglobulin (HRP-linked Ig; Amersham Life Science, Buckingham, UK). Twenty microlitres of HRP-Ig was added to 4.98 ml dH_2O and further diluted 1:1 to give a desired chemiluminescence output (3×10^7 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 to 150 $\mu\text{mol/l}$. The antioxidant capacity

of the seminal plasma was expressed in molar Trolox equivalents. With the luminometer in the kinetic mode, 100 μl of signal reagent and 100 μl of HRP were added to 700 μl of dH_2O and mixed. This solution was equilibrated to the desired level of chemiluminescence output (between 2 and 3×10^7 cpm) for 10 s. One hundred microlitres of the prepared seminal plasma was immediately 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.

In this study, the samples used as the reference point in the scaling of the ROS–TAC score were obtained from donors who were healthy, normal men. Other control groups should be within 1 standard deviation (SD) (40–60) of the ROS–TAC score approximately two-thirds of the time; any group falling outside this range cannot be employed for calculating the reference value.

Statistical analyses

Comparisons among diagnosis groups were performed with a one-way ANOVA and post-hoc *t*-tests for pairwise comparisons. A multiple comparison procedure (Dunnett, 1995) was used to adjust the *P* values. The ROS and TAC values from controls 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 log (ROS + 1) and TAC were standardized to the z-scores (mean = 0, SD = 1) so that both would have the same variability. These standardized scores were calculated by subtracting the mean value of the controls from each individual's observed value and dividing by the SD of the control population.

For log (ROS+1):

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

For TAC:

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

These two standardized variables were then analysed with principal component 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:

$$\text{Principal component} = (-0.707 \times \text{standardized ROS}) + (0.707 \times \text{standardized TAC})$$

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

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

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:

$$\begin{aligned} \text{Standardized ROS} &= [1.05 - 1.3885]/0.7271 \\ &= (-0.29/0.7272) = -0.46 \end{aligned}$$

$$\begin{aligned} \text{Standardized TAC} &= (2499.0 - 1650.93)/532.22 \\ &= (848.07/532.22) = 1.59 \end{aligned}$$

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

$$\begin{aligned} \text{ROS–TAC score} &= 50 + [(-0.707 \times -0.46) + (0.707 \times 1.59)] \times 10.629 \\ &= 50 + [(0.11) + (1.12)] \times 10.629 = 50 + 15.43 = 65.44 \end{aligned}$$

Table I. Mean and standard deviation of reactive oxygen species (ROS), total antioxidant capacity (TAC), and ROS–TAC scores in subgroups of clinical diagnosis patients and controls

Diagnosis	ROS		TAC		ROS–TAC	
	Log (ROS + 1)	<i>P</i> value versus controls*	Molar Trolox	<i>P</i> value versus controls*	Score	<i>P</i> value versus controls*
Control (<i>n</i> = 24)	1.39 ± 0.73		1650.93 ± 532.22		50.00 ± 10.00	
Varicocele (<i>n</i> = 55)	2.10 ± 1.21	0.02	1100.11 ± 410.30	0.0002	34.87 ± 13.54	0.0001
Varicocele with prostatitis (<i>n</i> = 8)	3.25 ± 0.89	0.0002	1061.42 ± 425.11	0.03	22.39 ± 13.48	0.0001
Vasectomy reversal (<i>n</i> = 35)						
Vasectomy reversal (infertile; <i>n</i> = 23)	2.65 ± 1.01	0.0004	1389.89 ± 723.92	0.30	33.22 ± 15.24	0.0002
Vasectomy reversal (fertile; <i>n</i> = 12)	1.76 ± 0.86	0.80	1876.93 ± 750.82	0.62	49.35 ± 12.25	1.00
Idiopathic infertility (<i>n</i> = 28)	2.29 ± 1.20	0.01	1051.98 ± 380.88	0.0003	32.25 ± 14.40	0.0001

*Pairwise *P* values from Student's *t*-tests, adjusted using Dunnett's method.

Table II. ROS–TAC score and area under the receiver operator characteristic (ROC) curve showing the predictive value of reactive oxygen species (ROS), total antioxidant capacity (TAC), and ROS–TAC score

Group	Area under ROC curve (% , 95% confidence interval)			<i>P</i> value	
	ROS	TAC	ROS–TAC score	ROS–TAC score versus ROS	ROS–TAC score versus TAC
Infertile varicocele (<i>n</i> = 55)	68.9 (57.0–80.7)	80.2 (70.0–90.5)	80.8 (71.2–90.3)	0.002*	0.92
Infertile varicocele with prostatitis (<i>n</i> = 8)	94.8 (86.8–100)	82.8 (64.2–100)	93.2 (80.4–100)	0.70	0.09
Infertile vasectomy reversal (<i>n</i> = 23)	84.6 (73.4–95.8)	67.0 (51.2–82.9)	80.8 (67.6–94.0)	0.43	0.04*
Idiopathic infertility (<i>n</i> = 28)	74.3 (60.4–88.1)	81.8 (70.5–93.2)	84.5 (74.1–94.9)	0.005*	0.68

DeLong's non-parametric method of comparing dependent ROC curves; **P* < 0.05 was considered significant.

By comparison, a patient with a high ROS value of 92436.36 and low TAC of 1106.85 would have an ROS–TAC score of 5.33.

Receiver operating characteristics (ROC) curves were used to examine the diagnostic ability of ROS–TAC score to predict fertility. ROC curves illustrate the sensitivity and specificity over the entire range of the ROS–TAC score. The area under the curve can range from 50% to 100% with diagnostic tests that approach 100% indicating a perfect predictor and 50% indicating random chance, or no predictive ability. ROS, TAC, and ROS–TAC score variables were compared using DeLong's non-parametric comparison (DeLong *et al.*, 1988).

Statistical significance was assessed at *P* < 0.05, and summary statistics presented as mean ± SD. Data were analysed by the SAS statistical software package (version 6.12, SAS Institute Inc., Cary, NC, USA).

Results

ROS, TAC, and ROS–TAC scores for the controls, infertile patients in all four clinical diagnostic groups, and fertile vasectomy reversal patients are shown in Table I. During the follow-up, 13 patients (12 vasectomy reversal, 1 varicocele) were found to have successfully initiated a pregnancy and were classified as fertile. Of the 35 vasectomy reversal patients, 23 were infertile and 12 fertile.

Reactive oxygen species

Compared to control (1.39 ± 0.73), all infertile patient groups had elevated ROS (*P* = 0.008). The varicocele with prostatitis group had the highest levels of ROS (3.25 ± 0.89) which was significantly higher than levels in the other infertile diagnostic groups (varicocele, *P* = 0.005 and idiopathic infertility, *P* =

0.03). The fertile vasectomy reversal group did not differ significantly from controls.

Total antioxidant capacity

The total antioxidant capacity was significantly higher in the control group (1650.93 ± 532.22) than in any other infertile groups (*P* < 0.006), with the exception of the infertile vasectomy reversal group. Levels of TAC were lowest in the idiopathic infertility group (1051.98 ± 380.88), and were significantly lower than levels in the infertile vasectomy reversal group (*P* = 0.02). The TAC in fertile vasectomy reversal patients did not differ significantly from the controls.

ROS–TAC score

ROS–TAC score was significantly higher in the control than in the infertile patient groups (*P* ≤ 0.0002; Table I). The lowest ROS–TAC score was observed in the varicocele with prostatitis group (22.39 ± 13.48). This score was significantly lower than the varicocele (*P* = 0.01), and infertile vasectomy reversal (*P* = 0.01) scores. The average ROS–TAC score for the fertile vasectomy reversal group was 49.35 ± 14.4, which did not differ significantly from the control group.

Receiver operating characteristic curves

The effectiveness of oxidative stress in discriminating fertile from infertile men with different clinical diagnoses was studied by generating receiver operating characteristic (ROC) curves. In addition, the percentage of area under the curve, and their 95% confidence intervals (95% CI) were also calculated (Table

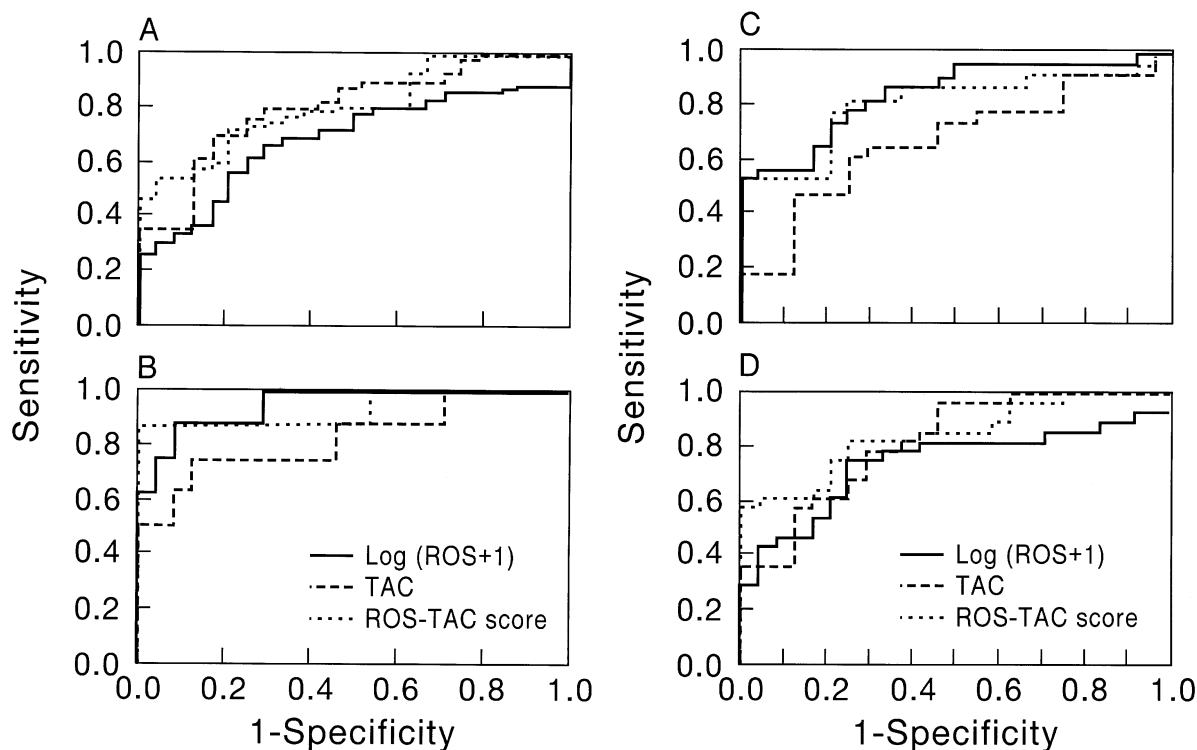


Figure 1. Receiver operator curves (ROC) illustrating that the ROS–TAC score generally has a greater area under the curve (diagnostic ability) than either log ROS or TAC alone for various clinical diagnoses: (A) varicocele, (B) varicocele with prostatitis, (C) vasectomy reversal, and (D) idiopathic infertility.

II; Figure 1A–D). Areas under the curve correspond to the percentage of men correctly classified to the proper fertility status. The area under the curve for ROS was 94.8% (95% CI: 86.8–100%) in discriminating controls from patients with varicocele with prostatitis. However, the area under the curve for ROS was <80% in both the infertile varicocele group (68.9%: 95% CI, 57.0–80.7%) and idiopathic infertility group (74.3%: 95% CI, 60.4–88.1%).

The TAC measure was >80% successful in discriminating infertile varicocele, varicocele with prostatitis, and idiopathic infertility groups. However, the area under the curve for infertile vasectomy reversal was 67.0% (95% CI, 51.2–82.9%). Therefore, for the infertile vasectomy reversal group, the level of the confidence interval is only slightly greater than random chance (50%).

The ROS–TAC score was significantly better at discriminating infertility among all diagnostic groups versus controls than either ROS or TAC alone. For all the infertile diagnoses, the area under the curve was >80%. The ROS–TAC score was better than ROS alone in identifying patients with varicocele ($P = 0.002$); and patients with idiopathic infertility ($P = 0.005$) (Table II, Figure 1A–D). The ROS and ROS–TAC scores were comparable in the varicocele with prostatitis and vasectomy reversal groups. Also, the ROS–TAC score was better than TAC alone in identifying the vasectomy reversal group ($P = 0.04$). The only group in which the ROS–TAC score did not perform significantly better than either ROS or TAC was the varicocele with prostatitis group, and this may be because of the small sample size ($n = 8$).

Within male factor diagnoses of varicocele, varicocele with

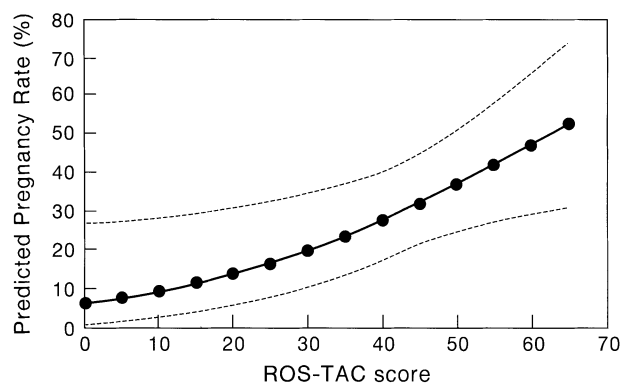


Figure 2. Predicted 1-year pregnancy rate (and 95% confidence interval) over the range of the ROS–TAC score for men with either varicocele or vasectomy reversal from logistic regression results.

prostatitis, and vasectomy reversal, 52 patients had a follow-up for an average of 17.4 ± 12.3 months (median, 12.6 months) to determine if subsequent pregnancies were achieved. Out of these 52, 13 (25%; 12 vasectomy reversal and 1 varicocele) patients eventually initiated normal pregnancies. Patients with partner pregnancies had significantly higher ROS–TAC scores (47.7 ± 13.2) compared to those who did not (35.8 ± 15.0 ; $P = 0.01$). The expected probability of the successful pregnancy during the average time frame of 12–18 months was calculated over a range of values of the ROS–TAC score based on logistic regression results (Figure 2). The probability of successful pregnancy was estimated at <10% for values of ROS–TAC score <30, but increased as the ROS–TAC score increased. Using logistic regression estimates, the

expected pregnancy rates for a patient with a ROS–TAC score of 30 would be 13.9, 21.0, and 31.6% for 12, 24, and 36 months respectively; whereas a score of 50 would have expected pregnancy rates of 35.1, 48.9, and 54.3% over the same intervals. Based on the expected probabilities, the estimated number of successful pregnancies among the 28 idiopathic infertility patients (assuming no female factor diagnoses) over a 12-month interval is 5.6 pregnancies (95% CI: 3.2–12.8).

Discussion

Oxidative injury to spermatozoa is a major cause of sperm dysfunction, and total non-enzymatic antioxidant defences in human seminal plasma are inversely related to lipid peroxidation (Smith *et al.*, 1996). Many studies have demonstrated the association of lipid peroxidation with mid-piece abnormality, decreased sperm count, motility, and loss of the capacity of the spermatozoon to undergo the acrosome reaction and fertilize (Sukcharoen *et al.*, 1996; Griveau and Le Lannou, 1997). The fertilizing ability of human spermatozoa is inversely related to the sperm ROS production (Sukcharoen *et al.*, 1996). Studies have demonstrated that infertile men are more likely than fertile ones to have depressed TAC and lower levels of individual antioxidants (Lewis *et al.*, 1995, 1997; Smith *et al.*, 1996). Sperm DNA is vulnerable to oxidative stress, in part because semen has a weak antioxidant system (Zini *et al.*, 1993; Aitken *et al.*, 1996) and in part because spermatozoa lack DNA repair enzyme activity (Matsuda *et al.*, 1989). Reactive oxygen species such as superoxide anion and hydrogen peroxide can lead to DNA fragmentation in somatic cells (Sun *et al.*, 1997).

The clinical significance of seminal oxidative stress is suggested by several independent studies indicating a link between peroxidative damage to human spermatozoa and the incidence of male infertility (Jones *et al.*, 1979; Aitken and Clarkson, 1987; Iwasaki and Gagnon, 1992; Weese *et al.*, 1993; Mazzilli *et al.*, 1994). Oxidative stress at the testicular level has also been implicated in the disruption of spermatogenesis observed during cryptorchidism, vitamin E depletion, and exposure to xenobiotics (Peltola *et al.*, 1994, 1995). Furthermore, it has been proposed that oxidative damage is a possible cause of idiopathic male infertility involving disruption of spermatogenesis (Lenzi *et al.*, 1993). Significantly high levels of free radicals (superoxide anion) were reported in the seminal fluid of infertile (87%) and fertile (55%) normozoospermic patients (Mazzilli *et al.*, 1994). Evidence of high levels of lipid peroxidation was also reported in 12% of normozoospermic men (Huszar and Vigue, 1994). This information suggests that a high incidence of ROS formation may be associated with reduced sperm fertilizing potential in both infertile and fertile males. In our study, we observed significantly higher levels of ROS in all infertile groups. The highest ROS levels were seen in the varicocele with prostatitis group followed by the infertile vasectomy reversal group and the idiopathic infertility group. The spontaneous formation of ROS has been associated with decreased sperm–egg interaction and reduced in-vivo fertility (Aitken and Clarkson, 1987;

Aitken *et al.*, 1989). The same group (Aitken and Clarkson, 1987) showed that the production of ROS by human spermatozoa is inversely related to their ability to exhibit sperm–oocyte fusion. High lipid peroxidation may reduce the capacity of the sperm to fertilize (Aitken *et al.*, 1989).

In our study, we found depressed TAC levels in all infertile patients. The idiopathic infertility group had the lowest levels of TAC, followed by the varicocele with inflammation, and varicocele groups. These levels were significantly different (>2 SD) compared to controls.

Whether TAC levels characterize the level of oxidative stress will depend on the source of ROS, i.e. whether it is produced by the abnormal spermatozoa and the neutrophils (extracellularly), or within the spermatozoa (intracellularly). Two ROS-generating systems have been proposed, an NADPH oxidase-like system at the sperm plasma membrane level, and a sperm diaphorase (an NADH-dependent oxido-reductase) located in the middle piece and integrated into the mitochondrial respiratory system of the spermatozoa (Aitken, 1997). ROS emanates in part from abnormal spermatozoa, which are characterized by the retention of excess residual cytoplasm as a result of defective spermiogenesis (Aitken *et al.*, 1994). Only one-third of the ROS produced by spermatozoa is released extracellularly (Plante *et al.*, 1994). The usual ROS scavengers such as catalase and superoxide dismutase, though very effective against ROS produced by an external source, are relatively ineffective when ROS production is intracellular. In a recent placebo-controlled study, patients with asthenozoospermia in association with peroxidative damage to the spermatozoa demonstrated a potential value of oral vitamin E supplementation (Suleiman *et al.*, 1996). In clinical situations where ROS production is extracellular, classical antioxidants would be very effective, and the TAC level may be indicative of the extent of oxidative stress. In the case of oligozoospermic males whose spermatozoa generate particularly high levels of ROS, the source of cytotoxic oxygen radicals is frequently intracellular (Aitken *et al.*, 1992; Gomez *et al.*, 1996) and TAC may not be able to differentiate the extent of oxidative stress. In such instances, albumin is reported to exhibit an excellent ability to sustain sperm motility (Twiggs *et al.*, 1998). A typical example of free-radical associated male pathology is oligozoospermia in which fertilization and pregnancy rates are low using in-vitro fertilization techniques. Indeed, half of these cases are associated with the generation of ROS above the normal fertile range (Aitken *et al.*, 1989, 1992). Even though it is now clear that oxidative stress is harmful to the spermatozoa, the debate on the use of antioxidants in improving semen quality continues (Geva *et al.*, 1998; Lenzi *et al.*, 1998; Ford and Whittington, 1998; Tarin *et al.*, 1998; Martin-Du Pan and Sakkas, 1998; Saez *et al.*, 1998; Twiggs *et al.*, 1998). Antioxidants may be beneficial only in those infertile cases where infertility is due to oxidative stress. More controlled, randomized double-blind studies are needed to validate this issue.

The hypotheses that we wanted to test were whether ROS and TAC levels were abnormal in infertile men with various clinical diagnoses, and, more importantly, whether we could identify a variable that could discriminate fertile from infertile

men better than ROS or TAC alone. The clinical diagnosis of infertility alone cannot identify the underlying pathophysiology, and routine semen analysis is inadequate to determine the underlying cause. Oxidative stress can be a major mediator of infertility, therefore any parameter that is able to quantify oxidative stress would also be able to predict whether the patients in a given clinical diagnosis are more likely to be infertile. It is important to recognize that there is a great deal of variability in the levels of oxidative stress even in infertile men (Alvarez *et al.*, 1987). The ROS–TAC score as proposed by us provides a measure derived both from the levels of ROS produced and the antioxidant levels present in a given set of patients. Furthermore, this score minimizes the variability present in the individual parameters of oxidative stress. The degree of oxidative stress can be effectively determined from the ROS–TAC score which provides a measure derived from both the levels of ROS produced and the antioxidant capacity in a given set of patients.

In our study, the ROS–TAC score was calculated from control comprising healthy normal men who had very low levels of ROS, had initiated pregnancies in the past 2 years, and were therefore fertile. The composite ROS–TAC score calculated for these men was representative of the fertile group, and any scores significantly below these were indicative of the patients being infertile. Furthermore, using the area under the curve for the three variables (ROS, TAC, and ROS–TAC score) indicated the probability of correctly identifying patients in any given clinical diagnosis as either fertile or infertile. As seen from our ROC curves, ROS levels effectively discriminate fertile and infertile populations in many clinical diagnoses. But across all clinical diagnoses, the ROS–TAC score was a superior discriminator between fertile and infertile men than either ROS or TAC alone. It is quite likely that neither variable alone adequately quantifies seminal oxidative stress and the combination of these two variables is better at quantifying the overall oxidative stress affecting spermatozoa.

Furthermore, analyses of patients with male factor diagnoses indicated that those with subsequent successful pregnancies had an average ROS–TAC score in the normal range compared to significantly lower ROS–TAC scores in those without subsequent pregnancies. In addition, we found that the average ROS–TAC score for the fertile vasectomy reversal group was nearly identical to the controls. It appears that individuals with ROS–TAC scores <30, the lower limits of normal range (2 SD from 50), are at particular risk for prolonged inability to initiate pregnancies. Our observations are based on the assumption that there were no female factors present.

In conclusion, our study describes the ROS–TAC score, a novel measure of oxidative stress as being superior to ROS or TAC alone in discriminating between fertile and infertile men. We found that infertile men with male factor or idiopathic diagnoses had significantly lower ROS–TAC scores than controls, and that men with male factor diagnoses that eventually were able to initiate a successful pregnancy had significantly higher ROS–TAC scores than those who failed. In addition, male partners of couples who achieved pregnancy did not have significantly different ROS–TAC scores than controls. Therefore, the ROS–TAC score may serve as an important

measure in identifying those patients with a clinical diagnosis of male infertility who are likely to achieve a pregnancy over a period of time.

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