

Relationship Between Seminal White Blood Cell Counts and Oxidative Stress in Men Treated at an Infertility Clinic

RAKESH K. SHARMA,* FABIO F. PASQUALOTTO,* DAVID R. NELSON,† ANTHONY J. THOMAS JR,* AND ASHOK AGARWAL*

From the *Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Urological Institute, and †Department of Biostatistics and Epidemiology, Cleveland Clinic Foundation, Cleveland, Ohio.

ABSTRACT: In semen, granulocytes are major producers of reactive oxygen species (ROS), which can damage sperm. The diagnosis of leukocytospermia is usually based on the World Health Organization (WHO) definition of 1×10^6 white blood cells per milliliter, but controversy remains over the minimum leukocyte level that impairs fertility. The goals of this study were to clarify the relationship between leukocyte count and oxidative stress and to establish the minimum leukocyte count associated with oxidative stress. To do so, we compared oxidative stress in semen samples with different leukocyte counts (by the Endtz test) after a simple wash-and-resuspend procedure and determined the correlation between leukocyte counts and oxidative stress (expressed as ROS-TAC score, a composite score calculated from ROS levels and total antioxidant capacity (TAC), both measured with chemiluminescence assays). ROS-TAC decreases as oxidative stress rises. We compared specimens from 271 men attending an infertility clinic and 28 healthy controls. About 9% of patients had WHO-defined leukocytospermia and an additional 16% had some leu-

kocytes. Samples with no seminal leukocytes had significantly lower ROS levels and significantly higher ROS-TAC scores than samples with any seminal leukocytes, even very low levels. Oxidative stress was correlated with rising white blood cell (WBC) count ($r = .39$; $P < .001$). Receiver operating characteristics curves showed that ROS-TAC score would be fairly accurate at distinguishing between patients with any leukocytes and those with no leukocytes (area under the curve, 75%). In conclusion, oxidative stress occurs even in patients with very low seminal WBC counts (between 0 and 1×10^6 /mL) and rises with an increase in WBC count. Therefore, we are unable to determine a safe minimum WBC count; the presence of any WBCs is associated with oxidative stress and may therefore impair fertility. Complete removal of WBCs from semen samples used for assisted reproduction may help reduce oxidative stress.

Key words: Leukocytospermia, male infertility, semen, reactive oxygen species, Endtz test.

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The white blood cell (WBC) count in semen is one of the classical measures of semen quality (World Health Organization [WHO], 1999), but the clinical relevance on fertility has been questioned repeatedly (Wolff et al, 1990; Tomlinson et al, 1993; Aitken et al, 1994; Aitken and Baker, 1995).

Reactive oxygen species (ROS) are known to impair sperm function, and granulocytes and macrophages are known to produce ROS. A substantial minority of infertile patients have leukocytospermia (Wolff, 1995), and granulocytes are known to impair the fertilizing ability of spermatozoa (Maruyama et al, 1985). Normally, the antioxidant-rich seminal plasma protects sperm from ROS; however, infertile men have reduced seminal antioxidant capacity (Lewis et al, 1995; 1997). Also, high leukocyte concentrations can overwhelm the defense mechanisms (Yanushpolsky et al, 1996). In addition, seminal plasma

is typically removed when sperm are prepared for assisted reproductive techniques (ART). DNA oxidation increases during ART procedures (Twigg et al, 1998).

The clinical significance of both ROS and leukocyte levels continues to be debated. Some authors have found ROS levels or leukocyte counts to be of little prognostic help in either in vivo or in vitro reproduction (Tomlinson et al, 1993), whereas others have found ROS levels to have prognostic value in both in vivo (Aitken et al, 1991) and in vitro reproduction (Krausz et al, 1994; Sukcharoen et al, 1994).

Much of the controversy centers on the best definition of pathological leukocytospermia and the correlation of WBCs with seminal oxidative stress is unclear. The WHO definition is more than 1×10^6 WBC/mL of semen. However, the minimum WBC count that impairs fertility may be lower or higher.

We designed this study to answer several related questions about leukocytospermia by comparing semen samples from healthy controls to specimens from patients undergoing fertility evaluation. We compared oxidative stress and other sperm characteristics in specimens with different WBC counts. We also assessed the ability of an oxidative stress measure to distinguish between patients

Correspondence to: Ashok Agarwal PhD HCLD, Urological Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, A19.1, Cleveland, OH 44195 (e-mail: agarwaa@ccf.org).

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with no leukocytes and patients with any leukocytes. ROS-TAC score measures oxidative stress; a composite measure derived from ROS and nonenzymatic total antioxidant capacity, or TAC (Sharma et al, 1999). The goals of this study were to clarify the relationship between leukocyte count and oxidative stress and to establish the minimum leukocyte count associated with oxidative stress.

Materials and Methods

Subjects

This study was approved by the Cleveland Clinic Institutional Review Board. Semen specimens were provided by 271 patients attending the male infertility clinic at an academic medical center for infertility evaluation. Patients were divided into three groups. Patients in group 1 had no seminal leukocytes ($n = 203$). Patients in group 2 had some seminal leukocytes but fewer than $1 \times 10^6/\text{mL}$ ($n = 43$). Patients in group 3 had WHO-defined leukocytospermia ($n = 25$). In addition, 28 normal healthy men with semen characteristics that met the WHO (1999) criteria for controls were enrolled in the study. Not all controls were of proven fertility, but none met the WHO criteria for leukocytospermia (1×10^6 WBC/mL seminal ejaculate).

Semen Analysis

Computer-assisted semen analysis was performed on all specimens with a VP 50 semen analyzer (Motion Analysis Corporation, Santa Rosa, Calif). For each measurement, a $5\text{-}\mu\text{L}$ aliquot was loaded on a counting chamber (MicroCell, Conception Technologies, La Jolla, Calif). Four to eight representative fields containing 200 or more spermatozoa were examined. Samples were analyzed for concentration, percent motility, and complex motion characteristics.

Quantification of White Blood Cells (Granulocytes) by Endtz Test

A $20\text{-}\mu\text{L}$ volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial (Allegiance Healthcare Corporation, McGaw Park, Ill) and $20\text{ }\mu\text{L}$ of phosphate-buffered saline (PBS; pH 7.0) and $40\text{ }\mu\text{L}$ of benzidine solution were added. The mixture was vortexed and allowed to sit for 5 minutes. Five microliters of this mixture was placed on a Makler chamber (Sefi Medical, Haifa, Israel) and examined for cells that had stained brown, indicating that they contained peroxidase and were therefore granulocytes (Shekarriz et al, 1995).

Sperm Morphology

Air-dried seminal smears stained with Diff-Quik (Baxter Scientific, Miami, Fla) were scored for sperm morphology according to WHO (1999) guidelines and Kruger's strict criteria (Kruger et al, 1986). A total of 200 cells were scored for normal and abnormal forms at $1000\times$ magnification. Smears were not evaluated for the presence of residual cytoplasmic mass.

Reactive Oxygen Species Measurement

Specimens were washed twice with PBS (pH 7.4) and resuspended in the same medium at a concentration of 20×10^6 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 (Sharma et al, 1999). Ten microliters of 5 mM luminol prepared in dimethyl sulfoxide was added to $400\text{ }\mu\text{L}$ 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 in 10^4 counted photons per minute (cpm) per 20×10^6 sperm.

Total Antioxidant Capacity Measurement

Nonenzymatic TAC was measured in seminal plasma using an enhanced chemiluminescence assay (Kolettis et al, 1999). Aliquots of the seminal plasma, which were stored at -20°C , were thawed at room temperature and assessed for their antioxidant capacity. Seminal plasma was diluted 1:10 with deionized water and filtered through a $20\text{-}\mu\text{m}$ Millipore filter (Allegiance Health Care). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckingham, England). A constant source of ROS was produced by horseradish peroxidase-linked immunoglobulin (HRP-Ig; Amersham Life Science). Twenty microliters of HRP-Ig was added to 4.98 mL deionized H_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 and $150\text{ }\mu\text{M}$. The antioxidant capacity of the seminal plasma was expressed in molar Trolox equivalents. With the luminometer in the kinetic mode, $100\text{ }\mu\text{L}$ of signal reagent and $100\text{ }\mu\text{L}$ of HRP were added to $700\text{ }\mu\text{L}$ of distilled water and mixed. This solution was equilibrated to the desired level of chemiluminescence output (between 2 to 3×10^7 cpm) for 10 seconds. One hundred microliters of the prepared seminal plasma was immediately added to the signal reagent and HRP, and the chemiluminescence was measured. The time of chemiluminescence suppression and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence were recorded.

Measures to Protect Samples Against Deterioration

Antioxidant capacity falls progressively during exposure to oxygen-rich environments. The deterioration is slow when the samples are stored at low temperatures and not subjected to repeated freezing and thawing. Ascorbic acid is labile and probably accounts for most of the initial deterioration (Whitehead et al, 1992). In our study, all samples were processed within 1 hour of collection and were stored at -70°C . In a separate pilot study, we investigated the effect of storage time on the total antioxidant capacity (of essentially vitamin E) and found no significant decline in the total antioxidant capacity using Trolox as the standard (unpublished data).

Table 1. Comparison of sperm characteristics (mean ± SE) in controls and three groups of patients classified according to their Endtz values*

Variable	Donors, n = 28 Mean ± SE	Patients, Group 1 Endtz = 0, n = 203 Mean ± SE	Patients, Group 2 Endtz >0 and <1 (n = 43) Mean ± SE	Patients, Group 3 Endtz >1, n = 25 Mean ± SE	P	
					Among all 4 groups	Among 3 patient groups
Volume, mL	2.50 ± 0.23	2.66 ± 0.11	2.45 ± 0.19	2.51 ± 0.42	.84	.70
Concentration, ×10 ⁶ /mL	62.15 ± 6.76	38.34 ± 2.84	38.47 ± 4.91	37.31 ± 10.71	.04†	.99
Motility, %	53.70 ± 3.29	38.73 ± 1.35	39.83 ± 3.15	40.37 ± 3.83	.003†	.89
Normal sperm morphology						
WHO, %	39.79 ± 2.50	33.05 ± 0.85	29.66 ± 2.04	28.86 ± 2.74	.02†	.12
Kruger's, %	12.07 ± 0.86	9.49 ± 0.35	8.74 ± 0.75	7.91 ± 0.96	.06	.28
Log (ROS + 1) cpm	1.34 ± 0.13	1.87 ± 0.08	2.62 ± 0.17	3.18 ± 0.20	<.001‡	<.001
TAC Trolox equivalents	1668.92 ± 107.06	1169.75 ± 38.32	1023.93 ± 75.71	906.80 ± 75.60	<.001§	.03
ROS-TAC score	50.70 ± 2.00	38.19 ± 1.02	29.01 ± 2.14	19.33 ± 2.09	<.001	<.001

* WHO indicates World Health Organization; ROS, reactive oxygen species; TAC, total antioxidant capacity; WBC, white blood cells; cpm, counted photons per minute.

† Donors significantly higher than patients over 3 semen parameters.

‡ Log (ROS + 1) significantly different among all 4 groups in pairwise comparisons ($P < .05$).

§ TAC significantly higher in donors ($P < .001$) compared to all patient groups; only patient differences between Endtz = 0 and Endtz $\geq 1 \times 10^6$ WBC/mL ($P = 0.03$).

|| ROS-TAC score significantly different among all four groups in pairwise comparisons ($P < .01$).

Calculating the ROS-TAC Score

ROS values were log-transformed (log [ROS + 1]) to normalize the data distribution. The ROS and TAC values from controls were used to create a scale of these two variables that used the control values as reference points (for details see Sharma et al, 1999). In brief, both log (ROS + 1) and TAC were standardized to their z-scores and then analyzed 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 standard deviation of 10 in normal donors, the ROS-TAC score was transformed as:

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

Statistical Analysis

Comparisons among groups were performed with a one-way analysis of variance (ANOVA), followed by pairwise comparisons with Scheffé's multiple range test. Spearman's correlations were used to assess the correlation between continuous measures (the three measures of oxidative stress and WBC count or other measures of semen quality). An ROC curve was calculated to determine whether ROS-TAC could be used to discriminate between various cutoff levels for Endtz values.

As a secondary analysis, the patients' ROS-TAC scores were compared with 52 male factor patients with known fertility outcomes described in an earlier study (Sharma et al, 1999). Using the long-term follow-up results from this group, we calculated logistic regression estimates of the probability of infertility (with 95% confidence intervals [CI]) for men with different levels of

leukocytospermia. We were then able to use these estimates to predict a patient's risk of infertility from his ROS-TAC score.

For all analyses, statistical significance was assessed at $P < .05$ and summary statistics are presented as means ± standard errors. Data were analyzed by the SAS statistical software package (version 6.12, SAS Institute Inc, Cary, NC).

Results

Control specimens had significantly better concentration, motility, and percentage of normal forms than patient specimens (Table 1). Semen characteristics did not differ significantly between the three patient groups. However, significant differences were observed among patient groups for ROS and TAC measures. In ANOVA and the subsequent pairwise comparisons, all 3 groups differed significantly in ROS and the ROS-TAC score, indicating that oxidative stress increased as the Endtz value increased, even when the Endtz was less than the WHO cutoff of 1×10^6 WBC/mL. Also, TAC values were significantly higher in group 1 than in group 3.

We then divided the patient groups into smaller categories (Table 2). Even the 25 patients with Endtz values between 0 and 0.04×10^6 /mL had significantly higher ROS and lower ROS-TAC scores than patients with Endtz values of zero. However, no significant differences were observed in TAC levels until the WBC count reached 2×10^6 WBC/mL.

Correlations between the 3 measures of oxidative stress (ROS, TAC, and ROS-TAC score) and the Endtz count, sperm concentration, percent motility, and morphology

Table 2. Comparison of ROS, TAC, and ROS-TAC score results using different cutoff values for Endtz*

Endtz cutoff value, ×10 ⁶ WBC/mL	Log (ROS + 1) cpm		TAC Trolox Equivalents		ROS-TAC score	
	Mean ± SE	P	Mean ± SE	P	Mean ± SE	P†
Endtz = 0, n = 203	1.87 ± 0.08	...	1169.7 ± 36.8	...	38.2 ± 1.0	...
0 < Endtz ≤ 0.4, n = 25	2.47 ± 0.22	.01	1073.8 ± 108.7	.40	32.4 ± 2.8	.05
0.4 < Endtz ≤ 0.8, n = 17	2.94 ± 0.28	<.001	977.1 ± 131.6	.16	23.0 ± 3.6	<.001
0.8 < Endtz ≤ 2.0, n = 13	3.20 ± 0.32	<.001	979.0 ± 147.2	.21	22.2 ± 3.9	<.001
Endtz > 2, n = 13	2.98 ± 0.33	.001	792.2 ± 161.2	.02	17.6 ± 4.6	<.001

* ROS indicates reactive oxygen species; TAC, total antioxidant capacity; WBC, white blood cells; cpm, counted photons per minute.

† Comparisons to Endtz = 0.

(both by WHO guidelines and Kruger's strict criteria) in patients are given in Table 3. In all patients, ROS levels were significantly negatively correlated with sperm concentration and motility, and positively correlated with Endtz value. ROS was also negatively associated with frequency of sperm with normal sperm morphology. A negative correlation was seen between TAC and percent motility and Endtz value. ROS-TAC was positively associated with sperm concentration and WHO morphology

and negatively with the Endtz value. Although most of these correlations are relatively small (between $-.5$ and $+.5$) and perhaps not of biological importance, the most significant correlations observed were between sperm concentration and ROS among patients with 0 to 1×10^6 WBC/mL ($r = -.56$) and patients with greater than 1×10^6 WBC/mL ($r = -.58$).

In samples with no leukocytes, ROS was negatively correlated with concentration, motility, and normal mor-

Table 3. Correlation of ROS and TAC levels with sperm characteristics for all patients and for patients classified according to Endtz*

Variable	Endtz, ×10 ⁶ WBC/mL	Sperm concentration,		Normal Sperm Morphology	
		×10 ⁶ /mL	Motility, %	WHO, %	Kruger's, %
All Patients					
ROS, r†	.35	-.35	-.16	-.29	-.19
(P)‡	(<.001)	(<.001)	(.01)	(<.001)	(.003)
TAC, r	-.18	-.025	-.19	-.13	-.10
(P)	(.003)	(.69)	(.002)	(.06)	(.15)
ROS-TAC score, r	-.39	.29	.05	.21	.13
(P)	(<.001)	(<.001)	(.47)	(.002)	(.06)
Endtz = 0					
ROS, r		-.25	-.20	-.23	-.15
(P)		(<.001)	(.005)	(.002)	(.06)
TAC, r		.01	-.20	-.17	-.16
(P)		(.90)	(.005)	(.02)	(.03)
ROS-TAC score, r		.21	.091	.13	.05
(P)		(.005)	(.25)	(.09)	(.50)
Endtz between 0 and <1 × 10 ⁶ WBC/mL					
ROS, r	.07	-.56	-.16	-.41	-.38
(P)	(.66)	(<.001)	(.32)	(.01)	(.02)
TAC, r	-.14	-.13	-.27	-.02	.04
(P)	(.39)	(.42)	(.10)	(.91)	(.80)
ROS-TAC score, r	-.24	.48	-.02	.36	.42
(P)	(.16)	(.004)	(.91)	(.05)	(.02)
Endtz ≥1 × 10 ⁶ WBC/mL					
ROS, r	-.15	-.58	.04	-.38	-.22
(P)	(.50)	(.006)	(.87)	(.12)	(.36)
TAC, r	-.39	-.20	.18	-.07	.17
(P)	(.08)	(.40)	(.47)	(.80)	(.51)
ROS-TAC score, r	-.13	.38	.10	.23	.13
(P)	(.61)	(.13)	(.70)	(.43)	(.66)

* ROS indicates reactive oxygen species; TAC, total antioxidant capacity; WHO, World Health Organization; WBC, white blood cells.

† Spearman correlation.

‡ P < .05 was considered significant.

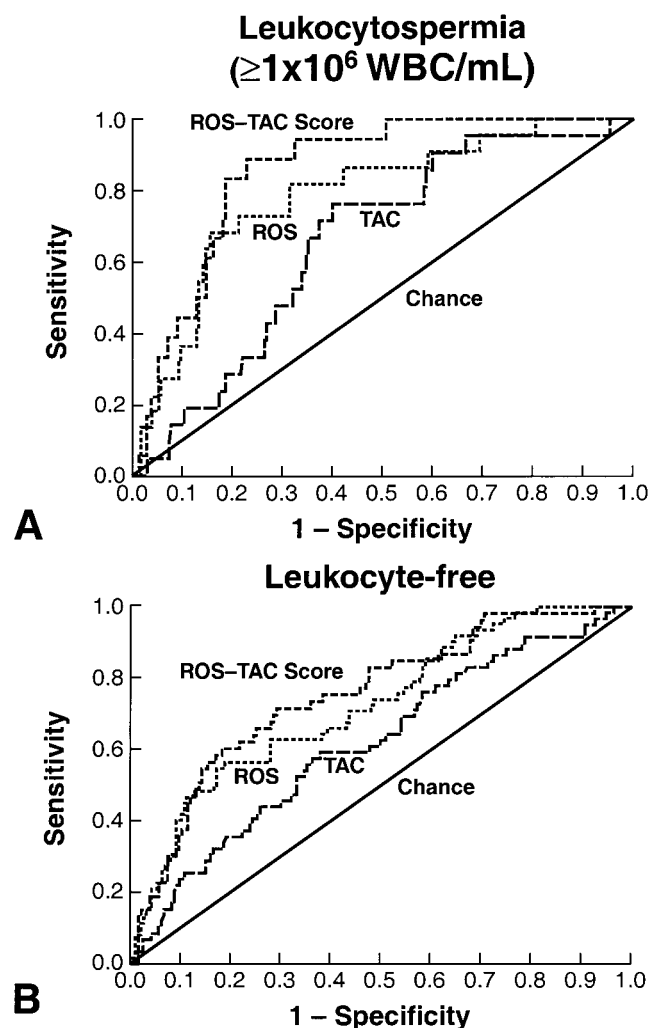


Figure. The ability of ROS-TAC score to distinguish samples with leukocyte contamination. (A) Leukocytospermia sample ($\geq 1 \times 10^6$ WBC/mL), area under the curve 85.6%. (B) Leukocyte-free sample, area under curve 75.3%.

phology. Similarly, TAC was negatively related with motility and morphology. In samples with fewer than 1×10^6 leukocytes/mL, ROS was negatively correlated with sperm concentration and normal morphology. Again, ROS-TAC score was related to sperm concentration and abnormal morphology. In samples with Endtz values above 1×10^6 WBC/mL, ROS was negatively correlated with concentration.

The area under the ROC curve was evaluated to determine how well ROS-TAC could distinguish patients with different Endtz values. When the Endtz cutoff was 1×10^6 WBC/mL, the area under the curve was higher, but not much higher (85.6%; Figure, A). When the cutoff was set at 0 Endtz (ie, when ROS-TAC was used to differentiate patients with any detectable leukocytes from those with no leukocytes), the area under the ROC curve was 75.3% (Figure, B). Thus, the ROS-TAC index of oxida-

tive stress can be used to identify patients with any seminal leukocytes as well as those with WHO-defined leukocytospermia. For detecting Endtz values greater than zero, a ROS-TAC score cutoff of 31.4 maximized both sensitivity (71.7%) and specificity (70.7%). Therefore, patients with ROS-TAC score values of less than 31.4 have increased rates of detectable WBCs. To detect the 1×10^6 WBC/mL Endtz cutoff, a ROS-TAC score cutoff of 24.5 provides a sensitivity of 83.3% and specificity of 81.3%.

Discussion

We were unable to establish the minimum level of WBCs associated with oxidative stress because it appeared that the presence of any seminal leukocytes, no matter how few, was associated with oxidative stress. Oxidative stress increased with an increase in Endtz value even when Endtz was much less than 1×10^6 WBC/mL. In addition, there was a significant negative correlation between oxidative stress and sperm concentration, motility, and sperm morphology, reflecting the strong association of ROS with impaired semen quality. The ROC curves showed that the ROS-TAC index of oxidative stress was best at identifying samples above a WBC count of 1×10^6 /mL; however, ROS-TAC was almost as accurate at discriminating leukocyte-free samples from those with very low levels of leukocyte contamination. Thus, no WBC count can be considered a safe cutoff in terms of oxidative stress. Our finding is in contrast to earlier reports (Aitken et al, 1994, 1995) that found no damage to spermatozoa in the ejaculate even in the presence of more than 10^6 leukocytes/mL.

The incidence of leukocytospermia ($>1 \times 10^6$ WBC/mL) as defined by WHO (1999) ranges between 10% to 20% among male infertility patients (Wolff, 1995). Investigators have used different methods to identify the leukocytes, ranging from cytology, immunocytology, peroxidase, and polymorphonuclear-elastase. The incidence of leukocytospermia reported in the literature ranged from 2% (Kung et al, 1993) to as high as 35.3% (Barrat et al, 1990) when the same methods of detection (immunocytology) were used. Similarly, in studies using peroxidase as the method of detection, the incidence of leukocytospermia ranged from 3% (Kovalski et al, 1992) to 23.7% (Endtz, 1974). Wolff et al (1992) found leukocytospermia in 9.2%, and Comhaire et al (1980) in 13.4% of the cases using peroxidase staining. Leukocytospermia as defined by WHO was demonstrated in fewer than 9% of the total subfertile samples in our study. An additional 16% of the patients had an Endtz value greater than 0 but less than 1×10^6 WBC/mL. Our results on the prevalence of leukocytospermia are in agreement with the findings of Ait-

ken et al (1994) and Wang et al (1994), but lower than those of Wolff and Anderson (1988), who observed a 23% prevalence of leukocytospermia among infertile men. Such disparities suggest that patient groups vary considerably, and warrants use of standardized definitions and methods of WBC quantitation.

Although specimens from control men in our study had significantly higher concentration, motility, and percentage of WHO normal morphology than specimens from patients ($P < .04$), the patients did not differ across Endtz classifications for semen characteristics ($P > .12$).

In our study, ROS was measured in artificial conditions, with the seminal plasma washed away. This measurement therefore reflects the production of ROS by granulocytes and macrophages only in the absence of seminal plasma. Such leukocyte-derived ROS would be free to attack spermatozoa when they are washed free of seminal plasma during the swim-up procedure (Krausz et al, 1994; Sukcharoen et al, 1994).

The etiology of seminal leukocytes is not clear. Antibiotic treatment has frequently been successful in reducing seminal WBCs (Berger et al, 1982; Haidl et al, 1990; Branigan and Muller, 1994). However, 80% of samples with WBCs are microbiologically negative (Hillier et al, 1990). Release of WBCs may be stimulated by nonspecific male genital tract inflammation, caused at first by bacteria but then continued in their absence by continued immunological activity. White blood cells may also play a physiologic role in immunosurveillance and eliminating abnormal sperm (Kiessling et al, 1995).

As the etiology of seminal leukocytes is unclear, it may be more useful to focus on their effects. A white blood cell concentration higher than $2 \times 10^6/\text{mL}$ reduces sperm motility (Millingos et al, 1996; Yanushpolsky et al, 1996). A reduced rate of in vitro fertilization was reported when the number of leukocytes exceeded $6 \times 10^6/\text{mL}$ of ejaculate (De Geyter et al, 1994). Other investigators have also confirmed that leukocytes have a negative effect on fertilization in vitro (Krausz et al, 1994; Sukcharoen et al, 1995).

The relevance of leukocytospermia in vivo is controversial (for an overview, see Wolff, 1995). Leukocytes are an important source of oxidative damage, which is clearly a key factor in the etiology of male infertility (Aitken and Fisher 1994; Cummins et al, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996). Oxidative injury to spermatozoa is a major cause of spermatozoal dysfunction, and the total level of nonenzymatic antioxidant defenses of human seminal plasma is inversely related to lipid peroxidation (Sikka et al, 1995; Smith et al, 1996). Oxidative stress is known to affect the integrity of the sperm genome, inducing high frequencies of single and double DNA strand breaks (Hughes et al, 1996; Twigg et al, 1998). Poor-quality semen samples

have a greater percentage of spermatozoa with DNA fragmentation than normal fertile samples (Sun et al, 1997; Lopes et al, 1998). Similarly, the percentage of spermatozoa with fragmented DNA is negatively correlated with fertilization rates in in vitro fertilization (Sun et al, 1997) and intracytoplasmic sperm injection (Lopes et al, 1998). Leukocyte contamination is an important factor in determining the fertilizing potential of human spermatozoa in vitro (Sukcharoen et al, 1995).

Seminal lymphocytes and macrophages appear to originate mainly from the epididymis and rete testis, whereas granulocytes seem to be contributed largely by the prostate and seminal vesicles (El-Demiry et al, 1985) under conditions of inflammation; secretions of the normal prostate very rarely contain granulocytes (Schaeffer et al, 1981). White blood cells in semen can also be an early sign of acute epididymitis (Wolff, 1995). If concentrations of activated granulocytes are elevated in the epididymis, prostate, or seminal vesicles during a silent genital tract infection, the released ROS could impair normal sperm function (Wolff et al, 1991).

However, leukocytes are not the only source of ROS. Defective spermatozoa with an excessive amount of residual cytoplasm can produce higher amounts of ROS (Zini et al, 1993; Aitken et al, 1994, 1995; Sikka et al, 1995; Ochsendorf, 1999). Nevertheless, leukocytes produce 1000 times more ROS than do spermatozoa at capacitation (de Lamirande and Gagnon, 1995).

Seminal plasma strongly quenches the oxidative bursts released by granulocytes in response to infection or inflammation. Men with higher antioxidant levels may tolerate larger numbers of ROS-producing WBCs, whereas men without adequate seminal plasma protection may suffer sperm damage by granulocyte numbers as low as $0.6 \times 10^6/\text{mL}$ (Kovalsky et al, 1992). During in vitro sperm preparation, ROS have the potential to be more damaging because the protective effect of the antioxidants in the seminal plasma is removed. Whether TAC levels reflect the level of oxidative stress will depend on the source of ROS; that is, whether they are produced by abnormal spermatozoa and neutrophils (extracellularly) or within the spermatozoa (intracellularly).

In our study, significant differences were seen in TAC only when the Endtz values were significantly elevated ($>2 \times 10^6$ WBC/mL). High levels of certain antioxidants have been positively correlated with semen quality (Fraga et al, 1991, 1996; Kobayashi et al, 1991; Suleiman et al, 1996). The epididymis is a rich source of antioxidant enzymes that scavenge any excess reactive oxygen metabolites released by the spermatozoa during epididymal transit (Aitken and Vernet, 1998). Ascorbate, urate, and thiols are the major individual antioxidants present in human semen (Lewis et al, 1997). Ascorbate is relatively unstable at room temperature and even when frozen at

–20°C (Fraga et al, 1991). Vasectomized men do not have lower concentrations of ascorbic acid and uric acid than men with normospermia, which indicates that these may not be the key antioxidants in the epididymis (Potts et al, 1999). The epididymis may possess only region-specific antioxidant activity (Potts et al, 1999).

Infertile men are more likely than fertile men to have depressed TAC levels and lower levels of individual antioxidants (Lewis et al, 1995, 1997; Smith et al, 1996). In clinical situations in which ROS production is extracellular, classical antioxidants would be very effective, and the TAC level may indicate the extent of oxidative stress. In this study, we measured the chain-breaking antioxidant vitamin E, using water-soluble α -tocopherol as the standard. Vitamin E is one of the major membrane protectants against ROS and lipid peroxidation (Halliwell and Gutteridge, 1989). It is lipid-soluble and acts mainly within cell membranes (Ford and Whittington, 1998). Because vitamin E is a chain-breaking antioxidant and not a scavenging antioxidant, it would be expected to offer protection to membrane components without influencing ROS generation. We however did not measure the relative contribution and relevance of other antioxidant systems.

In our earlier study (Sharma et al, 1999), we reported that the ROS-TAC score is superior to ROS or TAC alone, as neither variable alone adequately quantifies seminal oxidative stress. In the present study, ROS-TAC was associated with sperm concentration and percent of sperm with normal morphology. It decreased significantly with increasing contamination by WBCs, with the biggest difference in samples with Endtz scores higher than 2×10^6 WBC/mL. Oxidative stress was already known to be associated with leukocyte contamination; our study provides further information about the correlation between the two (the Spearman's correlation between ROS-TAC and WBC count was $r = -.39$). In addition, our ROC curve analysis shows that the ROS-TAC measure of oxidative stress is fairly accurate in distinguishing between samples with no leukocytes and samples with even very small numbers of leukocytes.

The clinical effect of this increased oxidative stress was evaluated by comparing their ROS-TAC scores to the ROS-TAC scores of 52 men in our earlier study (Sharma et al, 1999), who were followed to determine fertility status. Based on comparisons to these 52 men of known fertility status, men without any detectable Endtz values are projected to have a 23.7% chance of initiating a pregnancy (95% CI, 12.4%–40.8%) during 12 months of follow-up, based solely on their ROS-TAC levels. Men with some leukocytes but fewer than 1×10^6 /mL are projected to have a 15.5% fertility rate (95% CI, 7.3%–32.3%) during 1 year of follow-up, which is a 35% reduction in success rates. Men with Endtz findings of more than 1×10^6 /mL are projected to have a fertility rate of 8.6% (95%

CI, 2.7%–26.3%), which is 64% less than men without any leukocytes. Obviously, infertility is related to many factors aside from ROS-TAC; however, these comparisons are meant to illustrate the potential clinical implications of the observed increases in oxidative stress. Increases in oxidative stress may be useful in identifying the subsets of patients within a clinical diagnosis as either fertile or infertile.

Our study has several limitations. First, we measured only the basal levels of ROS and not the activation status of the leukocytes. Second, we did not examine the smears for spermatozoa with excessive residual cytoplasm; that is, defective spermatozoa that could also be contributing ROS. Finally, we did not follow these patients for long enough to relate our results to long-term fertility outcome. Men with chronic or episodic leukocytospermia should be enrolled in future studies to further define the etiology and long-term clinical effects of leukocytospermia and to develop improved prevention or treatment strategies.

Semen specimens prepared for ART include washing of sperm to remove seminal plasma that is rich in antioxidants. Washed sperm are resuspended in a simple culture media lacking antioxidants, which are sometimes supplemented with transition metals known to promote lipid peroxidation (Armstrong et al, 1998). Removing WBCs from seminal ejaculates during preparation for ART would seem to be a prudent measure. These WBCs can be removed with paramagnetic beads coated with antibodies to CD45 antigen, and confirmed with the formyl-methionyl-leucyl-phenylalanine provocation test (Krausz et al, 1992; Aitken et al, 1995). Adding antioxidants to the culture medium may also be beneficial (Aitken et al, 1996; Baker et al, 1996).

In conclusion, our study suggests that the WHO definition of leukocytospermia (1×10^6 /mL) may be too high. We were unable to identify a safe lower limit for WBC count in semen because the presence of any leukocytes, no matter how few, was associated with elevated oxidative stress. We showed that oxidative stress is positively correlated with WBC count in vitro. Oxidative stress (ROS-TAC score) can be used not only to identify patients who meet the WHO definition of leukocytospermia, but also to distinguish patients with no leukocytes from patients with any WBCs, providing further evidence that oxidative stress begins to rise with even minimal leukocyte contamination.

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