Journal of Andrology, Vol. 24, No. 6, November/December 2003

Utility of the Nitroblue Tetrazolium Reduction Test for Assessment of Reactive Oxygen Species Production by Seminal Leukocytes and Spermatozoa

NAVID ESFANDIARI,* RAKESH K. SHARMA,† RAMADAN A. SALEH,‡ ANTHONY J. THOMAS, JR.,† AND ASHOK AGARWAL†

*From *IVF and Andrology Laboratories, Toronto Center for ART, Toronto, Ontario, Canada; the †Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute, The Cleveland Clinic Foundation, Cleveland, Ohio; and the ‡Department of Dermatology and Venereology, Faculty of Medicine, South Valley University, Sohag, Egypt.*

ABSTRACT: The purpose of this study was to evaluate the ability of spermatozoa and leukocytes in semen to produce reactive oxygen species (ROS) by using nitroblue tetrazolium (NBT) staining and to examine the association between NBT staining and levels of ROS as measured by chemiluminescence. Twenty-one infertility patients (leukocytospermia; n = 8; nonleukocytospermia, n = 13) and 9 healthy donors were included. Standard semen analysis and density gradient centrifugation were performed to test NBT staining, ROS, and total antioxidant capacity. A ROS–total antioxidant capacity (ROS-TAC) score was calculated by using principal component analysis. In the leukocytospermic group, after separation on a density gradient, the percentage of NBT-positive staining was significantly higher in sperm suspensions contaminated with leukocytes (median [25th, 75th percentiles]; 70% [61%, 79%]) compared to the nonleukocytospermic group (14.5% [9%, 25.5%]; $P = .03$) and donors (7% [3%, 11%]; $P = .02$), respectively. A strong positive correlation was seen between levels of ROS in whole ejaculates and NBT-positive staining in leukocytes ($r = 0.59$; $P < .0006$) and in leukocyte fractions ($r = 0.72$; $P < .0001$) after density gradient separation. Similarly, ROS was positively correlated with excessive cytoplasmic retention in spermatozoa from whole ejaculates and abnormal spermatozoa after separation on density gradients ($r = 0.72$; $P < .0001$). The ROS-TAC score was inversely correlated with NBT staining in leukocytes in whole ejaculates ($r = -0.960$, $P < .0007$) and in both leukocyte fractions ($r = -0.39$; $P < .04$) and spermatozoa with cytoplasmic retention ($r = -0.38$; $P < .04$). Our results indicate that the NBT reduction test can be used to assess the contribution of seminal leukocytes and defective spermatozoa towards ROS generation in semen. Levels of ROS assessed by chemiluminescence assay are strongly correlated with the results of NBT staining.

Key words: Male infertility, oxidative stress.

J Androl 2003;24:862–870

Recent reports have indicated that high levels of reactive oxygen species (ROS) are present in semen samples of 25% to 40% of infertile men (de Lamirande et al, 1995; Padron et al, 1997). ROS production in the male reproductive tract is of concern because of the potential for pathological effects. Spermatozoa, like all cells living under aerobic conditions, constantly face the oxygen (O_2) paradox: O_2 is required to support life, but its metabolites such as ROS can modify cell functions, endanger cell survival, or both (de Lamirande and Gagnon, 1995). Therefore, it is not surprising that seminal plasma contains a battery of different antioxidants that help protect spermatozoa against such oxidants (Sies, 1993). Seminal oxidative stress (OS) develops as a result of an imbalance between ROS-generating and -scavenging activities (Sikka et al, 1995; Sharma and Agarwal, 1996; Sikka, 2001; Saleh and Agarwal, 2002). The individual measures of oxidative stress such as ROS and total antioxidant capacity (TAC) may not be adequate markers of oxidative stress. A novel score called ROS-TAC score has been shown to be a better discriminator of oxidative stress (Sharma et al, 1999).

Spermatozoa are particularly susceptible to OS-induced damage because their plasma membranes contain large quantities of polyunsaturated fatty acids (Alvarez and Storey, 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Aitken and Fisher, 1994; de Lamirande and Gagnon, 1995; Sharma and Agarwal, 1996). Oxidative stress attacks not only the fluidity of the sperm plasma membrane, but also the DNA integrity (Aitken, 1999; Saleh et al, 2002). Morphologically abnormal spermatozoa and seminal leukocytes are the main source of high ROS production in human ejaculates (Aitken and

Presented at the 59th Annual Meeting of the American Society for Reproductive Medicine, October 12–17, 2002, Seattle, Washington.

Correspondence to: Dr Ashok Agarwal, HCLD, Director, Center for Advanced Research in Human Reproduction, Infertility, and Sexual Function, Glickman Urological Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave, Desk A19.1, Cleveland, OH 44195 (e-mail: agarwaa@ccf.org).

Received for publication April 2, 2003; accepted for publication July 14, 2003.

West, 1990; Kessopolou et al, 1992). It is important to determine the source of ROS in a given semen sample because the clinical implications of infiltrating leukocytes are quite different from those of pathological conditions in which spermatozoa themselves are the source of ROS (Aitken, 1995). Methods that are currently used for assessment of seminal OS, such as chemiluminescence assays, help measure the total amount of ROS in semen. However, such assays do not provide information on the differential contribution of spermatozoa and leukocytes to ROS production in semen or on the state of activation of individual cells.

The myeloperoxidase or the Endtz test is used to differentiate granulocytes such as neutrophils, polymorphonuclear leukocytes, and macrophages from germinal cells. Peroxidase-positive leukocytes (neutrophils and macrophages) are the main leukocytes present in semen and these are also the source of ROS formation by phagocytosis. Consequently, this test can be used an indicator of excessive ROS formation in semen (Shekkariz et al, 1995). A disadvantage of the peroxidase test is that it cannot be used to detect ROS generation by spermatozoa. On the other hand, nitroblue tetrazolium (NBT) is a yellow water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form formazan derivative that can be monitored spectrophotometrically (Baehner et al, 1976; Armstrong et al, 2002). The cytoplasmic NADPH, which is produced by oxidation of glucose through the hexose monophosphate shunt, serves as an electron donor. The oxidase system available in the cytoplasm helps transfer electrons from NADPH to NBT and reduce NBT into formazan (Baehner et al, 1976). Thus, the NBT reaction indirectly reflects the ROS-generating activity in the cytoplasm of cells and therefore can help determine the cellular origin of ROS in a heterogeneous suspension such as the seminal ejaculate. This was the reason to justify development of the NBT test and use of NBT staining in individual cells, that is, spermatozoa and the leukocytes, and measurement of the formazan precipitation due to NBT reduction.

The objectives of our study were to determine the contribution of spermatozoa and leukocytes toward the amount of ROS production in semen, to examine the state of activation of leukocytes in semen of infertile men with various levels of leukocyte contamination, and to determine the correlation between levels of ROS and TAC in semen by chemiluminescence, ROS-TAC score and the NBT staining in spermatozoa and leukocytes.

Materials and Methods

Standard Semen Analysis

After approval from the Institutional Review Committee, semen samples were collected from infertile men (n = 21) attending the infertility clinic and healthy donors (n = 9). Based on leukocyte concentrations in semen, patients samples were classified into 2 groups: leukocytospermic group (leukocytic count $> 1 \times 10^6$ peroxidase-positive leukocytes [PPL]/mL semen; n = 8) and nonleukocytospermic group (leukocytic count $\leq 1 \times 10^6$ PPL/mL; n = 13).

Donors were selected on the basis of normal semen parameters according to World Health Organization (WHO) guidelines (WHO, 1999). All specimens were collected by masturbation at the clinical andrology laboratory after an abstinence period of 48–72 hours. After liquefaction, manual semen analysis was performed to measure sperm concentration and percentage of motility by using the WHO classification. Smears of neat semen were prepared for sperm morphology assessment. The smears were fixed and stained by using the Diff-Quik kit (Baxter Healthcare Corporation, Inc, McGaw Park, Ill). Immediately after staining, the smears were rinsed in distilled water and air-dried. Smears were scored for sperm morphology by using WHO criteria.

Quantification of Seminal Leukocytes

We assessed the presence of granular leukocytes (neutrophils and macrophages) in semen by myeloperoxidase staining or the Endtz test (Shekkariz et al, 1995). Briefly, a 20-μL volume of liquefied semen specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar Corp, Cambridge, Mass) with 20 μL of phosphate-buffered saline (PBS) (pH 7.2) and 40 μL of benzidine solution. The solutions were mixed and allowed to sit at room temperature for 5 minutes. PPL staining brown were counted in all 100 squares in a Makler's chamber under the bright-field objective (magnification, 20×) in 5 different fields and an average count was determined. Leukocytospermia was defined as counts higher than 1×10^6 PPL/mL of semen.

Density Gradient Centrifugation

Aliquots of 1 mL of the liquefied semen were loaded onto a 47% and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, Calif) and centrifuged at $500 \times g$ for 20 minutes at room temperature. The resulting interfaces between seminal plasma and 47% (containing leukocytes) and 47% and 90% (containing abnormal spermatozoa) were aspirated and transferred to separate test tubes. Both fractions (leukocytes and abnormal spermatozoa) were diluted in 1 volume of human tubal fluid (HTF) medium and centrifuged at $500 \times g$ for 7 minutes. The pellet was then resuspended in 1 mL of HTF. An aliquot from whole ejaculate and each fraction (leukocytes and abnormal spermatozoa) was examined for sperm and leukocyte concentration, ROS production, and NBT staining.

Measurement of Reactive Oxygen Species

Levels of ROS were measured in the whole ejaculate and immature and mature spermatozoa by chemiluminescence assay by using luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma Chemical Co, St Louis, Mo) as a probe (Kobayashi et al, 2001). Measurements were made with a Berthold luminometer (Autolumat: LKB 953, Wallace Inc, Gaithersburg, Md). Eight microliters of horseradish peroxidase (HRP) (12.4 U of HRP type VI, 310 U/mg; Sigma) were added to 400 μL of the whole

ejaculate, leukocytes, and abnormal spermatozoa to allow sensitization of the assay for measurement of extracellular hydrogen peroxide. Ten microliters of luminol prepared as 5-mM stock in dimethylsulfoxide was added to the mixture. Ten microliters of 5-mM luminol was added to 400 μL of PBS as a negative control. Levels of ROS were determined by measuring chemiluminescence in the integrated mode for 15 minutes. Results were expressed as $\times 10^6$ counted photons per minute (cpm) per 20×10^6 cells/mL.

Measurement of Total Antioxidant Capacity

Total nonenzymatic antioxidant capacity was measured in the seminal plasma by using the enhanced chemiluminescence assay (Saleh et al, 2002). The liquefied semen samples were centrifuged at $300 \times g$ for 7 minutes, and the seminal plasma was removed and placed into a test tube. The seminal plasma was centrifuged at $300 \times g$ for 10 minutes to remove all cellular contaminants. Aliquots of the seminal plasma were stored at −80°C until use. The frozen seminal plasma was thawed at room temperature and immediately assessed for nonenzymatic antioxidant capacity. The seminal plasma was diluted 1:10 with deionized water (dH_2O) and filtered through a 0.20-μm Millipore filter (Allegiance Healthcare Corporation, McGaw Park, Ill). Signal reagent was prepared by adding 30 μL of H_2O_2 (8.8 molar/L), 10 μL of para-iodophenol stock solution (41.72 μM), and 110 μL of luminol stock solution (3.1 mM) to 10 mL of Tris buffer (0.1 M, pH 8.0). HRP working solution was prepared from an HRP stock solution by making a dilution of 1:1 of dH_2O to give a luminescence output of 3×10^7 cpm.

Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was added as a standard solution at concentration of 25, 50, and 75 μM for TAC calibration. The antioxidant capacity of the seminal plasma was then expressed in molar Trolox equivalents. With the luminometer in the kinetic mode, 100 μL of signal reagent and 100 μL of HRP working solution were added to 700 μL of dH_2O and mixed. The mixture was equilibrated to the desired level of chemiluminescent output (between 2.8 and 3.2×10^7 cpm) for 100 seconds. One hundred microliters of the prepared seminal plasma was immediately added to the mixture, and the chemiluminescence was measured. Suppression of luminescence 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.

Calculating the ROS-TAC Score

Reactive oxygen species values were log-transformed (log(ROS + 1)) to normalize the data distribution. The ROS and TAC values from the controls were used to create a scale of these 2 variables that used the control values as reference points as discussed in our earlier work (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 accounted 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 the normal donors, the ROS-TAC score was transformed as:

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

Nitroblue Tetrazolium Test

Nitroblue tetrazolium (NBT) (0.1%) was prepared in PBS by adding 10 mg of NBT powder (Sigma) to 100 mL of PBS (pH 7.2) and stirred at room temperature for 1 hour. NBT solution was filtered with a 0.2-μm filter (Allegiance Health Care). NBT staining was done for whole ejaculate, leukocytes, and abnormal spermatozoa by adding equal volumes of 0.1% of NBT solution and incubated for 30 minutes at 37°C. The tubes were centrifuged at $250 \times g$ for 5 minutes and smears were prepared from the pellet and air-dried. The air-dried smears were stained with Wright stain and a total of 100 spermatozoa and 100 leukocytes were scored under 100× magnification. Three skilled observers (N.E., R.A.S., and R.S.) scored the NBT-stained slides in a blinded manner. When the scoring differences were comparable between these observers, 1 person (N.E.) scored the actual test samples in a blinded fashion.

Leukocytes in the smears were scored as follows: no detectable formazan (−), scattered or few formazan granules (+), intermediate density (++), and cells filled with formazan (+++) (Figure 1). Spermatozoa in the smears were scored as follows: formazan occupying 50% or less of the cytoplasm (+) and more than 50% of the cytoplasm (++) (Figure 2).

Statistical Analysis

Comparisons between groups and comparison of immature and mature spermatozoa within the same individuals were performed by Wilcoxon rank-sum test. The Spearman method was used to calculate the correlation between peroxidase test and the NBT test and Dunn's pairwise comparison comparing the 3 groups for peroxidase and NBT test. The ROS-TAC score was compared with NBT staining in the 3 groups. Receiver operating characteristic (ROC) curves were used to predict a peroxidase level greater than 1 by NBT test. ROC curves illustrate the sensitivity and specificity over the entire range. The area under the curve (AUC) also was calculated. This 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. The AUC is interpreted as the probability that a randomly drawn leukocytospermic sample (peroxidase values $> 1 \times 10^6$ PPL/mL) has a higher value of NBT than a randomly drawn nonleukocytospermic sample (peroxidase value $< 1 \times 10^6$ PPL/mL). We also examined the various cutoff values for NBT test to identify the best NBT cutoff points associated with sensitivity and specificity.

All summary statistics are presented as median and interquartile values (25th and 75th percentiles). All statistical tests were 2-tailed with statistical significance considered at $P < .05$, and computed by using SAS version 8.1 (SAS Institute Inc, Cary, NC).

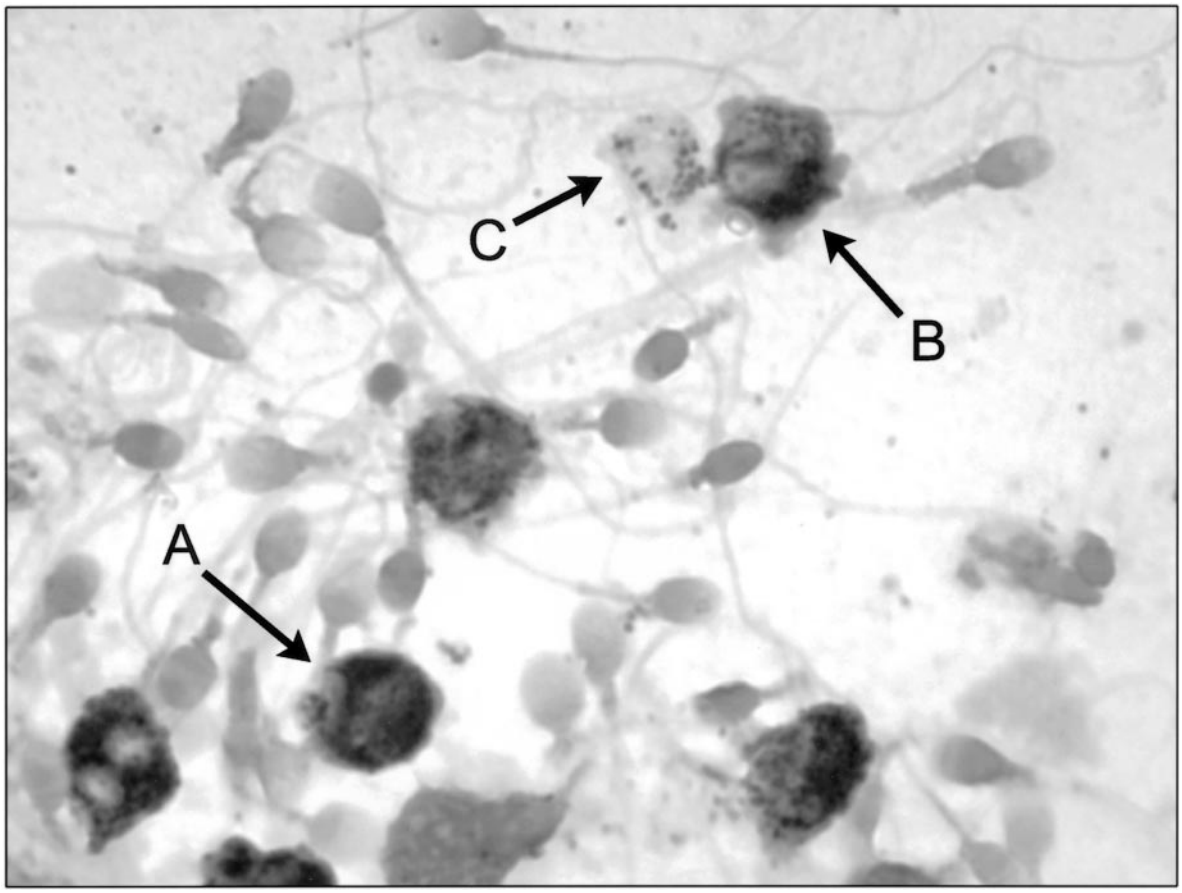

Figure 1. Positive nitroblue tetrazolium staining in leukocytes: Leukocytes were scored as follows: **(A)** cells filled with formazan (+++), **(B)** intermediate density (++), **(C)** scattered or few formazan deposit (+).

Results

Semen Characteristics

Comparisons of sperm concentration, percentage motility, and morphology for the donors and patients are shown in Table 1. Sperm quality was significantly better in healthy donors than in patients except for WHO morphology. Sperm characteristics did not differ significantly between the 2 patient groups. Median and interquartile values (25th and 75th percentiles) of seminal leukocyte concentrations ($\times 10^6$/mL) in donors were 0.06 (0, 0.18) compared to 0.06 (0.0, 0.06; $P = .81$) in the nonleukocyto-

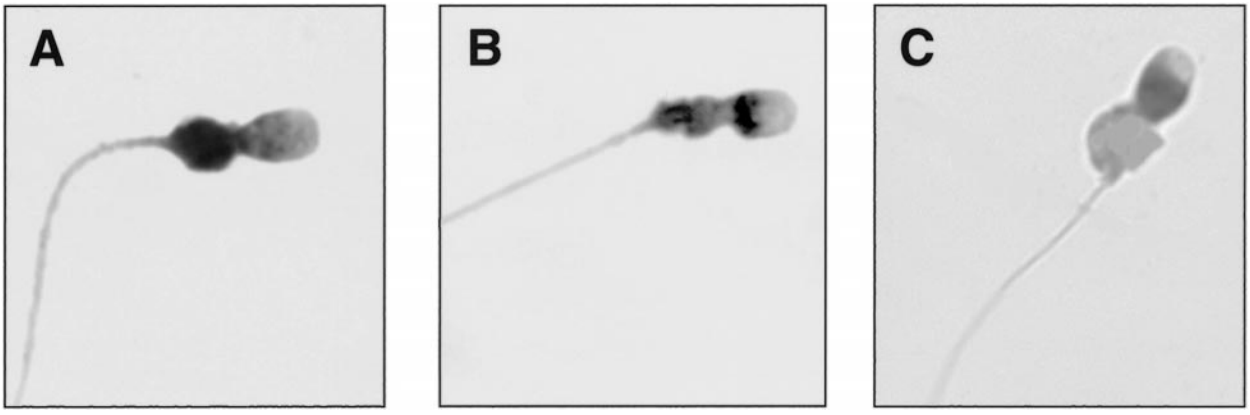

Figure 2. Positive nitroblue tetrazolium staining in morphologically abnormal sperm. Spermatozoa were scored as follows: **(A)** formazan occupying ≤50% of cytoplasm (+); **(B)** >50% of cytoplasm (++); **(C)** no formazan deposit.

Table 1. *Semen characteristics in donors and leukocytospermic and nonleukocytospermic patients**

Variable	Donors (n = 9)	Leukocytospermic Patients (n = 8)	Nonleukocyto-spermic Patients (n = 13)	*P* Value†		
				A	B	C
Concentration, 10^6/mL	112 (72, 136)	54.5 (31.55, 81.60)	54 (32, 60)	.06	.00	.42
Motility, %	69 (50, 80)	48.5 (38.0, 53.50)	47 (34, 57)	.05	.02	.97
Morphology, %	24 (21, 31)	21 (10, 29)	25 (22, 31)	.58	.90	.64

* Values are median and 25th, 75th percentiles.
† A indicates comparison between donors and leukocytospermic group; B, comparison between donors and nonleukocytospermic group; and C, comparison between leukocytospermic and nonleukocytospermic group. $P < .05$ was considered significant.

spermic group and 2.25 (1.63, 3.10; $P = .003$) in the leukocytospermic group.

Reactive Oxygen Species

Production of ROS in whole ejaculate, leukocytes, and abnormal spermatozoa among donors and patients is shown in Table 2. The differences for amount of ROS in whole ejaculate were statistically significant across all 3 groups. The highest levels of ROS were seen in whole ejaculate of leukocytospermic patients compared to donors ($P = .04$) and nonleukocytospermic group ($P = .06$). In leukocytospermic group, ROS levels showed significant positive correlation with the concentration of round cells in leukocytospermic group ($r = 0.88$, $P = .006$).

Seminal Total Antioxidant Capacity and ROS–TAC Score

The median (25th, 75th percentiles) seminal plasma TAC and ROS-TAC scores for the whole ejaculate are shown in Table 2. TAC levels were comparable among the 3 groups. The lowest median (25th, 75th percentiles) ROS-TAC score was observed in the leukocytospermic patients compared to the donors ($P = .04$). The ROS-TAC score for the nonleukocytospermic group did not differ significantly from the donors. A significant negative correlation was found between the ROS-TAC score and the leukocyte concentrations ($r = -0.46$, $P = .01$), levels of ROS in the fraction containing leukocytes ($r = -0.39$, $P = .04$), and levels of ROS in fraction containing abnormal spermatozoa ($r = -0.57$, $P = .002$).

NBT Test Results

Median interquartile range (25th and 75th percentiles) of NBT results for leukocytes and spermatozoa with cytoplasmic retention are shown in the Table 3. In leukocytospermic samples, 51% (41%, 67%) of leukocytes stained positive for NBT, of these 2.5% (1%, 7%) were classified as (+++), 10.5 % (8%, 19%) as (++), and 36.5% (23%, 44.5%) as (+). In nonleukocytospermic samples, 6% (2%, 7%) were classified as (+++), 1% (0, 3%) as (++), and 4% (1%, 6%) as (+). A strong positive correlation was seen between ROS levels in whole ejaculate and an NBT-positive response in leukocytes in whole ejaculate ($r = 0.59$; $P = .0006$) and leukocytes ($r = 0.7$; $P < .0001$; Figure 3). Similarly, positive correlation was seen between spermatozoa with cytoplasmic retention in neat semen ($r = 0.5$; $P = .008$) and abnormal spermatozoa ($r = 0.72$; $P < .001$; Figure 4). A strong negative correlation was seen between ROS-TAC scores and NBT staining in leukocytes in whole ejaculate ($r = -0.60$; $P = .0007$) and leukocytes ($r = -0.39$, $P = .04$); and in sperm with cytoplasmic retention in abnormal

Table 2. *Levels of ROS, TAC, and ROS-TAC score in samples from donors and infertile patients (leukocytospermic and nonleukocytospermic)**

Variable	Donors (n = 9)	Infertile Patients (n = 21)		*P* Value†		
		Leukocytospermia (n = 8)	Nonleukocytospermic (n = 13)	A	B	C
Whole ejaculate						
ROS	0 (0, 0.01)	0.28 (0.3, 0.36)	0.05 (0.02, 0.07)	.01	.04	NS
TAC (trolox equivalents)	551 (463, 644)	622 (492, 824)	754 (453, 886)	NS	NS	NS
ROS-TAC score	53.2 (47.5, 55.0)	35 (30.5, 50.2)	46.2 (39.2, 51.5)	.04	NS	NS
Density gradient separation						
Leukocytes	0 (0, 0.57)	34.65 (29.34, 49.47)	0.2 (0.04, 1.42)	.02	NS	.02
Abnormal spermatozoa	1.55 (0, 2.07)	20.37 (8.17, 125.82)	10.71 (3.84, 29.85)	.04	.05	NS

* ROS indicates reactive oxygen species; TAC, total antioxidant capacity. ROS was measured as $\times 10^6$ counted photons per minute per 20×10^6 cells. Values are median and 25th, 75th percentiles.
† A indicates comparison between donors and leukocytospermic group; B, comparison between donors and nonleukocytospermic group; and C, comparison between leukocytospermic and nonleukocytospermic group. $P < .05$ was considered significant; NS indicates nonsignificant.

Table 3. *Percentage of cells positive for nitroblue tetrazolium staining in whole ejaculate, and in leukocytes and spermatozoa separated after density gradient in samples from donors and infertile patients (leukocytospermic and nonleukocytospermic)**

Variable	Donors (n = 9)	Infertile Patients (n = 21)		*P* Value†		
		Leukocytospermia (n = 8)	Nonleukocytospermic (n = 13)	A	B	C
Whole ejaculate						
Leukocyte	0 (0, 2)	51 (47, 73)	7 (6, 14)	.02	.02	.03
Immature spermatozoa	13 (9, 22)	49 (43, 53)	32.5 (12, 46)	.06	.4	.24
Density gradient separation						
Leukocytes	7 (3, 11)	70 (61, 79)	14.5 (9, 25.5)	.02	.1	.03
Abnormal spermatozoa	11 (9, 31)	52 (37, 67)	53.5 (13, 68)	.05	.2	.67

* Values are median and 25th, 75th percentiles.

† A indicates comparison between donors and leukocytospermic group; B, comparison between donors and nonleukocytospermic group; and C, comparison between leukocytospermic and nonleukocytospermic group. $P < .05$ was considered significant.

spermatozoa in abnormal spermatozoa fraction ($r = -0.38$; $P = .04$).

Comparison Between Peroxidase and NBT Test

Overall comparison of the 3 groups (donors and patients with and without leukocytes) showed significant correlation between peroxidase and the NBT test ($P < .001$; Figure 5A). The AUC was 0.96 with 95% confidence interval (CI) of 0.89, 1.0 ($P < .001$). An NBT test with a cutoff of 19% had a sensitivity of 1.000 (0.631, 1.000) and specificity of 0.864 (0.651, 0.971). Similarly, a cutoff of 23% and 27% had a sensitivity of 1.000 (0.631, 1.000) and 0.875 (0.473, 0.997) and specificity of 0.909 (0.708, 0.989), respectively.

Donors (median and 25th and 75th percentiles; 0.66 [0, 1.0]) could be identified correctly from patients with leukocytospermia (2.25 [1.3, 11.7]; $P < .001$), both by peroxidase and NBT test (0 [0, 27] versus 51 [23, 77]; $P < .001$). Similarly, patients with and without leukocytospermia (0.06 [0, 0.4] versus 2.25 [1.3, 11.7]; $P < .001$) could be identified both by peroxidase and by NBT test (6.0 [0, 71] versus 51 [23, 77]; $P < .004$). Thus, both peroxidase and NBT test are sensitive to identify leukocytes in a given specimen. The high sensitivity (1.0; CI of 0.63, 1.0) and specificity (0.91; CI of 0.71, 0.99) of the NBT test provides the ability of predicting specimens with peroxidase values $>1 \times 10^6$ PPL/mL of the seminal ejaculate (Figure 5B).

Discussion

Clear evidence suggests that human spermatozoa produce oxidants (Aitken et al, 1992; Hendin et al, 1999; Gil-Guzman et al, 2001). Spermatozoa may generate ROS in 2 ways: the NADPH oxidase system at the level of the sperm plasma membrane (Aitken et al, 1992), and the NADH-dependent oxido-reductase system at the mito-

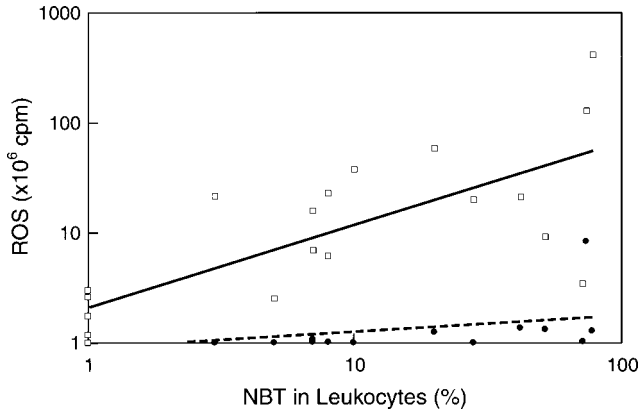

Figure 3. Correlation between nitroblue tetrazolium (NBT) in leukocytes and both reactive oxygen species (ROS) in whole ejaculate and immature spermatozoa. (□): whole ejaculate; (●): immature spermatozoa; (—): regression line for correlation between ROS and NBT in whole ejaculate; and (– – –): regression line for correlation between ROS and NBT in immature spermatozoa.

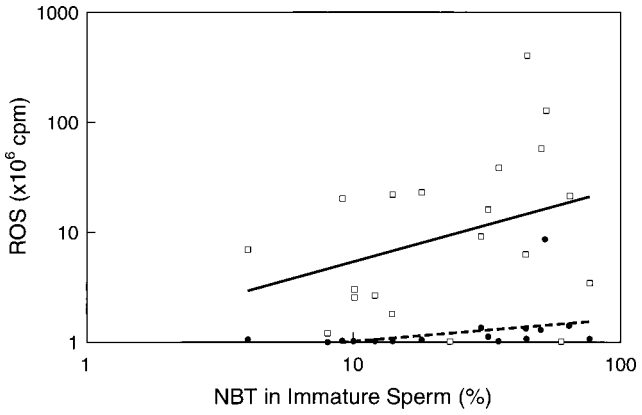

Figure 4. Correlation between nitroblue tetrazolium (NBT) staining in sperm with cytoplasmic retention and reactive oxygen species (ROS) in whole ejaculate and mature spermatozoa. (□): whole ejaculate; (●): mature spermatozoa; (—): regression line for correlation between ROS and NBT in whole ejaculate; and (– – –): regression line for correlation between ROS and NBT in mature spermatozoa.

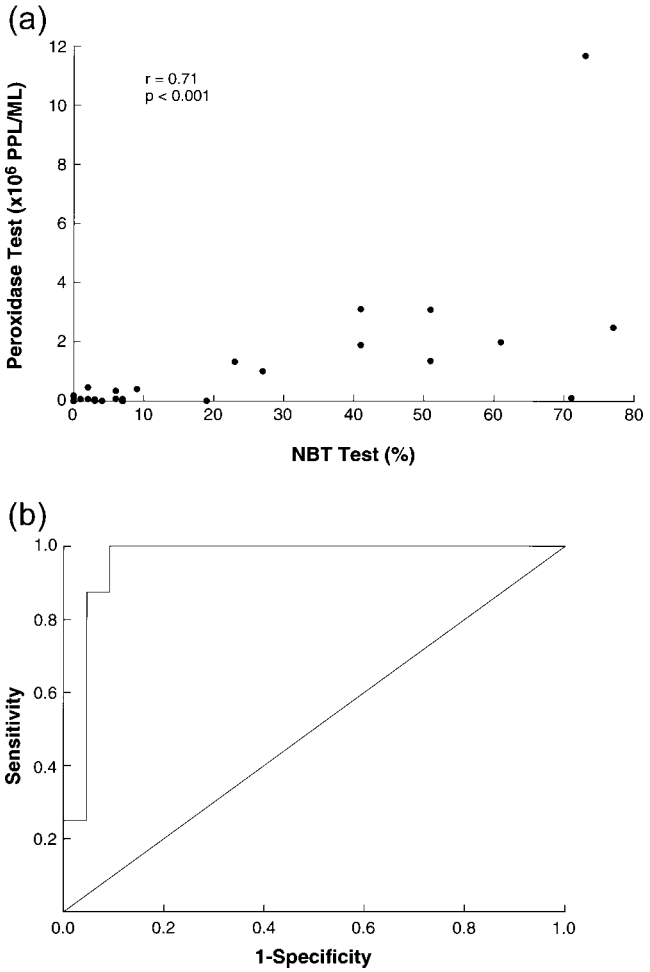

Figure 5. Correlation between myeloperoxidase and nitroblue tetrazolium (NBT) staining in the 3 combined groups, that is,donors and patients with and without leukocytospermia ($r = 0.71$, 95% CI, 0.43, 0.98). **(a)** Receiver operating characteristic curve predicting peroxidase value $>1 \times 10^6$ peroxidase-positive leukocytes per milliliter by NBT test. **(b)** The area under the curve is 0.96 with 95% CI of 0.89, 1.0 ($P < .001$).

chondrial level (Gavella and Lipovac, 1992). The mitochondrial system is the major source of ROS in spermatozoa from infertile men (Plante et al, 1994). Peroxidase-positive leukocytes in semen also have been identified for their capacity to generate high levels of ROS (Aitken et al, 1995; Rajasekaran et al, 1995; Shekarriz et al, 1995; Ochsendorf, 1999; Sharma et al, 2001). Peroxidase-positive leukocytes include polymorphonuclear (PMN) leukocytes, which represent 50% to 60% of all seminal leukocytes, and macrophages, which represent another 20% to 30% (Fedder et al, 1993; Thomas et al, 1997). Peroxidase-positive leukocytes in semen are contributed largely by the prostate and the seminal vesicles (Wolff, 1995).

Activated leukocytes are capable of producing 100-fold higher amounts of ROS than nonactivated leukocytes (Plante et al, 1994). Leukocytes may be activated in response to a variety of stimuli including inflammation and infection (Pasqualotto et al, 2000). Activated leukocytes increase NADPH production via the hexose monophosphate shunt. The myeloperoxidase system of both PMN leukocytes and macrophages also is activated, which leads to respiratory burst and production of high levels of ROS (Blake et al, 1987). Such an oxidative burst is an early and effective defense mechanism in cases of infection for killing the microbes (Saran et al, 1999).

Sperm damage from ROS that is produced by leukocytes occurs if seminal leukocyte concentrations are abnormally high, that is, leukocytospermia (Shekarriz et al, 1995) or if seminal plasma is removed during sperm preparation for assisted reproduction (Ochsendorf, 1999). Excessive ROS production that exceeds critical levels can overwhelm all antioxidant defense strategies of spermatozoa and seminal plasma, causing OS (Sikka et al, 1995; Sharma and Agarwal, 1996; de Lamirande et al, 1997; Sikka, 2001). Despite the fact that OS has been established as a major factor in the pathogenesis of male infertility, consensus is lacking as to the clinical utility of seminal OS testing in an infertility clinic. One important reason for the inability to utilize an OS test in clinical practice may be the lack of a simple method that can reliably measure ROS in semen.

One of the limitations of the myeloperoxidase test is its inability to stain spermatozoa unlike the leukocytes, and hence its inability to indicate ROS levels produced by the spermatozoa. Earlier studies showed that NBT reduction and formazan deposition in blood neutrophils are related to their phagocytic activity (Park et al, 1968; Segal and Levi, 1973). In the same studies, the state of activation of neutrophils was determined by scoring the blue-black formazan granules deposited in the cytoplasm. Therefore, we attempted to evaluate the NBT assay as an indicator of ROS production in both leukocytes and spermatozoa.

To the best of our knowledge, this is the first study examining ROS-generating activity in individual cells (sperm and leukocytes) in semen based on their morphological characteristics acquired by deposition of formazan granules in ROS positive cells. Myeloperoxidase or other cytochemistry tests such as determination of granulocyte-elastase level and immunocytochemistry with monoclonal antibodies currently are used for identifying the leukocytes in semen (Wolff, 1995). These tests yield a static value for leukocyte concentration, but they do not provide any clear indication of leukocyte viability or activity. However, the NBT reduction test can be used for 2 purposes: to determine the ROS-generating activity and to detect and identify the neutrophils. We believe that the NBT has this advantage over the peroxidase test, in that the NBT test, as a histochemical method, can assess the ROS-generating activity in morphologically abnormal and immature spermatozoa with cytoplasmic retention. The

NBT test can detect neutrophils at a concentration of 0.5 $\times$ 10^6/mL or higher (Kovalski et al, 1991). This level of sensitivity is appropriate for detecting the cutoff value of 1.0 $\times$ 10^6/mL established by the WHO for leukocytospermia. The NBT assay described is an indirect reflection of ROS generation. The mechanism of ROS generation in human sperm recently was found to depend upon a novel NADPH-oxidase (NOX5) resembling the multicomponent NADPH-oxidase of white blood cells (WBC). A recent study with electron spin resonance analysis, chemiluminescence, and NBT reduction indicated that the ROS-producing activity of spermatozoa may be different and significantly lower than the WBC-NADPH-oxidase (Armstrong et al, 2002).

Examination of our results indicated that the density of formazan deposition in spermatozoa and seminal leukocytes was directly correlated with the state of activation of these cells. In addition, a strong positive relationship was seen between results of the NBT test, expressed as percent NBT-positive cells, and the levels of ROS in the same cell suspensions as measured by chemiluminescence assay. Although luminol-dependent chemiluminescence assay helped measure the total amount of ROS in semen, the NBT test provided information on the differential contribution of spermatozoa and leukocytes to ROS production in semen and on the state of activation of these cells. It is true that NBT reacts with cellular superoxide ions to form formazan derivatives that can be monitored spectrophotometrically or as demonstrated in our study. However, many cellular reductases also can donate an electron to NBT, forming its radical, which under aerobic conditions reacts with environmental oxygen to form monoformazan. This formazan formation can be inhibited by superoxide dismutase (SOD), and the SOD-rich seminal plasma. In addition, changes in cellular content of various oxidoreductases also are responsible for alterations in rates of NBT reduction (Fridovich, 1997). However, we did not examine this aspect as this was not the focus of the present study.

In our study, overall comparison of the 3 groups (donors and patients with and without leukocytospermia) showed significant correlation between peroxidase and the NBT test. We examined the ROC curve predicting peroxidase level greater than 1 $\times$ 10^6 PPL/mL by the NBT test. The AUC was 0.96 with a 95% CI of 0.89, 1.0 ($P < .001$). We also examined the various cutoff values for the NBT test to identify the best NBT cutoff points associated with sensitivity and specificity. Our results indicate a strong positive relationship between the results of the NBT test, expressed as percent NBT-positive cells, and the level of ROS of the same cell suspension as measured by chemiluminescence.

Furthermore, results of the NBT test were inversely correlated with the ROS-TAC score, which has been recently introduced as an accurate measure of oxidative stress in infertile men (Sharma et al, 1999). The ROS-TAC score minimizes the variability present in the individual parameters of oxidative stress (ROS alone or TAC alone). In our earlier studies we stressed the importance of ROS-TAC scores as an index of oxidative stress that may explain previously unexplained cases of male infertility (Pasqualotto et al, 2001). In addition, both abnormal spermatozoa and leukocyte contamination influence ROS-TAC score, and lowest scores are seen in patients with leukocytospermia (Sharma et al, 2001). Examination of our results suggests that the NBT test can be used as a simple alternative to the ROS-TAC score for assessment of the levels of seminal oxidative stress. Therefore, the use of the NBT test in semen may have important implication both for the andrology research and clinical practice.

In conclusion, identification of particular cells in semen that produce ROS in excess may be the first step towards detection of the underlying inherent or acquired defect behind such abnormality. In addition, the NBT reduction test is readily available, easily performed, inexpensive, and has high sensitivity. This test can be used for assessment of seminal oxidative stress, and the differential contribution of cells to ROS generation, and to determine the state of activation of seminal leukocytes. Such a test can be added to the routine clinical andrology workup for assessment of seminal OS without the need for expensive equipment such as the luminometer.

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