Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa

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Objective: To investigate the role of increased seminal leukocytes in enhancing reactive oxygen species (ROS) production by human spermatozoa.

Design: A prospective study.

Setting: Male infertility clinic.

Patient(s): Forty-eight infertile men.

Intervention(s): Standard semen analysis. Assessment of sperm nuclear DNA damage by sperm chromatin structure assay. Incubation of spermatozoa from nonleukocytospermic samples with blood neutrophils.

Main Outcome Measure(s): Spontaneous and phorbol 12-myristate 13-acetate (PMA)-induced ROS production in pure-sperm suspensions (after removal of leukocytes) as measured by a chemiluminescence assay.

Result(s): Levels of spontaneous and PMA-induced ROS production in pure-sperm suspensions from the infertile men with a diagnosis of leukocytospermia (n = 16) were significantly higher compared with the case of infertile men without leukocytospermia (n = 32) and with the case of a control group of healthy volunteers (n = 13). A similar pattern of increased ROS was observed when spermatozoa were incubated with blood neutrophils. Leukocytospermia was associated with a significant decrease in sperm motility and increase in DNA damage.

Conclusion(s): Increased seminal leukocytes may play a role in stimulating ROS production by human spermatozoa. Such stimulation may be mediated via direct cell–cell contact or by soluble products released by leukocytes. Poor sperm quality in leukocytospermic samples may be due to leukocyte-mediated oxidative stress. (Fertil Steril® 2002;78:1215–24. ©2002 by American Society for Reproductive Medicine.)

Key Words: Leukocytospermia, male infertility, nuclear DNA, oxidative stress, spermatozoa

Leukocytes are present throughout the male reproductive tract and are found in almost every human ejaculate (1, 2). However, the clinical significance of increased leukocyte infiltration in semen, that is, leukocytospermia, is currently the subject of controversy (3). The World Health Organization (WHO) defines leukocytospermia as the presence of peroxidase-positive leukocytes in concentrations of >1 × 10^6 per mL of semen (4). Peroxidase-positive leukocytes include polymorphonuclear (PMN) leukocytes, which represent about 50–60% of all seminal leukocytes (5), and macrophages, which represent another 20–30% (3). Several studies have shown that peroxidase-positive leukocytes are the major source of reactive oxygen species (ROS) in semen (6–9).

In addition to ROS production by seminal leukocytes, clear evidence suggests the production of these powerful oxidants by spermatozoa (10–13). A study by Fisher and Aitken (14) has indicated that male germ cells at various stages of differentiation, from pachytene spermatocytes to mature caudal epididymal spermatozoa, from mature male rats, mice, hamsters, and guinea pigs, have the potential to generate ROS. In the same study, the authors found that superoxide production could be dramatically enhanced by the addition of exogenous NADPH, in a manner that was closely correlated with the stage of epididymal development, being maximal for
immature cells recovered from the caput epididymis in all species.

A recent study has shown a negative correlation between levels of ROS production by spermatozoa and the quality of spermatozoa in the original semen (15). Huszar et al. (16) indicated that the link between poor sperm quality and increased ROS generation lies in the presence of excess residual cytoplasm. These investigators stated that when spermatogenesis is impaired, the cytoplasmic extrusion mechanisms become defective, and as a result spermatozoa are released from the germinal epithelium carrying surplus cytoplasm. In the same study, the investigators found a significant correlation between the presence of sperm with cytoplasmic retention and levels of ROS production in response to stimulation by phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (11–13, 15).

Spermatozoa are particularly susceptible to damage induced by ROS because their plasma membranes contain large quantities of polyunsaturated fatty acids (18), and their cytoplasm contains low concentrations of scavenging enzymes (19). In addition, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to supplement their limited intrinsic antioxidant defenses by dependence on the protection afforded by the seminal plasma that bathes these cells (20).

The lack of clinical significance of leukocytospermia reported in some studies is possibly a reflection of the powerful antioxidant properties of the seminal plasma, which may provide protection against leukocyte-mediated oxidative stress (7, 8). Oxidative stress develops when levels of ROS production by leukocytes, spermatozoa, or both, become high enough to overwhelm all antioxidant strategies, resulting in lipid peroxidation, impairment of sperm motility, and loss of fertilizing potential (21). Oxidative stress also affects the integrity of the sperm genome by causing high frequencies of single- and double-strand DNA breaks (22). Therefore, it is extremely important to identify the source of any excessive ROS generation activity in the human ejaculate. The potential role of excessive leukocyte infiltration into semen, in other words, leukocytospermia, in stimulating ROS production by human spermatozoa has never been investigated. It is unclear from the existing literature whether the interaction between leukocytes and spermatozoa implies a direct or indirect stimulatory effect, which may enhance the capacity of spermatozoa to generate excessive ROS.

The objectives of this study were as follows:

1. Investigate the correlation between excessive leukocyte infiltration into semen, that is, leukocytospermia, and the capacity of sperm to produce ROS spontaneously and after stimulation with PMA.
2. Assess the levels of oxidative stress in a group of infertile men with leukocytospermia vs. infertile men without leukocytospermia and a group of healthy donors by measuring basal levels of ROS production in original cell suspensions (containing both spermatozoa and leukocytes) and levels of total antioxidant capacity (TAC) in seminal plasma and by calculating the composite value of a ROS-TAC score.
3. Compare sperm quality in infertile men with leukocytospermia with that of infertile men without leukocytospermia by evaluating standard sperm parameters (sperm concentration, motility, and morphology) and levels of sperm nuclear DNA damage.

MATERIALS AND METHODS

The institutional review board of the Cleveland Clinic Foundation approved the study.

Study Groups

The study included 48 men attending the infertility clinic between August 2000 and August 2001 for a history of infertility of ≥1 year. Genital examination was performed by an experienced urologist (A.J.T.). Standard semen analysis was performed in the clinical andrology laboratory and repeated at 2 to 4 weeks. On the basis of the results of semen analysis, semen samples were classified as leukocytospermic (leukocyte concentrations >1 × 10⁹ peroxidase-positive cells per milliliter of semen, n = 16) and nonleukocytospermic (n = 32). A group of healthy donors (n = 13) served as a control. All donors had normal genital examination and normal standard semen parameters according to the WHO criteria (4).

Urine and semen cultures of leukocytospermic samples were negative for pathogenic organisms, including Chlamydia and Mycoplasma.

Standard Semen Analysis

Sperm concentration, motility, and morphology

Semen specimens were collected by masturbation after a period of 48 to 72 hours of sexual abstinence. After liquefaction, manual semen analysis was performed using a Microcell counting chamber (Conception Technologies, San Diego, CA) to determine sperm concentration and motility. Smears of the raw semen were stained using Diff-Quik kit (Baxter Healthcare Corporation, Inc., McGaw Park, IL) for assessment of sperm morphology. After staining, the smears were rinsed in distilled water, air dried, and scored using the WHO classification (4). Normal values for sperm parameters were considered to be sperm concentration of ≥20 × 10⁹/mL, motility of ≥50%, and normal sperm forms of ≥30% (4).
Quantification of seminal leukocytes

Leukocyte concentrations in semen were quantified by a myeloperoxidase-staining assay (23). A 20-μL volume of liquefied semen specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar Corp., Cambridge, MA) with 20 μL of phosphate-buffered saline (PBS; pH 7.0) and 40 μL of benzidine solution. The solutions were mixed and allowed to sit at room temperature for 5 minutes. Peroxidase-positive leukocytes staining brown were counted by a Microcell counting chamber (Conception Technologies, San Diego, CA) under the bright-field objective (magnification, ×20). The average of 5–10 fields was calculated. The results after correction for dilution were recorded as ×10^6 peroxidase-positive leukocytes per milliliter of semen.

Leukocytospermia was defined as concentrations of >1 × 10^6 peroxidase-positive leukocytes per milliliter of semen (4). The term leukocytes used throughout the text refers to PMN leukocytes and macrophages.

Paramagnetic-Bead Separation of Seminal Leukocytes

Removal of leukocytes from semen by using anti-CD45-coated paramagnetic beads is an effective means of purifying human sperm suspensions (24). In this study, magnetic Dynabeads (M-450; Dynal, Inc., Lake Success, NY) pre-coated with sheep anti-mouse immunoglobulin Ig were stored at 4°C until used. One milliliter of the above suspension was mixed with 1 mL of a 1:10 dilution of a monoclonal antibody against the common leukocyte antigen, CD45, in PBS. The beads were incubated overnight with the antibody at 20°C while being rotated at 36 rounds per minute (rpm) on a rotating machine (Dynal, Inc., Lake Success, NY). At the end of the incubation period, magnetic beads were collected by a magnetic particle concentrator.

The supernatant was aspirated and replaced with 4 mL of Biggers-Whitten-Whittingham (BWW) medium filtered through a 0.22-μm filter. After further rotation for 30 minutes at 36 rpm, the beads were once again held in the magnetic particle concentrator, while the supernatant was aspirated and replaced with an additional 4-mL volume of filtered BWW medium. This washing procedure was repeated once again, and the beads were suspended finally in 1 mL of filtered BWW medium and stored at 4°C. Liquefied semen was centrifuged at 300 × g for 7 minutes, and the seminal plasma was separated and stored at −80°C for measurement of TAC. The pellet was washed with PBS and resuspended in the same media at a concentration of 20 × 10^6 sperm per milliliter and finally divided into two aliquots.

One aliquot of the resulting original cell suspension (containing both sperm and leukocytes) was processed to remove leukocytes, and the second aliquot was used to assess basal levels of ROS production. Leukocytes were removed by incubating an aliquot of the original cell suspension with bead suspensions at optimum concentration, as recommended by the manufacturer (>4 beads per target cell). The beads were immobilized after 40 minutes of rotation at 36 rpm at 20°C using the magnetic particle concentrator, while the supernatant (leukocyte-free sperm suspension) was removed and divided into 400-μL aliquots for measurement of ROS production in pure-sperm suspensions with and without PMA stimulation.

Measurement of ROS

Reactive oxygen species levels were measured by a chemiluminescence assay using luminol (5-amino-2, 3, -dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as a probe (25). Levels of ROS were measured simultaneously in original cell suspensions (basal ROS), in pure-sperm suspensions (pure-sperm ROS), and in PMA-stimulated sperm suspensions (PMA-induced ROS). Four hundred-microliter aliquots of the original cell suspensions and pure-sperm suspensions were used to assess basal and pure-sperm ROS levels. Eight microliters of horseradish peroxidase (HRP) (12.4 U of HRP type VI, 310 U/mg; Sigma Chemical) were added to sensitize the assay so that it could measure extracellular hydrogen peroxide. Ten microliters of luminol, prepared as 5 mM stock in dimethyl sulfoxide, were added to the mixture. A negative control was prepared by adding 10 μL of 5 mM luminol to 400 μL of PBS.

We used the formyl-methionyl leucyl phenylalanine (FMLP) provocation test to assess the purity of sperm suspensions from residual leukocyte contamination (24). Formyl-methionyl leucyl phenylalanine (50 mM) was prepared from a 10 mM stock in dimethyl sulfoxide and 2 μL of 50 mM of FMLP, and 8 μL of HRP were added to 400-μL aliquots of pure-sperm suspensions. The residual capacity of the pure-sperm suspension for ROS generation was assessed by adding 4 μL of PMA, which was prepared as a 1 mM stock solution in dimethyl sulfoxide and diluted 1:100 to give a 10 μM working solution and a final concentration of 100 nM when added to 400 μL of the sperm suspension. Levels of ROS were assessed by measuring the luminol-dependent chemiluminescence with a luminometer (LKB 953, Wallac Inc., Gaithersburg, MD) in the integrated mode for 15 minutes. The results were expressed as ×10^6 counted photons per minute (cpm) per 20 × 10^6 sperm.

Measurement of TAC

Total nonenzymatic antioxidant capacity in the seminal plasma was measured with an enhanced chemiluminescence assay (26). Frozen samples of seminal plasma were thawed at room temperature and immediately assessed for TAC. Seminal plasma was diluted 1:20 with deionized water (dH2O) and filtered through a 0.2-μm filter (Allegiance Healthcare Corporation, McGaw Park, IL). Signal reagent was prepared by adding 30 μL of H2O2 (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). Horseradish peroxidase working solution was prepared from HRP stock solution by making a dilution of 1:1 of dH2O to give a chemiluminescence output of 3 × 10^7 cpm.

Trolox (6-hydroxy-1, 5, 7, 8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was prepared as a standard solution (25, 50, and 75 µM) for TAC calibration. 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 dH2O and mixed. The mixture was equilibrated to the desired level of chemiluminescence 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. The results were expressed as molar Trolox equivalent.

**Coincubation of Sperm with Peripheral Blood Neutrophils**

Semen samples from three nonleukocytospermic infertile men were subjected to a washing procedure using PBS as described above. The pellet was suspended in PBS at a concentration of 20 × 10^6 sperm/mL and finally divided into two aliquots. One aliquot was used as a leukocyte-free control by treating it with anti-CD45–coated magnetic beads. The resulting pure-sperm suspensions were tested for their capacity for spontaneous and PMA-induced ROS production. Neutrophils, derived from peripheral blood of normal healthy volunteers, were added to the second aliquot at concentrations of 5 × 10^6 neutrophils per milliliter and incubated at 37°C for 20 minutes.

Neutrophils were then removed using CD45-coated magnetic beads, and pure-sperm suspensions were tested for their capacity for spontaneous and PMA-induced ROS production. Measurement of ROS in pure-sperm suspensions from the two aliquots was performed at the same time, using a chemiluminescence assay as described above.

**Sperm Chromatin Structure Assay**

Assessment of sperm chromatin structure assay (SCSA)–defined sperm nuclear DNA damage was carried out as described elsewhere (27). The SCSA measures the susceptibility of sperm nuclear DNA to acid-induced denaturation in situ (28). Semen samples, frozen at −196°C, were thawed in a 37°C water bath and immediately diluted with buffer (0.15 M NaCl, 0.01 M Tris, 0.001 M EDTA, pH 7.4) to obtain a sperm concentration of 1 to 2 × 10^6 sperm cells per milliliter. The sample was sonicated for 30 seconds with a Branson Sonifier 450 (VWR Scientific) operating at a power setting of 3 and using 70% of 1-second pulses. Under these conditions, >95% of the sperm tails were separated from their heads.

A 200-µL aliquot was removed and mixed with 400 µL of a low-pH detergent solution (0.08 N HCl, 0.15 M NaCl, 0.01% Triton X-100, pH 1.2). After 30 seconds, spermatozoa were stained by adding 1.20 mL of acridine orange staining solution containing 6 µg/mL of AO (chromatographically purified; Polysciences, Warrington, PA) per milliliter of buffer (0.037 M citric acid, 0.126 M Na2 HPO4, 0.0011 mM EDTA [disodium], 0.15 M NaCl, pH 6.0). Immediately after staining, the sample was placed into an Ortho Cytocounter (Ortho Diagnostic, Inc., Westwood, MA) sample chamber.

The flow cytometer was equipped with a Lexel 100-mW argon ion laser operated at 35-mW and interfaced to a Cicer data-handling unit with PC-based Cyclops Software (Cytometry, Fort Collins, CO). Green (515–530 nm) and red (>630 nm) fluorescence, corresponding to amounts of native DNA and denatured DNA, respectively, were collected for each sperm measured at a rate of approximately 250 cells per second. Computer-generated means and standard deviations (SD) of green and red fluorescence values, derived from a population of approximately 5000 cells, were analyzed.

Alpha t (α̅) was calculated as the ratio of the red fluorescence to the total of red and green fluorescence of an individual sperm cell (α̅ = red/total [red + green] fluorescence). X̅̅ represents the mean population of α̅. Standard deviation of α̅ (SDα̅) represents the variability of chromatin structure abnormalities within the sperm population. The DNA fragmentation index (DFI) represents percentage of spermatozoa with abnormal chromatin structure. Spermatozoa with abnormal chromatin structure are susceptible to acid-induced DNA denaturation in situ and have a higher X̅̅, SDα̅, and DFI. The fourth parameter, percentage of high–DNA-stainable sperm, identifies the cells with immature nuclei by the characteristic pattern of increased green fluorescence.

**Statistical Analysis**

Continuous variables among the three groups were compared using Kruskal-Wallis tests. Pairwise comparisons between the three groups were performed with Wilcoxon rank-sum tests. Fisher’s exact test was used for the categorical variables. Correlation between variables was calculated using Spearman’s nonparametric method. The correlation was considered clinically meaningful when r > 0.1. All hypothesis testing was two-tailed, and the results were considered to be statistically significant when P<.05.

Multiple regression was used to determine the relationship of several variables with ROS levels, and because of nonnormality, the base-10 logarithms of the values (after adding 1 to all values to account for the possibility of observed values of zero) of both the independent and dependent variables were used. The amount of variation explained was based on the R^2 from these regressions. As a secondary analysis, ROS-TAC score was calculated as described in an
earlier study (26). The sample size of 61 individuals was selected to detect a correlation of \( r > 0.4 \) with a 90% power. All analyses were calculated with the SAS statistical software package (version 8.1; SAS Institute Inc., Cary, NC). Summary statistics are presented as median and interquartile values (25th and 75th percentiles).

**RESULTS**

In the leukocytospermic group, 19% (3/16) of patients had varicoceles, 19% (3/16) were diagnosed with chronic prostatitis (clinically and confirmed by ultrasound), and the remaining 62% (10/16) had no apparent abnormalities in their genital examinations. In the nonleukocytospermic group, 28% (9/32) of the patients had varicoceles whereas the remaining 23 (72%) patients had no apparent abnormality. There was no statistically significant difference in median age between the leukocytospermic (median, 33 [25th, 75th percentiles: 19, 38] years) and the nonleukocytospermic group (median, 33 [25th, 75th percentiles: 25, 38] years) and the nonleukocytospermic patients (median, 32 [30, 35]; \( P = .47 \)). Similarly, no statistically significant differences were seen in the length of infertility between the leukocytospermic (median, 2 [1, 4]) and the nonleukocytospermic group (median, 1.5 [1, 2]; \( P = .37 \)).

**Leukocytospermia and Standard Semen Parameters**

Leukocytospermia was associated with an abnormality in one or more of the standard sperm parameters in 94% (15/16) of cases. Abnormal sperm morphology was observed in 87% (14/16) of the leukocytospermic samples vs. 69% (22/32) of nonleukocytospermic samples. Sperm concentration and percentage of normal sperm forms in the leukocytospermic group were significantly lower compared with the donors (\( P = .008 \) and .0003, respectively) but were not significantly different from the case of the nonleukocytospermic group (\( P = .31 \) and .06, respectively). Sperm motility was significantly lower in the leukocytospermic group compared with the nonleukocytospermic (\( P = .04 \)) and donor groups (\( P < .001 \)). Leukocyte concentrations in semen were negatively correlated with percentages of sperm motility (\( r = 0.31; P = .01 \)) and normal sperm forms (\( r = 0.28; P = .03 \)), but not with sperm concentration (\( P = .19 \)).

**Leukocytospermia and SCSA-Defined DNA Damage**

The four SCSA parameters in the donors and in the two patient groups are shown in Table 1. The \( X_m \), SD\(_{at} \), and DFI were all significantly higher in the leukocytospermic group compared with in the nonleukocytospermic (\( P = .02 \), .01, and .02, respectively) and donor groups (\( P = .002 \), .001, and .002, respectively). Levels of DFI of \( > 27 \% \) were seen in 10 of 16 (62%) leukocytospermic samples compared with in 8 of 32 (25%) nonleukocytospermic samples. Leukocyte concentrations in semen were positively correlated with \( X_m \) (\( r = 0.33 \); \( P = .03 \)), SD\(_{at} \) (\( r = 0.35 \); \( P = .006 \)), and DFI values (\( r = 0.32 \); \( P = .01 \)). The percentage of high-DNA-stainable sperm was significantly higher in the leukocytospermic group compared with the case of donors (\( P = .006 \)) but was not significantly different from the case of the nonleukocytospermic group (\( P = .39 \)).

**Leukocytospermia and Oxidative Stress Indices**

Levels of various oxidative stress measures are provided in Table 2. Complete removal of leukocytes from the original cell suspensions was confirmed by negative response to the leukocyte-specific FMLP probe. Levels of ROS were significantly reduced after leukocyte removal in the leukocytospermic group (\( P < .001 \)), as well as in the nonleukocytospermic (\( P = .04 \)) and donor groups (\( P = .04 \)). However, ROS levels in pure-sperm suspensions (after leukocyte removal) remained significantly higher in the leukocytospermic group compared with in the nonleukocytospermic group (\( P = .002 \)) and donor groups (\( P = .001 \)). In addition, PMA-induced ROS production in pure sperm from the leukocytospermic group was significantly higher than in the nonleukocytospermic (\( P = .0003 \)) and donor groups (\( P = .0002 \)). In 5 of the 16 leukocytospermic samples,
levels of PMA-induced ROS in pure-sperm suspensions were even higher than the levels of ROS in the original cell suspensions (containing both sperm and leukocytes) from the same samples (Table 3).

Seminal leukocyte concentrations were positively correlated with ROS levels in original cell suspensions ($r = 0.70$, $P < 0.0001$), in pure-sperm suspensions ($r = 0.54$, $P < 0.0001$), and after PMA stimulation ($P < 0.0001$). Phorbol 12-myristate 13-acetate–induced ROS levels were negatively correlated with sperm concentration ($P < 0.0001$), sperm motility ($P = 0.0003$), and normal sperm forms ($P < 0.0001$; Fig. 1). In addition, a significant positive correlation was seen between pure-sperm and PMA-induced ROS production and percentage DFI ($P = 0.01$ and $P = 0.02$; respectively; Fig. 2). After adjusting for seminal leukocyte concentrations, only sperm concentration was significantly correlated with the three measures of oxidative stress (basal, pure sperm, and PMA-induced ROS; Table 4). Multiple regression analysis indicated that seminal leukocyte concentrations might explain 47.9% to 66.5% of the amounts of ROS in a semen specimen, whereas sperm concentrations account for 4.8% to 9.8% of ROS variability. Low sperm motility or poor morphology added no additional information.

Results of ROS production after coincubation of blood neutrophils with sperm from three nonleukocytospermic samples are demonstrated in Table 5. Coincubation of sperm with blood neutrophils resulted in an increased spontaneous and PMA-induced ROS production, a finding similar to that observed in leukocytospermic samples. Levels of TAC in the leukocytospermic group were significantly lower compared with nonleukocytospermic ($P = 0.04$) and donor groups ($P = 0.01$). The composite value of ROS-TAC score, using basal ROS and seminal TAC levels, was significantly lower in the leukocytospermic group compared with the nonleukocytospermic ($P = 0.0001$) and donor groups ($P = 0.0003$). Seminal leukocyte concentrations were negatively correlated with levels of TAC ($r = -0.34$; $P = 0.007$). Seminal leukocytes also showed significant negative correlation with ROS-TAC score ($r = -0.56$; $P < 0.0001$; Fig. 3).

### Table 2

Median (25% and 75% interquartile value) ROS levels in original cell suspension (basal), in nonstimulated leukocyte-free sperm suspension (pure sperm), and in leukocyte-free suspension after stimulation with PMA; levels of seminal TAC; and ROS-TAC score in the three groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Donors (n = 13)</th>
<th>Nonleukocytospermic (n = 32)</th>
<th>Leukocytospermic (n = 16)</th>
<th>P valuesa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal ROS ($\times 10^6$ cpm)</td>
<td>0.4 (0.1, 2.5)</td>
<td>2.7 (0.53, 12)</td>
<td>178 (32, 430)</td>
<td>.06</td>
</tr>
<tr>
<td>Pure sperm ROS ($\times 10^6$ cpm)</td>
<td>0.06 (0.01, 0.2)</td>
<td>0.31 (0.09, 1.2)</td>
<td>3.3 (0.5, 7.4)</td>
<td>.05</td>
</tr>
<tr>
<td>PMA-induced ROS ($\times 10^6$ cpm)</td>
<td>0.1 (0.03, 0.1)</td>
<td>0.94 (0.3, 2)</td>
<td>9.2 (2.374)</td>
<td>.003</td>
</tr>
<tr>
<td>TAC (Trolox equivalent)</td>
<td>989 (863, 1534)</td>
<td>986 (847, 1199)</td>
<td>636 (437, 982)</td>
<td>.33</td>
</tr>
<tr>
<td>ROS-TAC score</td>
<td>54.5 (52, 60)</td>
<td>50.3 (42, 54.8)</td>
<td>27.8 (23.7, 35)</td>
<td>.01</td>
</tr>
</tbody>
</table>

a A = Donors vs. nonleukocytospermic patients; B = donors vs. leukocytospermic patients; C = nonleukocytospermic vs. leukosytospermic patients. Wilcoxon rank-sum test was used for comparison, and statistical significance was assessed at $P < 0.05$ level.

### Table 3

Seminal leukocyte concentration, sperm concentration and basal, pure-sperm, and PMA-induced ROS levels in semen samples from five leukocytospermic samples exhibiting high levels of PMA-induced ROS production by sperm.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Leukocyte concentration ($\times 10^9$/mL)</th>
<th>Sperm concentration ($\times 10^6$/mL)</th>
<th>WHO morphology (%)</th>
<th>Basal ROS ($\times 10^6$ cpm)</th>
<th>Pure-sperm ROS ($\times 10^6$ cpm)</th>
<th>PMA-induced ROS ($\times 10^6$ cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.2</td>
<td>12</td>
<td>2</td>
<td>24</td>
<td>8.4</td>
<td>707</td>
</tr>
<tr>
<td>2</td>
<td>4.8</td>
<td>58</td>
<td>30</td>
<td>89</td>
<td>5.18</td>
<td>955</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>12</td>
<td>15</td>
<td>321</td>
<td>12.7</td>
<td>202</td>
</tr>
<tr>
<td>4</td>
<td>4.8</td>
<td>28</td>
<td>12</td>
<td>118</td>
<td>6.33</td>
<td>545</td>
</tr>
<tr>
<td>5</td>
<td>16</td>
<td>26</td>
<td>9</td>
<td>572</td>
<td>8.91</td>
<td>1484</td>
</tr>
</tbody>
</table>

Note: WHO, World Health Organization.

DISCUSSION

It is of paramount importance to establish the source of ROS in a given semen sample because the clinical implications of infiltrating leukocytes is quite different from that of pathological conditions in which spermatozoa themselves are the source of ROS (8). Overall, our results agree with those of earlier studies that identified peroxidase-positive leukocytes as the major source of ROS in semen (6–8, 23). In addition, the results of our study indicate the following: [1] levels of spontaneous and PMA-induced ROS production by spermatozoa from infertile men with a diagnosis of leukocytospermia was significantly higher compared with the infertile men without leukocytospermia and to the normal

![Figure 1: Correlation of seminal leukocyte concentrations with ROS-TAC score in donors (○), nonleukocytospermic patients (●), and leukocytospermic patients (△).](image)

![Figure 2: Correlation of DFI with (A) pure-sperm ROS and (B) PMA-induced ROS in donors (○), nonleukocytospermic patients (●), and leukocytospermic patients (△).](image)

TABLE 4

Multiple regression analysis showing the amount of ROS accounted for by leukocyte and sperm concentration in semen.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Leukocyte concentration alone</th>
<th>Sperm concentration alone</th>
<th>Both variables together</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (%)</td>
<td>P value</td>
<td>B (%)</td>
</tr>
<tr>
<td>Basal ROS (×10⁶ cpm)</td>
<td>64.8</td>
<td>&lt;.0001</td>
<td>8.4</td>
</tr>
<tr>
<td>Pure-sperm ROS (×10⁶ cpm)</td>
<td>47.9</td>
<td>&lt;.0001</td>
<td>9.8</td>
</tr>
<tr>
<td>PMA-induced ROS (×10⁶ cpm)</td>
<td>66.5</td>
<td>&lt;.0001</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Note: A = percentage of ROS explained by leukocyte concentration alone; B = percentage of ROS explained by sperm concentration alone; C = percentage of ROS explained by both leukocyte and sperm concentrations together. Wilcoxon rank-sum test was used for comparison, and statistical significance was assessed at P<.05 level.

donors; [2] a similar pattern of increased spontaneous and PMA-induced ROS production by human spermatozoa was observed when spermatozoa from nonleukocytospermic samples were incubated with peripheral blood neutrophils; and [3] seminal leukocyte concentrations were significantly correlated with levels of spontaneous and PMA-induced ROS production by spermatozoa.

These findings led to the hypothesis that seminal leukocytes may play a role in stimulating excessive ROS production by spermatozoa. However, the mechanism of such stimulation is unclear and may be due to a direct sperm–leukocyte contact or mediated by soluble products released by the leukocytes. Further research is required to investigate this hypothesis. Another potential explanation for the increased ROS production in pure-sperm suspensions from leukocytospermic samples may be related, in part, to the relative increase in the percentage of spermatozoa with abnormal morphology compared with the case of the nonleukocytospermic samples. Morphologically abnormal spermatozoa, especially those with cytoplasmic retention, have been shown for their capacity to generate high levels of ROS (16).

A recent study has indicated failure of human spermatozoa with cytoplasmic retention to express a heat shock protein chaperone known as HspA2, which is necessary both for cytoplasmic extrusion and plasma membrane remodeling (29). In addition, Huszar and Vigue (30) have found a significant correlation between sperm morphological irregularities, specifically higher retention of cytoplasm and abnormal head and high CK activity in human spermatozoa. Similarly, recent studies found an inverse relationship between CK levels and sperm morphological forms and suggested that CK level can be used as a reliable marker for sperm quality and fertilizing potential in subfertile men (31, 32). A positive relationship was found between CK activity and the rate of lipid peroxidation, as measured by malondialdehyde formation, in sperm fractions separated by Percoll (33).

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>ROS 1 (×10^6 cpm)</th>
<th>ROS 1 + PMA (×10^6 cpm)</th>
<th>ROS 2 (×10^6 cpm)</th>
<th>ROS 2 + PMA (×10^6 cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.01</td>
<td>0.1</td>
<td>0.9</td>
<td>144</td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>7.7</td>
<td>0.8</td>
<td>487</td>
</tr>
<tr>
<td>3</td>
<td>0.06</td>
<td>0.7</td>
<td>0.9</td>
<td>15.5</td>
</tr>
</tbody>
</table>

Note: ROS 1 = ROS in pure-sperm suspension; ROS 1 + PMA = PMA in pure-sperm suspension after PMA stimulation; ROS 2 = ROS in pure-sperm suspension after 20 minutes, incubation with blood neutrophils; ROS 2 + PMA = PMA-induced ROS in pure-sperm suspension after co-incubation.


Correlation between PMA-induced ROS levels (× 10^6 cpm) with sperm concentration (A); sperm motility (%; B); and normal sperm (according to WHO criteria; C) forms (%) in donors (○), nonleukocytospermic patients (●), and leukocytospermic patients (△).

The high PMA response displayed by spermatozoa from leukocytospermic samples, observed in our study, may be a marker of the poor sperm quality in leukocytospermic samples. The PMA-stimulated ROS production by human spermatozoa was found to be inversely correlated with the quality of sperm in original semen and may be mediated by
protein kinase C (15). A recent report has indicated that PMA-induced ROS production by sperm with large-head abnormalities could be 100-fold higher than basal level (12).

Leukocytospermia, in this study, was associated with a significant reduction of seminal TAC levels. Whether reduced levels of antioxidants in seminal plasma in leukocytospermic patients is an inherent characteristic or is a consequence of increased use of these antioxidants in the process of neutralizing excess ROS is not known.

The lack of antioxidant defenses in leukocytospermic samples, however, is an additional factor that may increase the susceptibility of sperm to oxidative stress in vivo. This condition would even become worse under in vitro conditions, where spermatozoa are washed free from the protective environment provided by the seminal plasma and resuspended in simple culture media that are generally devoid of antioxidant factors (34). The carryover of leukocytes into the washed sperm preparations profoundly influenced the fertilizing potential of the spermatozoa via mechanisms that were associated with the production of ROS (38). The composite value of ROS-TAC score is an accurate measure of seminal oxidative stress (26). In our study, leukocytospermic samples showed significantly high levels of oxidative stress, as indicated by low ROS-TAC scores.

Oxidative stress is known to play a key role in male infertility via mechanisms involving lipid peroxidation and altered membrane function together with impaired metabolism, motility, and fertilizing potential of sperm (18, 35, 36). Oxidative stress has also been shown to attack the integrity of the sperm nuclear DNA (22). A recent study has indicated that DNA fragmentation commonly observed in spermatozoa of infertile patients may be mediated by ROS (37). In this study, leukocytospermia was associated with reduced sperm motility, increased incidence of abnormal sperm morphology, and increased SCSA-defined nuclear DNA damage.

The mechanisms underlying this set of findings is not clear and may indicate a causal relationship or a circumstantial one reflecting a common underlying pathology. Reduced motility, together with increased DNA damage observed in sperm from leukocytospermic samples, may be explained, at least in part, by the leukocyte-mediated oxidative stress.

Another explanation for the positive correlation between leukocytospermia, abnormal sperm morphology, and DNA damage may be related to an association between leukocytospermia and defective spermiogenesis. Defective spermiogenesis results in increased production of sperm with defective nuclear and membrane remodeling (16). In this case, leukocytic infiltration into the male reproductive tract may play a protective role by eliminating defective spermatozoa and keeping a balance between normal and abnormal spermatozoa (38). In conclusion, the results of our study indicate that leukocytospermia is associated with increased capacity of spermatozoa for spontaneous and PMA-induced ROS production. Spermatozoa from leukocytospermic patients are at higher risk for leukocyte-mediated oxidative stress.

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