Positive Myeloperoxidase Staining (Endtz Test) as an Indicator of Excessive Reactive Oxygen Species Formation in Semen[1]

M. SHEKARRIZ,[2] R. K. SHARMA,[2] A. J. THOMAS, Jr., and A. AGARWAL[3,4]

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Purpose: *Although the significance of leukocytospermia in semen remains controversial, evidence exists that white blood cells (WBCs) may adversely affect sperm function and act as a potential cofactor in male infertility. Nevertheless, the mechanisms by which leukocytes may alter sperm function* in vitro *is unknown. Recent investigations suggest that reactive oxygen species (ROS) generated by the polymorphonuclear (PMN) granulocytes could adversely affect sperm function. The objective of this study was to investigate the relationship between the presence of leukocytospermia as determined by the Endtz test and the excessive formation of ROS.*

Methods: *The WBC concentration and ROS formation in human semen, obtained from men consulting for infertility, were assessed and compared to that of normal donors. ROS was measured by a chemiluminescence assay using luminol and a Berthold luminometer. The WBC concentration was determined with a myeloperoxidase staining technique (Endtz test). Specimens were obtained from 94 subjects (20 donors, 74 patients).*

Results: *Of the 20 donors, 2 were Endtz positive and ROS positive; 18 were Endtz negative with 2 (11%) ROS positive. In the patient group* (n = *74), 26 (35%) were ROS positive, and 17 were Endtz positive and found to be ROS positive. Of the 57 Endtz-negative patients, 9 (15%) were ROS positive. The positive Endtz test results correlated strongly with positive ROS formation in patients and donors* ($P < 0.001$).

Conclusions: *Our results indicate that the simple, cost-efficient Endtz test could be used as an indicator of excessive ROS formation in semen.*

KEY WORDS: spermatozoa; infertility; ROS; leukocytospermia; myeloperoxidase staining; Endtz test.

INTRODUCTION

Investigations of the significance of leukocytospermia in male infertility have yielded conflicting results (1). The presence of leukocytes in semen has been associated with poor sperm quality (2). Furthermore, results of the sperm penetration assay, a predictor of male infertility, have been shown to have a negative correlation with the number of leukocytes in human semen (3). White blood cell (WBC) presence can compromise the fertilizing potential of spermatozoa in the zona-free hamster ova penetration tests (4). However, El-Demiry *et al.* found no association between conventional semen parameters and leukocyte concentration in human semen (5). The presence of immature germ cells but not leukocytes is associated with decreased fertilizing capacity in spermatozoa *in vitro* (6,7).

Certainly, the different techniques used to determine leukocyte concentration and the lack of agreement on the definition of subclinical genital tract infection are partly responsible for this dilemma. However, recent studies (1) suggest that the role of leukocytes in the human reproductive tract is more complex than previously thought, and it appears that other unknown factors might contribute to leukocyte effects on spermatozoa. Experimental and clinical data indicate that WBC products may significantly affect sperm function (2,3,8). The ability to form large amounts of reactive oxygen species (ROS) by polymorphonuclear neutrophils (PMN)

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[2] Andrology Laboratory, Department of Urology, A100, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195.

[3] Supported by a grant from the Cleveland Clinic Foundation, Cleveland, Ohio.

[4] To whom correspondence should be addressed.

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and macrophages (9,10), which are the main leukocytic components of the seminal fluid (11), suggest that ROS may potentially induce adverse leukocytic effects on sperm function (8). However, evidence is conflicting in regard to the correlation of WBCs in semen and the level of ROS formation (12,13). The disagreement between studies has several explanations. In earlier studies contribution of leukocytes to ROS formation did not receive adequate attention and the methods for quantifying WBCs in semen were not clearly stated. Furthermore, different techniques have been used to determine leukocytic contamination in human semen (10,12), making the comparison of data difficult. Although the immunohistochemistry and monoclonal antibody tests have been shown to be highly sensitive (1), these techniques are expensive and labor intensive (11).

The aim of this study was to investigate the correlation between ROS formation in semen and the leukocytospermia as determined by a peroxidase staining technique (Endtz test).

MATERIALS AND METHODS

Patients

Semen samples were obtained from 94 men (donors: $n = 20$; patients: $n = 74$). The specimens were obtained from 20 normal volunteers (donor specimens) selected on the basis of normal semen analysis results (volume of at least 2.0 ml, sperm count $>20 \times 10^6$/ml, motility of at least 50%, and morphology of at least 30% normal spermatozoa). A second group of specimens were collected from 74 patients who came to our laboratory because of suspected subfertility (patient specimens). Patient or donor specimens with sperm concentrations of 15×10^6/ml or less were not included in this study.

Semen Collection and Assessment of Semen Variables

Semen specimens were collected by masturbation after at least 2–3 days of sexual abstinence and liquefied at 37°C for 30 min. Five microliters of specimen were loaded on a 20-μl microcell chamber (Conception Technologies, San Diego, CA) and analyzed on a model IVOS Hamilton-Thorn motility analyzer, HTM version 10 (Hamilton-Thorn Research, Beverly, MA).

Quantitation of White Blood Cells

The presence of granulocytes in semen specimens was assessed by the Endtz test (14). A 20-μl volume of liquefied specimen was placed in a Corning 2.0-ml cryogenic vial (Corning Costar Corp., Cambridge, MA); 20 μl of phosphate-buffered saline (pH 7.0) and 40 μl of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 min. Peroxidase-positive WBCs staining dark brown were counted in all 100 squares of the grid in a Makler chamber (Sefi Medical, Haifa, Israel) under the bright-field objective (magnification, ×20). The results after correction for dilution were recorded as counts $\times 10^6$/ml. Significant leukocytospermia was defined as at least 1×10^6 WBC/ml.

Measurement of ROS Activity

Sperm concentration in Endtz-negative specimens was adjusted to 15 to 20×10^6/ml before ROS measurement. Adjustment of sperm concentration in Endtz-positive semen specimens was not done to avoid reducing the WBC count that could in turn change the ROS levels in semen. A modified (human tubal fluid [HTF], Irvine Scientific, Santa Ana, CA) medium with human serum albumin (5.0 mg/ml) was used to adjust the concentration. Each specimen was then divided in 2 equal aliquots of 0.5 ml and placed in 17 × 120 mm polystyrene tubes (Falcon, Lincoln Park, NJ).

ROS formation was measured by a chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). Luminol was prepared as a 100-mmol/l stock solution by dissolving 100 mg of luminol powder (Bio Orbit, Turku, Finland) with 5.64 ml of dimethyl sulfoxide (DMSO). The working solutions of luminol (5 ml of 5 mmol/l luminol) was prepared by further dilution (1:20) with DMSO before measurement. Twenty microliters of this working solution was then added to each specimen for the ROS analysis.

Luminol is a sensitive chemiluminescent probe that reacts with a variety of free radicals (hydrogen peroxide, hydroxyl radicals, and superoxide anions) (9). The reaction of luminol with free radicals results in light emission that is proportional to the ROS level in the sample. We used a Berthold (Autolumat LB 953, Wallace Incorporated, Gaithersburg, MD) luminometer. Chemiluminescence was measured in the integration mode at 37°C for 10 min

after addition of luminol. ROS production was expressed as counted photons per minute (cpm). One aliquot was used to measure the background luminescence for each specimen before adding luminol. The background readings were subtracted from the actual test value to calculate ROS activity. The second aliquot was used to determine the basal ROS level.

The ROS level was considered abnormal (positive) when the luminescence curve (Fig. 1) rose and peaked 1 to 4 min after addition of luminol. A positive response was consistent with a value of at least 10×10^4 cpm in the integration mode, so all values of 10×10^4 cpm or greater were considered as abnormal or positive.

Statistical Analysis

Fisher's exact test was used to compare the incidence of leukocytospermia and ROS formation between patients and donors as well as to compare Endtz test results with positive ROS formation. Student's t test was used to compare ROS values between Endtz-positive and Endtz-negative specimens as well as between patient and donor specimens. A P value of less than 0.05 was considered as significant. The statistical analysis was performed using the SAS statistical software package.

RESULTS

Of 74 patients 17 (23%) had leukocytospermia as determined by the Endtz test, whereas only 2 of 20 (10%) donors did. The difference was not significant ($P = 0.022$). In the Endtz-negative patients, out of 57, 9 were positive for ROS formation. Of the 18 Endtz-negative donors, 2 (11%) were positive for ROS formation. The difference was not statistically significant.

All specimens with a positive Endtz test ($n = 19$) were also positive for ROS formation. Comparison of ROS-positive specimens to Endtz-positive and Endtz-negative specimens in donors and patients showed a strong correlation between a positive Endtz test result and positive ROS formation ($P < 0.001$; Table I). Comparison of ROS levels between specimens both Endtz negative and ROS positive with specimens that were Endtz positive and ROS positive showed no significant difference (ROS median and interquartile range 41.7 (16–153.5) $\times 10^4$ cpm vs 109.9 (33.5–246) $\times 10^4$ cpm. ROS level in specimens defined as negative (less than 10×10^4 cpm) was 0.1 (0–1.15) $\times 10^4$ cpm in patients as compared to 0.9 (0–1.6) in donors. These differences were not statistically significant ($P = 0.12$).

Although a very high ROS formation was seen in some specimens with high leukocyte concentrations, a correlation between the leukocyte concentration and the ROS level at the time of measurement was not seen.

DISCUSSION

In our study, a high correlation was seen between a positive Endtz test and a positive chemilumines-

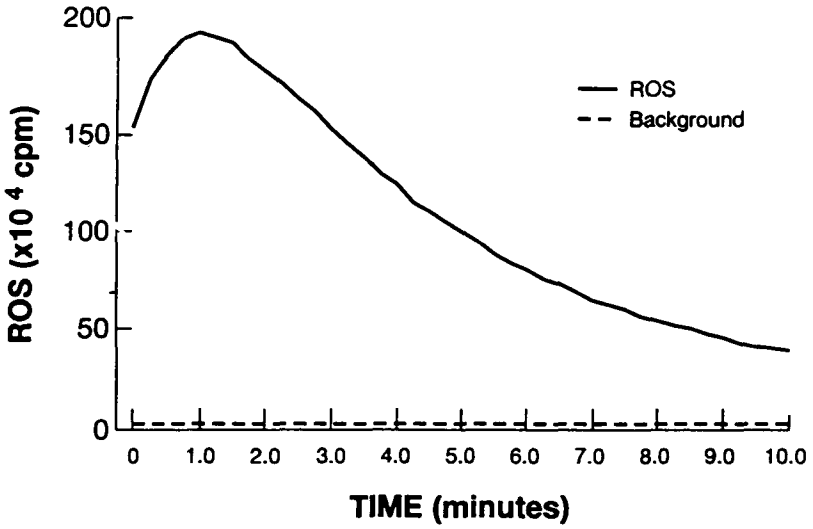

Fig. 1. A positive ROS curve in an Endtz-positive specimen. The chemiluminescence peaks 1 to 2 min after adding luminol, indicating a positive test. The baseline curve shows the background luminescence.

Table I. Comparison of ROS Positive with Endtz Negative Specimens and ROS Positive with Endtz Positive Specimens[a]

Specimen	Endtz (−) subjects	Endtz (+) subjects
ROS (−)	64 (85.3%)	0 (0%)
ROS (+)	11 (14.6%)	19 (100%)
Total (n = 94)	75	19

[a] The correlation of a positive Endtz test with positive ROS formation was highly significant ($P < 0.001$).

cence response for ROS in whole semen ($P < 0.001$). However, semen specimens which were positive for ROS in patients and donors were not always positive for Endtz test. This may support earlier reports of the dual source of ROS in human semen (10). Although previous studies have shown a linear relationship between the level of ROS generated from artificially activated PMN and the PMN cell concentration (13,15), the present study found no relationship between WBC concentration as determined by the Endtz test and the level of ROS generation in whole semen. This lack of association may have occurred because WBCs were not artificially activated to generate ROS in current study. Furthermore, this lack of correlation between the level of ROS activity and leukocyte concentration in semen may also be due to different sperm concentration in Endtz-positive specimens. Because patients consulting for infertility in this study were asymptomatic and leukocytospermia was detected incidentally on routine semen analysis, it appears that ROS generation is not limited to activated leukocytes as would be expected during a genital tract infection. Second, ROS measurements were performed in whole semen. The seminal plasma contains scavengers (8,16), such as superoxide dismutase and glutathione reductase, that may substantially influence ROS levels. Although the ROS levels in Endtz-positive specimens were higher as compared to those that were ROS positive and Endtz negative, the differences were not significant.

The contribution of spermatozoa to ROS formation in Endtz-positive specimens remains uncertain since separation of leukocytes from semen involves centrifugation, which has been shown to increase ROS formation (17). As pointed out by previous studies, it is difficult to determine the relative contribution of leukocytes and spermatozoa to ROS generation in semen (9,10).

The diagnosis of leukocytospermia has been made with cytochemical, immunohistochemical, and morphological techniques (1,11,18). The Endtz test (14) is based on the peroxidase activity of PMN and has been recommended by the World Health Organization (19) for determination of WBCs in semen. The advantage of this method is its simplicity and low cost compared to more complicated and expensive methods, such as the use of monoclonal antibodies (4). For clinical purposes, an ideal test must be easy to perform, fast, and inexpensive. This technique is limited in that it cannot detect lymphocytes in semen (11). However, peroxidase-positive leukocytes (neutrophils and macrophages) are the main leukocytes present in semen. These cells are also the source of ROS formation by phagocytosis. Consequently, these factors make the Endtz test suitable for screening for high levels of ROS in semen and also explain the high correlation of the Endtz test results with the formation of ROS in sperm ($P < 0.001$).

In conclusion, a positive Endtz test is an indicator of positive chemiluminescence for ROS in unprocessed human semen regardless of WBC activity or presence of seminal plasma. This test is inexpensive and easy to perform, and its results can be used to predict possible peroxidative damage to spermatozoa, which may adversely affect their fertilizing potential.

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