ABSTRACT

Objectives. An association between prostatitis and male infertility has been suspected, yet is poorly understood. Prostatitis is often associated with granulocytes in the prostatic fluid that generate reactive oxygen species (ROS), known to impair male fertility. We compared ROS, the total antioxidant capacity (TAC), and a novel index of oxidative stress (ROS-TAC score) in patients with chronic prostatitis and in healthy controls.

Methods. Semen specimens from 36 men with chronic prostatitis (National Institutes of Health category IIIa), 8 men with prostatodynia (National Institutes of Health category IIIb), and 19 controls attending our urologic clinic were examined according to the World Health Organization criteria. Leukocytospermia was measured by the Endtz test (myeloperoxidase assay). ROS and TAC production was measured by chemiluminescence assay. A composite ROS-TAC score was also calculated in patients and controls.

Results. The sperm concentration, percentage of motility, and morphology among the groups did not differ. The mean ± standard error log-transformed ROS level was significantly higher in patients with leukocytospermia (3.2 ± 0.6) than in patients without leukocytospermia (1.8 ± 0.2; P = 0.04) and controls (1.3 ± 0.3, P = 0.01). TAC was significantly lower in patients with or without leukocytospermia (859.69 ± 193.0 and 914.9 ± 65.2, respectively) than in controls (1653.98 ± 93.6, P = 0.001). The mean ROS-TAC score of controls (50.0 ± 4.1) was significantly higher than those of patients with chronic prostatitis and leukocytospermia (8.2 ± 9.2) and those without leukocytospermia (34.2 ± 2.9; P <0.001).

Conclusions. Men with chronic prostatitis or prostatodynia have seminal oxidative stress, irrespective of their leukocytospermia status. These observations may help shed light on the long-standing controversy surrounding prostatitis and infertility. UROLOGY 55: 881-885, 2000. © 2000, Elsevier Science Inc.
Symptoms in patients with chronic prostatitis may last several weeks or more and may recur periodically for many years.\textsuperscript{5} The chronicity and relapses characteristic of this syndrome may have deleterious effects on the male reproductive system. To diagnose prostatitis, prostatic-seminal vesiculitis, male accessory gland infection, or epididymo-prostatic-vesiculitis among asymptomatic infertile men, clinicians order a culture of seminal fluid.\textsuperscript{2,6-8} However, even in the presence of inflammatory cells, a causative organism is often not identified.\textsuperscript{7} Leukocytic infiltration itself, however, may have a negative impact on the semen profile and sperm function.\textsuperscript{8,9} Leukocytes, particularly neutrophils, have been associated with excessive reactive oxygen species (ROS) production, and leukocytospermia alone may explain this relationship.\textsuperscript{8}

Free radicals have been demonstrated to play an important role in the etiology of defective spermatozoa.\textsuperscript{9,10} As many as 40\% to 88\% of semen samples from infertile men have increased levels of ROS, suggesting that excessive seminal ROS impairs sperm function.\textsuperscript{11}

The purpose of this study was to determine whether seminal oxidative stress was higher in men with chronic prostatitis than in healthy men and whether seminal oxidative stress was affected by leukocytospermia. Levels of ROS, total antioxidant capacity (TAC), and a composite ROS-TAC score were measured in patients with chronic prostatitis both with and without leukocytospermia and also in controls.

**MATERIAL AND METHODS**

Our study was approved by the Cleveland Clinic Foundation Institutional Review Board, and all participants granted informed consent. Patients with chronic prostatitis (NIH category III) attending our prostatitis clinic were recruited for semen evaluation between March 1997 and June 1998. Chronic prostatitis was defined as a history of 3 months or more of pelvic or genital pain, or both, associated with voiding and/or sexual dysfunction. The diagnosis was confirmed by physical examination, including digital rectal examination of the prostate. In all patients, urine and semen localization cultures were negative, as were cultures for Chlamydia trachomatis, Ureaplasma urealyticum, and Mycoplasma hominis. Patients treated with antimicrobial agents during the previous 2 months were excluded.

Our study group included 44 patients who met the diagnostic criteria for chronic prostatitis; 36 met the criteria for NIH category III\textsubscript{a} and 8 met that for NIH category III\textsubscript{b}, classified clinically on the basis of EPS/VB3 results. All 44 patients were later compared on the basis of Endtz test results. Nineteen healthy donors with normal semen characteristics according to the World Health Organization (WHO) criteria\textsuperscript{12} were recruited as controls. Donors with a genitourinary history of infection, symptoms, or instrumentation were excluded from the study.

**SEmen ANALYSIS**

Semen samples were collected by masturbation into sterile containers after at least 48 hours of sexual abstinence. After liquefaction, semen specimens were evaluated for semen volume, appearance, and viscosity. Semen characteristics (concentration, motility, and morphology) were examined according to the WHO criteria.

Semen samples were analyzed on a computer-assisted semen analyzer (CASA, Motion Analysis, Cell-Trak, model VP 110, version 4.22B, Santa Rosa, Calif). For each measurement, 5-µL aliquots were loaded on a counting chamber (MicroCell, Conception Technologies, La Jolla, Calif). Four to eight representative fields containing 200 or more motile cells were examined.

**WHITE BLOOD CELLS**

The presence of WBCs in semen specimens was assessed by the myeloperoxidase (Endtz) test.\textsuperscript{13} A 20-µL volume of liquefied semen was placed in a 2.0-mL cryogenic vial, followed by 20µL of phosphate-buffered saline (pH 7.0) and 40 µL of benzidine solution. The sample was mixed, allowed to sit for 5 minutes, and examined for cells that had stained brown, indicating cells positive for peroxidase. Leukocytospermia was defined as 1 x 10\textsuperscript{6} or more WBC/mL of semen.\textsuperscript{13}
REACTIVE OXYGEN SPECIES

Aliquots of liquefied semen were centrifuged at 300g for 7 minutes. Seminal plasma was separated into aliquots and frozen at -20°C for later measurement of total antioxidant levels. The sperm pellet was washed twice with phosphate-buffered saline (pH 7.4) and resuspended in the same medium at a concentration of 20 x 10^6 sperm/mL. Levels of ROS were measured by chemiluminescence assay. Ten microliters of Luminol (5 mM, 5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemical, St. Louis, Mo) prepared in dimethyl sulfoxide (Sigma Chemical) were added to 400 µL of the washed sperm suspension, which was then mixed.

Levels of ROS were determined by measuring chemiluminescence with a luminometer (model LKB 953, Wallac, Gaithersburg, Md) in the integrated mode for 15 minutes, and the results were expressed as 10^4 counted photons per minute (cpm) per 20 x 10^6 sperm. On the basis of the analysis of the controls, negative controls, and interassay variability, variance components indicated that within an assay, measurement reliability was approximately 98%.

TOTAL ANTIOXIDANT ACTIVITY

Total antioxidant activity was measured in the seminal plasma using the enhanced chemiluminescence assay. Aliquots of the seminal plasma stored at -20°C were thawed at room temperature and immediately assessed for their antioxidant capacity as follows. Seminal plasma was diluted 1:10 with deionized water (dH_2O) and filtered through a 0.2-µm Millipore filter (Allegiance Healthcare, McGaw Park, 111). Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckinghamshire, United Kingdom). Twenty microliters of horseradish peroxidase-linked immunoglobulin (Amersham Life Science) was added to 4.98 mL of dH_2O. The solution was further diluted 1:1 to give a working solution with the desired luminescence output (3 x 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 µM. With the luminometer in the kinetic mode, 100 µL of signal reagent and 100 µL of horseradish peroxidase were added to 700 µL of dH_2O and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between 2 and 3 x 10^7 cpm) for 100 seconds. One hundred microliters of the prepared seminal plasma was added immediately to the signal reagent and horseradish peroxidase, and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence were recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls (n = 19)</th>
<th>CP (Endtz Negative) (n = 39)</th>
<th>CP (Endtz Positive) (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (x 10^6/mL)</td>
<td>69.4 ± 14.1</td>
<td>43.9 ± 9.3</td>
<td>48.4 ± 26.1</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>55.5 ± 4.9</td>
<td>44.2 ± 3.2</td>
<td>30.4 ± 9.0*</td>
</tr>
<tr>
<td>WHO morphology (%)</td>
<td>39.8 ± 3.3</td>
<td>32.8 ± 2.2</td>
<td>31.7 ± 7.0</td>
</tr>
<tr>
<td>Log (ROS+1)</td>
<td>1.3 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>3.2 ± 0.6†</td>
</tr>
<tr>
<td>TAC</td>
<td>1654.0 ± 93.6</td>
<td>914.9 ± 65.2*</td>
<td>859.7±193.0*§</td>
</tr>
<tr>
<td>ROS-TAC score</td>
<td>50.0 ± 4.1</td>
<td>34.2 ± 2.9‡</td>
<td>8.2 ± 9.2*§</td>
</tr>
</tbody>
</table>

KEY: CP = chronic prostatitis (NIH category III); WHO = World Health Organization; ROS = reactive oxygen species; TAC = total antioxidant capacity; NIH = National Institutes of Health.

Data presented as the mean ± SE.
* P <0.05, controls vs. Endtz positive.
‡P <0.05, Endtz negative vs. Endtz positive.
§P <0.05, controls vs. Endtz negative.
*P <0.05, Endtz positive vs. Endtz negative.
ROS-TAC Score

The ROS and TAC values from the controls were used to create a scale of these two variables that uses the control values as reference points. The log of (ROS + 1) was used in calculations so that both values were normalized to the same distribution. First, both TAC and log (ROS + 1) were standardized to z scores so that both would have the same variability. These standardized scores were calculated by subtracting the mean value for the controls from the mean value for the patients and dividing by the standard deviation of the control population.

For log (ROS + 1): Standardized ROS = [log (ROS + 1) - 1.38851/0.7271
For TAC: Standardized TAC = (TAC - 1650.93)/532.22

These two standardized variables were then analyzed using the principal components analysis which provided linear combinations (or weighted sums) that account for the most variability among the correlated variables. The first principal component provided the following linear equation: Principal component = (-0.707 x standardized ROS) + (0.707 x standardized TAC).

To ensure that the distribution of the ROS-TAC score would have a mean of 50 and standard deviation of 10 in the controls, the ROS-TAC score was transformed as: ROS-TAC score = 50 + (principal component X 10.629).

RECEIVER OPERATING CHARACTERISTIC CURVES

Receiver operating characteristic (ROC) curves illustrating the sensitivity and specificity over the entire range of the three values described above (ROS, TAC, and ROS-TAC score) were generated. The area under an ROC curve ranged from 50% to 100%, with 100% indicating a perfect predictor and 50% indicating random chance, or no predictive ability. The ROC curve was used to compare the ability of ROS, TAC, and the ROS-TAC score to discriminate patients with prostatitis from controls.

STATISTICAL ANALYSIS

We compared three classifications of men: those with chronic prostatitis and leukocytospermia, those with chronic prostatitis without leukocytospermia, and controls. Analysis of variance and pairwise t tests (using Dunnett’s method) were used to compare the groups for continuous variables. ROC curves were used to evaluate the significance and diagnostic ability to identify the patients with chronic prostatitis.

Statistical significance was assessed with two-tailed tests at P <0.05. Summary statistics are presented as the mean + standard error. Statistical tests were performed using Statistical Analysis Systems, version 6.12 software (SAS Institute, Cary, NC).

RESULTS

Comparisons of sperm concentration, motility, and morphology in the patients with chronic prostatitis and the controls are listed in Table 1. No differences were found among the three groups with respect to the total sperm count and sperm morphology. Sperm motility was significantly lower in patients with leukocytospermia (30.4% ± 9.0%) than in the controls (55.5% ± 4.9%, P = 0.02). No statistically significant differences in semen characteristics were evident between patients without leukocytospermia and the controls or between the patients with and without leukocytospermia.

ROS levels (using the log-transformation) were significantly higher in patients with leukocytospermia (3.2 ± 0.6) than in patients without leukocytospermia (1.8 ± 0.2, P = 0.04) or controls (1.3 ± 0.3, P = 0.01) (Table 1). However, ROS levels did not differ between patients without leukocytospermia and controls (Table 1). The area under the ROC curve measuring the ability of ROS levels to identify patients with chronic prostatitis was 61.2%(95% confidence interval 46.9% to 75.6%, P = 0.06), indicating that ROS alone cannot discriminate between patients with chronic prostatitis and controls.
Although no differences were found between semen characteristics or ROS, seminal plasma TAC levels were significantly lower in both patients with chronic prostatitis and leukocytospermia and patients with chronic prostatitis without leukocytospermia than in the control group (Table I). The TAC levels between patients with and without leukocytospermia did not differ. The area under the ROC curve that discriminates between controls and patients with chronic prostatitis was 89.4% (95% confidence interval 80.7% to 98.0%, P <0.001). The mean ROS-TAC score was significantly different between controls and patients, irrespective of the leukocytospermia status (P <0.001). Also, this score differed significantly between patients with and without leukocytospermia (P = 0.01). The area under the ROC curve was 79.2% (95% confidence interval 66.7% to 91.7%, P <0.001), which did not differ significantly from the diagnostic ability of TAC alone (P = 0.08).

COMMENT

It is estimated that 50% of all men will experience symptoms of prostatitis at least once during their lifetime, accounting for 8% of urologic office visits. Even though its etiology remains unclear, most experts would agree that the vast majority of all cases are associated with negative bacterial cultures. Even less well understood is the possible relationship between chronic prostatitis and infertility. It has been theorized that the higher incidence of chronic prostatitis among infertile couples may be related to an autoimmune source. Antisperm antibodies have been found with greater frequency among men with a history of bacterial and abacterial prostatitis. Deterioration of sperm head morphology and motility has also been associated with chronic prostatitis. Although no differences in sperm morphology were found among the three groups in our study, sperm motility was lower in patients with leukocytospermia than in patients without leukocytospermia or in controls. It is possible that our sample size was insufficient to detect abnormalities in sperm morphology among the patients with chronic prostatitis. The sample sizes in this study could detect a 20% difference in abnormal (WHO) morphology between the two prostatitis groups, with 80% power, greater than the accepted level of clinical significance. The duration of symptoms beyond 3 months was not recorded in our study, and perhaps greater differences would be found among those with longer histories of chronic prostatitis.

The importance of the presence of leukocytes in semen remains highly controversial. The ability of polymorphonuclear neutrophils and macrophages to produce large amounts of ROS suggests that ROS may be responsible for leukocyte-induced impairment of sperm function. We observed higher ROS levels in patients with prostatitis and positive Endtz test results than in those with negative Endtz test results, although it should be noted that we studied only a relatively small number of patients with positive Endtz results. Even though the WHO defines leukocytospermia as greater than 1 X 10^6 WBC/mL of semen, there has always been a debate as to the cutoff value from a clinical standpoint. Earlier studies have indicated the need for raising this value to higher than 2 x 10^6 WBC/mL. However, in our unpublished work, we observed that WBC values greater than 0 were capable of influencing both ROS and ROS-TAC scores. This indicates that spermatozoa, especially the functionally abnormal ones, are capable of producing substantial amounts of ROS. In this unpublished study, we found a positive correlation between the amount of WBCs present and the ROS-TAC score in these patients.

Our finding of high levels of ROS in patients with leukocytospermia is consistent with reports confirming leukocytes as the primary source of ROS production in semen. Under normal conditions, seminal plasma contains low levels of ROS, contributed by both leukocytes and spermatozoa. Depending on the nature and concentration of the particular ROS involved, they can have either beneficial or detrimental effects on sperm function. Even though ROS play a physiologic role in spermatozoa hyperactivation, capacitation, and acrosome reaction, elevated levels are more frequent among infertile men. Elevated levels of ROS in seminal plasma have been found in 40% to 88% of infertile men. Excessive ROS levels disrupt human sperm function by inducing peroxidative damage to the unsaturated fatty acids within the sperm plasma membrane, diminishing motility and leading to incompetence for sperm-oocyte fusion. Damage caused by ROS may also be targeted at DNA, and ROS can cause chromatin crosslinking, DNA base oxidation, and DNA strand breaks.
Our findings demonstrated oxidative stress in men with chronic prostatitis (NIH category III) irrespective of leukocytospermia status, suggesting that empiric supplementation with antioxidants may benefit this category of patients. Levels of TAC and the ROS-TAC score were significantly different between healthy men and men with chronic prostatitis, regardless of leukocytospermia status.

Oxidative injury is a major cause of spermatozoa dysfunction, and the total nonenzymatic antioxidant defenses in human seminal plasma is inversely related to lipid peroxidation. TAC in seminal plasma is lower in infertile men than in fertile men. In our study, patients with chronic prostatitis, irrespective of the leukocytospermia level, had depressed TAC levels. Empirical trials of antioxidant supplementation among infertile men have shown promising results. Improved semen characteristics and high rates of fertilization in vitro have been observed with the use of oral vitamin E or intramuscular glutathione.

On the basis of our findings, we hypothesize that patients with chronic prostatitis with leukocytospermia may be at greater risk of infertility than men with the same disease but without leukocytospermia. We theorize that one of the possible mechanisms contributing to the infertility in this patient population could be an increase in ROS produced by leukocytes combined with the depressed TAC found in all patients with chronic prostatitis. We consider our study a pilot study, and some of our findings need to be confirmed in larger populations of patients.

The results of our study demonstrated high levels of ROS and low TAC in the seminal plasma of patients with chronic prostatitis. On the basis of our previous studies that demonstrated higher oxidative stress in the semen of men seeking fertility treatment than in donors, we may assume that high ROS levels and low TAC might contribute to infertility. In the future, a prospective, randomized, double-blind study should help determine whether treating infertile patients with chronic prostatitis with antioxidants would lower seminal oxidative stress and restore fertility. Further research is warranted to determine whether differences in oxidative stress levels are primarily attributable to high levels of ROS or a lack of ability to scavenge them, or a combination of the two.

CONCLUSIONS

Elevated seminal ROS levels are evident in men with chronic abacterial prostatitis, but the levels are particularly high in men with leukocytospermia. TAC and the ROS-TAC score were lower in all patients with chronic prostatitis than in controls in our study. These observations may help explain the possible relationship between prostatitis and infertility and suggest that empiric antioxidant supplementation may benefit this category of patients.

REFERENCES


