RELATIONSHIP OF SPERM PARAMETERS WITH LEVELS OF REACTIVE OXYGEN SPECIES IN SEMEN SPECIMENS

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ABSTRACT

The levels of reactive oxygen species were compared in semen specimens from suspected subfertile men and from normal volunteers, and correlated with other semen parameters. Reactive oxygen species formation was measured in semen samples that had no white blood cells by a chemiluminescence assay with a Luminometer. The relationship of seminal reactive oxygen species to sperm parameters was evaluated. A total of 84 specimens from 69 suspected subfertile men and 15 normal volunteers was tested for reactive oxygen species production. Comparison of reactive oxygen species levels in white blood cell-negative patient and donor specimens showed significantly higher values (p < 0.005) in the patient group. Similarly, levels in patient and donor specimens with normal sperm motility were significantly lower (p < 0.005) than those in specimens showing poor motility. The seminal reactive oxygen species levels of white blood cell-negative patients with abnormal morphology were significantly higher (p < 0.005) than those in white blood cell-negative patients with normal morphology. Our results show that seminal reactive oxygen species levels in suspected subfertile men are significantly higher than in normal men, and that the presence of excess reactive oxygen species in semen is positively correlated with low sperm concentration, poor motility and poor morphology. In conclusion, the evaluation of reactive oxygen species levels in cases of idiopathic male infertility could serve as an important marker of sperm dysfunction.

KEY WORDS: semen; spermatozoa; infertility, male

The effects of reactive oxygen species, such as hydrogen peroxide, oxygen ion and the hydroxyl radical, on sperm function and the toxicity of the fatty acid peroxides generated by their attack on the cell membrane phospholipids were recognized only a decade ago.1 Human sperm are especially sensitive to oxygen-induced damage.2,3 It was recently shown that as many as 25% of semen samples from infertile men produce high levels of reactive oxygen species.4 The higher levels of reactive oxygen species produced by damaged or deficient spermatozoa were associated with a loss of motility and a decreased capacity for sperm-oocyte fusion.5-7 Lipid peroxidation of the sperm membrane and the high toxicity of the generated fatty acid peroxides were proposed as being responsible for the decreased sperm functions observed after exposure to reactive oxygen species.1,3,4 Several investigators have shown that reactive oxygen species levels are significantly higher in idiopathic male infertility patients4,8,9 and it is well known that activated white blood cells in semen produce excessive amounts of reactive oxygen species.5,10 Earlier studies did not provide definite proof to link sperm cells with the generation of reactive oxygen species, since semen samples were not tested for the presence of white blood cells. We compared reactive oxygen species levels in white blood cell-negative semen specimens from suspected subfertile men and semen specimens from normal donors. We studied the correlation among reactive oxygen species formation and sperm concentration, velocity, motility, morphology, hypsosemotic swelling test and bovine-cervical mucus penetration test results.

MATERIALS AND METHODS

Subject selection. Semen samples were obtained from 69 suspected subfertile men randomly selected from patients who presented to our laboratory for infertility evaluation. Specimens from 15 volunteers with normal semen analysis1 served as a control for these studies.

Semen collection and assessment of semen parameters. Semen specimens were collected by masturbation after at least 2 days of sexual abstinence and liquefied at 37°C. Then, 5 μl. were loaded on a 20 μl Microcell® chamber and analyzed on a Hamilton-Thorn Motility (HTM) Analyzer, version 7.1

HTM gate settings. The main gate settings for the HTM analyzer were number of frames—20, frame rate—30 frames per second, minimum contrast—7, minimum size—5, low/high size gates—0.5 to 1.8, low/high intensity gates—0.5 to 1.8, nonmotile head size—10, nonmotile intensity—200, medium path velocity value—25, low path velocity value—10, slow cells motile—no, threshold straightness—80, path velocity—10 to 500, progressive velocity—0 to 500, curvilinear velocity—0 to 500, straightness—0 to 100, linearity—0 to 100 and lateral head displacement—0 to 0.

Reactive oxygen species determination. Reactive oxygen species formation was measured with a computer-driven Luminometer, model 1251.1 Luminescence was recorded after the addition of 20 μl of 4 mM luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) to sperm samples suspended in 1.0 ml human tubal fluid medium. Measurement of reactive oxygen species activity was started 3 minutes after the addition of luminol and continued for 30 cycles. The readings were taken in the integration mode with constant mixing of analyzed sample for 10 seconds. Human tubal fluid medium acted as a control for each specimen analyzed for reactive oxygen species activity. Mean reactive oxygen species was expressed (mv. per second per 10⁶ sperm) as the mean ± standard error of reactive oxygen species value for 30 cycles. The difference between experimental and control readings was considered significant at p <0.05. Peak reactive oxygen species denotes the maximum

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value for each sample from among 30 readings and it was calculated by subtracting the corresponding control values.

Quantitation of white blood cells. The presence of granulocytes in semen specimens was identified by the Endtz test. \(^{15}\)Liquified specimens (20 \(\mu\)l) were placed in a Corning 2.0 ml cryogenic vial, and 20 \(\mu\)l phosphate buffered saline and 40 \(\mu\)l benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 minutes. Peroxidase-positive white blood cells staining dark brown were counted in all 100 squares of the grid in a Makler chamber under the 20X bright field objective. The results after correlation for dilution were recorded as 10\(^3\)/ml.

Sperm morphology. Seminal smears were prepared from liquefied semen for assessment of sperm morphology by standard methods and stained by Hemacolor.* A total of 100 cells was scored according to World Health Organization guidelines\(^{11}\) for normal forms; head, neck and tail defects, and undifferentiated round cells at 400X magnification.

Hypsoosmotic swelling test. The hypsoosmotic swelling test began with the addition of 1 ml hypsoosmotic solution (150 mOsm./L, 0.025M sodium citrate [2 water] + 0.075M \(\beta\)-D [--] fructose) to 0.1 ml liquefied semen.\(^{12}\) Following incubation at 37C for 30 minutes the sperm sample was examined and the percentage of swollen sperm (number of swollen sperm tails divided by the total number of sperm examined times 100) was calculated with use of an Olympus model BH 2 phase-contrast microscope.

Bovine cervical mucus penetration test. The bovine cervical mucus penetration test was performed, with some modification of the method of Alexander\(^ {14}\) using the Serono Penetrak set. Each capillary tube was thawed at room temperature for approximately 30 minutes and snapped at the red score mark above the mucous meniscus. The cut end was inserted into a small plastic beaker containing approximately 200 \(\mu\)l semen and incubated at room temperature for 90 minutes. The tubes were removed from the semen reservoir after the incubation period, wiped clean of any residual specimen and placed on a glass slide on a microscope stage. The distance (in millimeters) covered by the vanguard sperm was measured with the phase-contrast microscope. For each specimen a mean of the 2 capillary tube readings was taken.

Statistical analysis. Analysis of statistical differences in reactive oxygen species levels between patient and donor populations, or among various semen parameter groups was done by the unpaired Wilcoxon rank sum test using the InStat program† on an IBM PS/2 computer. The 0.05 level of probability was used as the criterion for significance.

RESULTS

A total of 84 specimens (69 patients and 15 donors) was tested for reactive oxygen species (mean and peak) generation. Among 69 patients 51 specimens were negative and 18 were positive for white blood cells (greater than 1 \(\times\) 10\(^6\)/ml). Among donors 13 specimens were negative and 2 were positive for white blood cells. Of 51 white blood cell-negative patient specimens 21 (41%) showed reactive oxygen species levels of greater than 10 mv. per second per 10\(^9\) sperm compared to only 2 of the white blood cell-negative donor specimens. Our results show that 27 of 69 patients (39%) (positive and negative for white blood cells) tested in this study generated reactive oxygen species of greater than 10 mv. per second per 10\(^9\) sperm.

Comparison of reactive oxygen species levels between patient and donor specimens. Reactive oxygen species levels were significantly higher in specimens from patients (mean reactive oxygen species p <0.01, table 1) than in those from donors. However, there was no significant difference in reactive oxygen species values in white blood cell-negative specimens from patients and donors, and white blood cell-positive specimens from both groups.

Reactive oxygen species and sperm motility. A total of 37 specimens with normal sperm motility from patients and donors showed significantly lower reactive oxygen species formation (mean 2.83 ± 1.07, peak 4.02 ± 1.18, fig. 1) than did 44 specimens with abnormal sperm motility (mean 12.31 ± 3.06, peak 14.04 ± 3.48). White blood cell-negative patient and donor specimens with greater than 50% sperm motility (27) showed significantly lower reactive oxygen species formation (mean 5.20 ± 2.99, peak 8.27 ± 4.82) than did 37 white blood cell-negative specimens with less than 50% sperm motility (mean 8.27 ± 4.82, peak 17.33 ± 4.08, fig. 2).

Reactive oxygen species and sperm morphology. Reactive oxygen species generation (mean and peak) in 27 white blood cell-negative semen specimens with abnormal morphology was significantly higher (p <0.05) than that in 20 white blood cell-negative semen specimens with normal morphology (table 2). White blood cell-negative patient specimens with abnormal morphology and motility (24) had significantly higher reactive oxygen species values than did the 10 specimens with normal morphology and motility (p <0.05).

Reactive oxygen species, sperm concentration and velocity. Reactive oxygen species generation in semen with normal sperm concentration (mean 9.33 ± 1.99 mv. per second per 10\(^9\) sperm) was significantly lower (p = 0.0003) than that in specimens with abnormal sperm concentration (mean 128.32 ± 45.74 mv. per second per 10\(^9\) sperm). No significant difference was noted in reactive oxygen species formation between specimens with normal velocity (mean 20.25 ± 6.07 mv. per second per 10\(^9\) sperm) and those with abnormal velocity (mean 23.89 ± 10.01 mv. per second per 10\(^9\) sperm).

Reactive oxygen species, hypsoosmotic swelling and bovine cervical mucus penetration test results. Reactive oxygen species generation in specimens with normal (greater than 60% tail swelling) hypsoosmotic swelling test results (mean 6.10 ± 2.02 mv. per second per 10\(^9\) sperm) was not significantly different from that in the group with abnormal (less than 50%) hypsoosmotic swelling test results (mean 14.38 ± 6.52 mv. per second per 10\(^9\) sperm). Similarly, reactive oxygen species formation in semen of normal (greater than 30 mm.) bovine cervical mucus penetration test subjects (mean 7.27 ± 2.69 mv. per second per 10\(^9\) sperm) was not significantly different from those in the group with abnormal (less than 30 mm.) bovine cervical mucus

<table>
<thead>
<tr>
<th>Reactive Oxygen Species Level (mv./sec./10^9 sperm)</th>
<th>All Subjects*</th>
<th>All White Blood Cell Neg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>17.66 ± 4.74</td>
<td>15.55 ± 3.79</td>
</tr>
<tr>
<td>Probability</td>
<td>0.0078</td>
<td>Not significant</td>
</tr>
<tr>
<td>Peak</td>
<td>23.44 ± 0.07</td>
<td>8.76 ± 4.42</td>
</tr>
<tr>
<td>Probability</td>
<td>0.0078</td>
<td>10.42 ± 4.46</td>
</tr>
</tbody>
</table>

Values are mean ± standard error.

* Includes patients and donors.

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* EM Diagnostics, Inc., Gibbstown, New Jersey.
† GraphPAD Software, Inc., San Diego, California.
TABLE 2. Reactive oxygen species values for white blood cell-negative semen specimens from infertile patients according to sperm morphology and motility

<table>
<thead>
<tr>
<th>Reactive Oxygen Species Level (mV/sec/10^9 sperm)</th>
<th>White Blood Cell Neg.</th>
<th>Morphology and Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean</strong></td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Probability</td>
<td>6.08 ± 1.43</td>
<td>24.64 ± 7.99</td>
</tr>
<tr>
<td>Peak Probability</td>
<td>4.90 ± 1.41</td>
<td>17.21 ± 5.45</td>
</tr>
<tr>
<td><strong>Probability</strong></td>
<td>0.0491</td>
<td>0.0389</td>
</tr>
<tr>
<td><strong>More Than 50%</strong></td>
<td>7.24 ± 2.05</td>
<td>16.89 ± 3.38</td>
</tr>
<tr>
<td><strong>Less Than 50%</strong></td>
<td>9.21 ± 2.15</td>
<td>19.4 ± 4.15</td>
</tr>
<tr>
<td><strong>Not significant</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± standard error.

Figure 1. Comparison of reactive oxygen species (ROS) levels between all subjects with normal and abnormal motility. a, p < 0.01 compared to reactive oxygen species levels in subjects with abnormal motility.

Figure 2. Comparison of reactive oxygen species (ROS) levels between white blood cell-negative subjects with normal and abnormal motility. a, p < 0.01 compared to white blood cell-negative, abnormal motility population.

penetration test results (mean 32.87 ± 20.61 mV. per second per 10^9 sperm). In white blood cell-negative patient and donor specimens reactive oxygen species levels showed no correlation with hypooosmotic and bovine cervical mucus penetration results.

**DISCUSSION**

Our data indicate that 30% of the patients attending our infertility clinic have seminal reactive oxygen species formation of greater than 10 mV. per second per 10^9 spermatozoa. These results are in agreement with previous reports in which 25% of the patients showed reactive oxygen species generation of greater than 10 mV. per second per 10^9 spermatozoa.4 Our results strongly suggest that sperm cells and white blood cells act as dual sources of reactive oxygen species generation in semen. Furthermore, a strong positive correlation noted in our study with excess reactive oxygen species formation in semen with abnormal sperm concentration, motility and morphology is in agreement with earlier observations.8,13

Sperm have been shown to be able to produce reactive oxygen species.3,15,16 The hyperproduction of reactive oxygen species may be directly responsible for the loss of sperm function through the peroxidation of unsaturated fatty acids1 or the denaturation of proteins in the sperm plasma membrane.2 An alternative possibility is that the excess generation of reactive oxygen species may be a consequence (rather than a cause) of a primary defect in the differentiation of the sperm plasma membrane, such that the control mechanisms regulating this activity are no longer effective.

Failure to detect high levels of reactive oxygen species formation in semen of normal volunteers is consistent with the hypothesis that abnormal sperm are the primary source of reactive oxygen species production.1,2,17 Considering that the population of patients who presented for infertility was unselected, the observation that 30% of the patients have significant reactive oxygen species formation is interesting. Further studies are needed to determine which subgroups of infertile men are especially affected by reactive oxygen species, to investigate the type of reactive oxygen species involved in sperm dysfunctions, and to evaluate the balance of intracellular and extracellular scavenging systems in semen samples affected by reactive oxygen species.

In conclusion, our results show that seminal reactive oxygen species levels in suspected subfertile men are significantly higher than those in normal men, and that presence of excess reactive oxygen species in semen shows a strong positive relationship with poor sperm concentration, motility and morphology. Evaluation of reactive oxygen species levels in cases of idiopathic male infertility may provide valuable information regarding the functional status of the sperm.

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**REFERENCES**

6. Aitken, R. J., Clarkson, J. S. and Fischel, S.: Generation of reactive


