VARICOCELE IS ASSOCIATED WITH ELEVATED SPERMATOZOAL REACTIVE OXYGEN SPECIES PRODUCTION AND DIMINISHED SEMINAL PLASMA ANTIOXIDANT CAPACITY

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ABSTRACT

Purpose: Because varicocele is seen often in infertile men and oxidative stress has been implicated in sperm dysfunction, we assessed spermatozoal reactive oxygen species and seminal total antioxidant capacity in men with and without varicocele.

Materials and Methods: Levels of reactive oxygen species and total antioxidant capacity were measured in the semen of 21 infertile men with varicocele, 15 men with incidental varicocele and 17 normal donors without varicocele (controls). Men with leukocytospermia (more than 1x10^6 white blood cells per ml.) were excluded from study. Reactive oxygen species were measured in washed spermatozoa with a luminol dependent chemiluminescence assay. Total seminal antioxidant capacity was measured with an enhanced chemiluminescence assay, and the results were expressed as trolox equivalents. Sperm characteristics were assessed with a computer assisted semen analyzer, and sperm morphology was assessed using World Health Organization and Kruger's strict criteria.

Results: Patients with varicocele had significantly higher reactive oxygen species levels than controls (p = 0.02). Reactive oxygen species levels did not differ significantly between infertile and men with incidental varicocele. Total antioxidant levels were significantly lower among men with varicocele (p = 0.02) and those with incidental varicocele compared to controls (p = 0.05). Reactive oxygen species and total antioxidant capacity levels did not correlate in any group.

Conclusions: Our results suggest that elevated reactive oxygen species and depressed total antioxidant capacity levels are associated with varicocele. These changes may be related to functional sperm abnormalities and infertility seen commonly in these patients. These findings support a possible rationale for controlled clinical trials of antioxidant supplementation in infertile men with varicocele.

KEY WORDS: varicocele; infertility, male; reactive oxygen species; antioxidants

Varicocele has long been implicated as a cause of male infertility. The pathophysiological mechanism by which varicocele produces sperm dysfunction and infertility is not completely understood. Although clinically evident varicocele has been reported in 8 to 23% of men, not all men with varicocele are infertile. For example, 16.7% of men presenting for vasectomy who had fathered children within the preceding 3 years had palpable varicoceles. Nonetheless, varicocele is strongly associated with male infertility and present in 19 to 41% of men presenting for infertility evaluation.

Research during the last decade has implicated oxidative stress as a mediator of sperm dysfunction. Reactive oxygen species include hydrogen peroxide and highly unstable free oxygen radicals (the hydroxyl radical and superoxide anion). While normal spermatozoa produce low levels of reactive oxygen species, the spermatozoal membrane, which is rich in polyunsaturated fatty acids, is susceptible to peroxidation in the presence of...
elevated seminal reactive oxygen species levels. Therefore, sperm dysfunction may be a consequence of elevated seminal reactive oxygen species. Antioxidants scavenge oxygen radicals to protect spermatozoa. Recent studies have investigated possible deficiencies in the seminal total antioxidant capacity among infertile men, and suggested an association between decreased total antioxidant capacity and male infertility. Because varicocele is a common cause of male infertility and its pathophysiological mechanism is poorly understood, we prospectively studied seminal reactive oxygen species production and total antioxidant capacity among men with varicocele. We hypothesized that infertility attributed to varicocele could be associated with increased oxidative stress due to excess reactive oxygen species generation and diminished antioxidant capacity.

**MATERIALS AND METHODS**

Between October 1996 and October 1997, 3 populations of men were recruited from the Male Infertility Clinic and the Andrology Laboratory of the Department of Urology at our tertiary care hospital. The study was approved by the Institutional Review Board. A total of 21 men with varicocele who presented for evaluation of infertility (infertile varicocele group) were recruited. The duration of infertility was at least 12 months and the presence of clinical varicocele was confirmed on examination by a male infertility specialist (A. J. T.). Additionally, 15 men who had clinically apparent varicocele but who were not clinically infertile (incidental varicocele group) were recruited, of whom 2 had achieved pregnancy, 4 had been attempting conception for less than 12 months and 9 were not actively trying to achieve pregnancy. Another 17 donors who had no history of infertility, no clinical varicocele and previously normal semen analyses (controls) were also recruited. Subjects whose ejaculate contained fewer than 15 million total spermatozoa (oligospermia) or who had greater than 1.0 x 10⁶ white blood cells per ml. were excluded from study. Specimens with low sperm counts were insufficient for the measurement of reactive oxygen species. Similarly, specimens from patients with leukocytospermia were excluded from study, as leukocytes are the main source of reactive oxygen species generation in the semen of these men.

Semen specimens were obtained by masturbation after 2 to 3 days of sexual abstinence and analyzed within 90 minutes of collection. Semen parameters, including volume, sperm concentration, percent motility and sperm morphology, were assessed according to World Health Organization (WHO) and Kruger’s strict criteria. For all specimens computer assisted semen analysis was done with a semen analyzer, and myeloperoxidase staining was performed to measure the granulocyte concentration.

Liquefied semen aliquots were centrifuged at 300 g for 7 minutes. The seminal plasma was aliquoted and frozen at 20°C for later measurement of total antioxidant capacity. The sperm pellet was washed twice with phosphate buffered saline, pH 7.4, and resuspended in phosphate buffered saline at a concentration of 20 x 10⁶ sperm per ml. Reactive oxygen species levels were determined by the chemiluminescence method using luminol as the probe. Then 10 µl. 5 mM. luminol (5-amino-2,3 dehydro-1,4 phthalazinedione) prepared in dimethyl sulfoxide were added to 400 µl. washed sperm suspension and centrifuged. The 10 µl. 5 mM. luminol added to 400 µl. phosphate buffered saline served as a negative control. The reactive oxygen species level was determined by measuring chemiluminescence in the integrated mode for 15 minutes and expressed as 10⁶ counted photons per minute per 20 x 10⁶ sperm.

Total antioxidant activity was measured in the seminal plasma using the enhanced chemiluminescence assay. Aliquots of the frozen seminal plasma were thawed at room temperature and antioxidant capacity was assessed immediately. The seminal plasma was diluted 1:10 with deionized water and put through a 0.20 gm. filter. The signal reagent was prepared using a chemiluminescence kit. Then 20µl. horseradish peroxidase linked Ig were added to 4.98 ml. deionized water, and this solution was further diluted 1:1 for a working solution with the desired luminescence output of 3 x 10⁷ cpm.

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water soluble tocopherol analogue, was added as the standard at concentrations from 50 to 250 µM. With the luminometer in the kinetic mode 100 µl. signal reagent and 100 µl. horseradish peroxidase were added to 700 µl. deionized water and mixed. This solution was equilibrated to the desired chemiluminescence output of 2 to 3 X 10⁷ cpm for 100 seconds. Then 100 µl. seminal plasma were immediately added to the solution and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemiluminescence level were recorded. Antioxidant capacity was expressed as molar trolox equivalents.
Reactive oxygen species values were log transformed (reactive oxygen species plus 1) to normalize the data distribution. Spearman rank correlation was used to determine whether reactive oxygen species and total antioxidant capacity significantly correlated with sperm characteristics. Non-normally distributed reactive oxygen species scores were compared among the 3 groups using analysis of variance and the Student-Newman-Kuels multiple range test. Summary statistics are presented as mean plus or minus standard error. All tests were 2-tailed and considered significant at p <0.05. Computer software was used for all analyses. The total sample had 88% power to find correlations of 0.4 and greater between the variables, and 85% power to find a difference of 1 ± standard deviation on the Wilcoxon rank sum test between men with varicocele and controls.

RESULTS

Mean age was 33.6 ± 1 years (range 26 to 44) for the infertile varicocele group, 29.9 ± 1.7 (range 21 to 35) for the incidental varicocele group and 31.1 ± 2.1 (range 20 to 56) for controls. Sperm motion characteristics and morphology indicated that the controls had significantly better semen quality than the infertile varicocele group (see table). Semen quality tended to decrease according to pathological condition. With regard to sperm concentration, percent motility, complex motion characteristics and percentage normal morphology by WHO and Kruger's criteria, controls had the best, the incidental varicocele group had intermediate and the infertile varicocele group had the poorest semen quality. Differences in semen quality between controls and the infertile varicocele group were significant for all semen criteria but not for all criteria when comparing the incidental varicocele group and either of the other 2 groups (see table).

Mean adjusted reactive oxygen species levels (log [reactive oxygen species plus 1]) were 1.30 ± 0.33 for controls, 1.99 ± 0.26 for the incidental and 2.18 ± 0.25 for the infertile group (see table). These differences were significant when comparing controls to the incidental (p = 0.02) and the infertile (p = 0.02) varicocele groups. Spermatozoal reactive oxygen species production did not significantly differ between the incidental and infertile varicocele groups.

Seminal plasma total antioxidant capacity levels varied significantly among the 3 groups (p = 0.008, see table). Controls had the highest total antioxidant capacity level (1,443.0 ± 105.0 trolox equivalents) and the incidental varicocele group had the lowest level (939.0 ± 107.0, p = 0.05). Total antioxidant capacity levels were significantly lower in the incidental and infertile varicocele groups compared to controls (p = 0.05) but the difference between the 2 varicocele groups was not significant (p = 0.09). Similarly, no significant difference was observed between non-infertile (controls and the incidental varicocele group) and infertile subjects. However, controls had significantly higher total antioxidant capacity levels compared to all men with varicocele (p = 0.02). Overall reactive oxygen species level did not correlate with total antioxidant capacity level (r^2 = 0.07, p = 0.64). Reactive oxygen species levels correlated negatively with the percentage of morphologically normal sperm for all study subjects (r^2 = 0.35, p = 0.046) and this correlation was significant for men with varicocele (r^2 = 0.49, p = 0.02) but not controls (r^2 = 0.33, p = 0.27).

DISCUSSION

We have demonstrated that the spermatozoal reactive oxygen species levels are higher in men with varicocele, irrespective of fertility status, suggesting a strong relationship between a potent end effect of sperm dysfunction and varicocele. This novel finding supports our hypothesis that infertility associated with varicocele is at least in part related to oxidative stress. The biochemical mechanism(s) by which varicocele induces spermatogenic and spermatozoal dysfunction have not been completely elucidated. While our study does not address whether elevated spermatozoal reactive oxygen species production is the cause or effect of abnormal spermatozoa in patients with varicocele, the observation that the reactive oxygen species level is elevated in these patients suggests that there is a common underlying pathophysiological process. Previous studies have reported increased reactive oxygen species generation in 40% of the general infertile population, whereas our select infertile varicocele population had a rate of 80%.
Comparison of semen characteristics reactive between controls and men with varicocele

<table>
<thead>
<tr>
<th></th>
<th>Controls (17 pts.)</th>
<th>Incidental Varicocele (15 pts.)</th>
<th>Infertile Varicocele (21 pts.)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs.)</td>
<td>31.1 ± 2.1</td>
<td>29.9 ± 1.7</td>
<td>33.6 ± 1.0</td>
<td>N.S.</td>
</tr>
<tr>
<td>Vol. (ML)</td>
<td>2.65 ± 0.83</td>
<td>3.65 ± 0.50</td>
<td>3.26 ± 0.23</td>
<td>N.S.</td>
</tr>
<tr>
<td>Concentration (X 10^6/ml)</td>
<td>69.4 ± 9.1</td>
<td>42.3 ± 10.2</td>
<td>37.0 ± 5.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>55.5 ± 4.9</td>
<td>48.6 ± 5.0</td>
<td>35.6 ± 3.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Curvilinear velocity (µm/sec.)</td>
<td>35.1 ± 2.0</td>
<td>32.3 ± 1.1</td>
<td>28.6 ± 1.3</td>
<td>N.S.</td>
</tr>
<tr>
<td>Straight line velocity (µm/sec.)</td>
<td>13.9 ± 0.8</td>
<td>13.0 ± 0.6</td>
<td>10.4 ± 0.6</td>
<td>N.S.</td>
</tr>
<tr>
<td>Av. path velocity (µm/sec.)</td>
<td>23.5 ± 1.3</td>
<td>21.0 ± 0.9</td>
<td>17.4 ± 0.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Linearity (%)</td>
<td>39.5 ± 1.0</td>
<td>39.0 ± 1.5</td>
<td>35.7 ± 1.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Amplitude lateral head motion (µm.)</td>
<td>2.24 ± 0.13</td>
<td>1.94 ± 0.09</td>
<td>1.85 ± 0.09</td>
<td>N.S.</td>
</tr>
<tr>
<td>WHO morphology (%)</td>
<td>39.8 ± 2.5</td>
<td>32.0 ± 3.2</td>
<td>30.6 ± 2.8</td>
<td>N.S.</td>
</tr>
<tr>
<td>Knuger's morphology (%)</td>
<td>12.1 ± 0.9</td>
<td>9.7 ± 1.3</td>
<td>9.9 ± 1.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Log (reactiv oxygen species + 1)</td>
<td>1.30 ± 0.33</td>
<td>1.99 ± 0.26</td>
<td>2.1 ± 0.25</td>
<td>0.02</td>
</tr>
<tr>
<td>Total antioxidant capacity (trolext: equivalents)</td>
<td>1,443.0 ± 105.0</td>
<td>939.0 ± 107.0</td>
<td>1,186.0 ± 96.9</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Includes controls and the incidental varicocele group.
N.S. - Not Significant

Furthermore, 77% of our incident varicocele group had elevated reactive oxygen species production compared to only 20% of our controls. The actual rate of elevated reactive oxygen species production in infertile men with varicocele may be higher because our study has an inherent selection bias, that is we excluded moderately to severely oligospermic men. Despite this selection bias toward varicocele subjects with better sperm counts, the prevalence of elevated reactive oxygen species generation in our infertile varicocele group was approximately double that reported in the general population of infertile men. These findings suggest that oxidative stress has a major role in the spermatozoal dysfunction seen in patients with varicocele. The association between elevated reactive oxygen species production and varicocele is further confirmed by the 4-fold increase in the frequency of elevated reactive oxygen species generation in the incident varicocele group compared to controls.

Previous studies have shown poor sperm motility in infertile men to be strongly related to lower antioxidant levels, and that antioxidant capacity and lipid peroxidation are inversely related. Our finding that total antioxidant capacity is lower in the seminal plasma of all varicocele subjects and does not correlate with the reactive oxygen species level is more difficult to explain. The total antioxidant capacity assay measures consumable, nonenzymatic antioxidants that are primarily extracellular, such as ascorbate, urate, tocopherol and glutathione. There may be variations in the intracellular enzymatic antioxidant species, such as catalase, superoxide dismutase, xanthine oxidase or glutathione peroxidase, in these patients. Furthermore, chain breaking antioxidants in seminal plasma may be associated with reactive oxygen species. Because of their short life span, which is a consequence of their highly reactive properties, the source of seminal reactive oxygen species must be cellular in nature.
infertility compared to 35% among men with primary infertility, supporting the hypothesis that varicocele can exert a progressive deleterious effect on fertility. It is possible that the infertility in some of our subjects may be secondary to imbalances between reactive oxygen species production and total antioxidant capacity, and not directly from varicocele. Our incidental varicocele group may include some subjects whose varicoceles are not truly benign with regard to fertility potential. These issues cannot be resolved yet and will require longer followup as well as further evaluation of the role of seminal antioxidants in male infertility.

Although few clinical trials have been performed to date, the results of supplementation with oral antioxidants, such as vitamin E, have been promising. Improved in vitro human spermatozoal function and pregnancy rates have been reported after antioxidant supplementation among men whose infertility was not attributed to varicocele.

CONCLUSIONS

Nonoligospermic men with varicocele had significantly elevated levels of spermatozoa reactive oxygen species. Furthermore, the concentration of seminal plasma antioxidants was lower in men with varicocele irrespective of fertility status. Seminal oxidative stress was strongly associated with varicocele and sperm dysfunction, and merits further investigation of the underlying pathophysiology in laboratory and clinical trials.

Dave Nelson, Department of Biostatistics and Epidemiology, conducted statistical analyses, and Debbie Garlak, Cheryl Fitzhugh and Karen Seifarth, Andrology Laboratory, provided technical assistance.
REFERENCES


