Semen characteristics and sperm DNA fragmentation in infertile men with low and high levels of seminal reactive oxygen species

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Objective: To examine sperm motility, total antioxidant level (TAC), DNA fragmentation, and medical history in infertile men with high seminal reactive oxygen species (ROS).

Design: Prospective study.

Setting(s): Tertiary care hospital.

Patient(s): Infertile men (n = 101).

Intervention(s): Group I (n = 57) included men with seminal ROS (<250 relative light units/sec/×10⁶ sperm) while group II (n = 44) included men with seminal ROS levels (≥250 relative light units/sec/×10⁶ sperm).

Main Outcome Measure(s): Seminal ROS, TAC, sperm DNA fragmentation, ROS/TAC score were measured.

Result(s): Group II had a higher incidence of sperm DNA fragmentation than group I. The odds ratio of 1.25 for elevated ROS levels corresponded to >10% greater DNA fragmentation in our patients (95% confidence interval 1.01–1.53). Group II showed poor motility, a higher incidence of leukocytospermia, and higher ROS-TAC scores compared with group I. ROS was negatively correlated with sperm curvilinear velocity (r = −.24), linearity (r = −.24), and sperm motility (r = −.31). Sperm motility was correlated with %TUNEL+ve sperm (r = −.39).

Conclusion(s): An increase in seminal ROS levels by 25% was associated with a 10% increase in sperm DNA fragmentation. Sperm motility was affected by seminal ROS and sperm DNA fragmentation. (Fertil Steril® 2010;94:2141–6. ©2010 by American Society for Reproductive Medicine.)

Key Words: Sperm motility, sperm motion kinetics, male infertility, sperm DNA fragmentation, oxidative stress, reactive oxygen species, total antioxidant capacity

Basal levels of reactive oxygen species (ROS) are essential for physiologic sperm functions (1, 2). However, high seminal ROS levels lead to oxidative stress (OS), which in turn, can reduce sperm motility, viability, and fertilizing potential and increase sperm DNA fragmentation rates (3–7). Some investigators report high ROS levels to be an independent marker for male factor infertility (3).

Introducing seminal ROS measurement in clinical andrology laboratory services may help clinicians in accurate diagnosis of their patients such as idiopathic cases or other diagnostic conditions associated with oxidative stress induced sperm damage (6, 8–10). New OS scores (such as ROS-total antioxidant level [TAC] score) have been added to easily diagnose infertile patients with OS; however, seminal ROS still remains the main parameter for measuring OS (11). High levels of seminal ROS may be an important indicator of further deterioration of sperm quality in men with OS.

In a preliminary study (unpublished data) we observed all seminal ROS values in the healthy donors (proven and unproven healthy) were <250 relative light units (RLU)/sec/×10⁶ sperm. We therefore took this cutoff to differentiate infertile men with values >250 RLU/sec/×10⁶ sperm and categorize these patients with high seminal ROS that are vulnerable to OS-induced sperm damage. The objective was to compare infertile men with normal and high levels of ROS in seminal ejaculates (as determined from unpublished data from our group) and examine their sperm parameters and clinical information.

MATERIALS AND METHODS

Subjects Selection

This prospective study was approved by the institutional review board of our hospital. Our patient population consisted of 101 infertile patients who attended our fertility clinic between 2008 and 2009 for male factor fertility. All patients underwent semen analysis according to World Health Organization (WHO) guidelines (12).

Semen Collection and Preparation

Semen specimens were collected and analyzed as per WHO 1999 guidelines. Briefly, 5 μL of liquefied specimen was loaded on a 20-μL Cell-Vu chamber (Millennium Sciences, Inc., New York, NY). Leukocytospermia was confirmed (using the Endtz test) in any specimen showing round cells >0.2 × 10⁶ white blood cells/mL of semen (13, 14). Sperm motion characteristics such as linearity (%) and curvilinear velocity (VCL, μm/sec) were examined by computer assisted sperm analysis (CASA, IVOS version 10, Hamilton Thorne, Inc., Beverly, MA) (15).

The remaining seminal ejaculate was divided into three aliquots for further use. One aliquot was used to measure seminal ROS levels. The second aliquot was used to assess DNA damage via the TUNEL (terminal dUTP nick-end labeling) assay. Spermatozoa were fixed and kept in 70% ethanol at −20°C.
for the TUNEL assay. The third aliquot was centrifuged at 1,000 × g for 7 minutes, and clear seminal plasma was aliquoted and stored at −70°C for the measurement of the total antioxidant capacity (TAC).

**Measurement of ROS**

Seminal ejaculates that had not undergone any additional processing (neat samples) were used for ROS measurement by chemiluminescence assay. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) was used as a probe. A 100-nmol/L stock solution of luminol was prepared in dimethyl sulfoxide. For the analysis, 10 μL of the working solution (5 mM) was added to 400 μL of a neat sperm sample (16 × 10⁶). Chemiluminescence was measured for 15 minutes using a luminometer (Autotumat LB 953; Berthold Technologies, LLC, Oak Ridge, TN). The results were expressed as RLU/sec × 10⁶ sperm.

Patients were divided into two groups based on their ROS levels. Group I consisted of patients with normal levels of seminal ROS (<250 RLU/sec × 10⁶ sperm; n = 57), which was used as a control group, and group II consisted of patients with high levels of seminal ROS (>250 RLU/sec × 10⁶ sperm; n = 44). This cutoff point was derived from an unpublished study performed by our group that showed all seminal ROS values in healthy donors (proven and unproven healthy) were <250 relative light units (RLU/sec × 10⁶ sperm).

**Total Antioxidant (TAC) Assay**

The TAC of the seminal plasma samples was measured using an antioxidant assay kit (Cayman Chemical Company, Ann Arbor, MI). Its principle is based on the ability of aqueous- and lipid-based antioxidants in seminal plasma to inhibit oxidation of the ABTS (2,2’-Azino-di-[3-ethylbenzthiazio-line sulphonate]) to ABTS⁺. Under the reaction conditions used, the antioxidants in the seminal plasma suppress absorbance at 750 nm to a degree that is proportional to their concentration. The capacity of the antioxidants in the sample to prevent ABTS oxidation was compared with that of Trolox, a water-soluble tocopherol analogue, and the results were reported as micro-sample antioxidant capacity.

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**Calculation of ROS-TAC Score**

The principal component (PC) was used to calculate the ROS-TAC score for all patients. This was derived from their seminal ROS and seminal plasma TAC values (19) with some modifications.

\[
\text{ROS - TAC} = 50 + (10/0.99672) \times (-0.707 \times \text{StdLogROS} + 0.707 \times \text{StdTAC})
\]

where \( \text{LogROS} = \log(\text{ROSRLU + 5}) \)

\[
\text{StdLogROS} = \text{Standardized Log ROS} = (\text{LogROS} - 2.3814)/(1.2357)
\]

\[
\text{StdTAC} = \text{Standardized TAC} = (\text{TAC} - 1976.56)/(641.68)
\]

**Sperm DNA Fragmentation**

Sperm DNA fragmentation was evaluated with the terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling (TUNEL) assay using Apo-Direct kit (Pharmingen, San Diego, CA) as established earlier (20, 21). Briefly, 1 to 2 million spermatozoa were washed in phosphate-buffered saline (PBS), resuspended in 3.7% paraformaldehyde with adjustment of the concentration to 1–2 × 10⁶ sperm/mL, and placed on ice for 30 to 60 minutes at 4°C. Thereafter, spermatozoa were again washed to remove the paraformaldehyde and then resuspended in 70% ice-cold ethanol. Specimens were kept at −20°C until the run time. In addition, we also included an internal test of samples that were tested negative or positive for DNA damage with each run. Following a second wash in PBS to remove the ethanol, sperm pellets were resuspended in 50 μL of the freshly prepared staining solution for 60 minutes at 37°C. According to the manufacturer’s instructions, the staining solution contains terminal deoxynucleotidyl transferase (TdT) enzyme, TdT reaction buffer, fluorescein isothiocyanate tagged deoxyuridine triphosphate nucleotides (FITC-dUTP), and distilled water. All specimens were further washed in rinse buffer to remove the unbound reaction solution, resuspended in 0.5 mL of propidium iodide/RNase solution, and incubated for 30 minutes in the dark at room temperature. Flow cytometric analysis was then performed. The percentage of positive cells (TUNEL⁺ve) was calculated on a 1023-channel scale using the flow cytometer software (FlowJo Mac version 8.2.4, FlowJo, LLC, Ashland, OR) (22).

**Review of Medical Charts and Related Parameters**

A complete medical history and clinical examination were performed for every patient. Their medical charts were reviewed for age, body mass index (BMI), primary or secondary infertility, and duration of infertility. A history of antioxidant and/or antibiotic prescription use was also verified.

**Statistical Analysis**

The differences in distributions of OS-induced damage markers and patient characteristics between patients with ROS levels below versus above 250 RLU/sec/10⁶ sperm were assessed using Wilcoxon rank sum and chi-square tests. Summaries of the distributions—mean, standard deviation, median, and interquartile ranges—were examined. Spearman correlations were used to assess associations between ROS levels and other measured parameters. Statistical significance was considered when \( P < .05 \). All analyses were performed using R version 2.3.1 (www.r-project.org) (23).

**RESULTS**

**The OS-Induced Sperm Damage Markers**

There were no significant differences between group I (normal seminal ROS levels) and group II (high seminal ROS levels) regarding seminal plasma TAC levels (\( P = .43 \)). However, group I had a significantly higher ROS-TAC score (\( P < .001 \)) than group II. There was a higher incidence of sperm DNA fragmentation expressed as %TUNEL⁺ve in group II (\( P = .036 \)) (Table 1). The odds ratio of elevated ROS corresponding to a 10% greater DNA fragmentation was estimated to be 1.25 (95% confidence interval [CI] 1.01–1.53).

**Semen Parameters**

Abstinence time (\( P = .32 \)), ejaculate volume (\( P = .35 \)), semen pH (\( P = .71 \)), and normal sperm morphology by WHO (\( P = .63 \)) and strict Kruger criteria (\( P = .41 \)) were comparable between the two patient groups.

The percentage of motile sperm (\( P = .001 \)), VCL μm/sec (\( P = .005 \)), and linearity (\( P = .026 \)) were all lower in group II (Table 1). The incidence of leukocytospermia was higher (73.9%) in group II (Table 2) versus group I (26.1%; \( P = .004 \)).

**Patient-Related Parameters**

There were no significant differences between infertile patient groups with regard to patient age, height, weight, or BMI. No significant differences were observed in infertile men in the two groups in regard to duration or type of infertility (Table 1).

Antibiotics were prescribed in 11 (19.3%) patients in group I versus 15 (34.1%) of patients group II (\( P = .1 \)). Antibiotic supplementation were recommended (\( P = .43 \)) and interquartile ranges—were examined. Spearman correlations were used to assess associations between ROS levels and other measured parameters. Statistical significance was considered when \( P < .05 \). All analyses were performed using R version 2.3.1 (www.r-project.org) (23).

**Correlation Studies**

As a secondary analysis, spearman correlation showed positive correlations between seminal ROS levels and the Endtz test...
(r = .41, P < .001) and with sperm DNA fragmentation (%TUNEL
tve) (r = .21, P = .06). ROS levels were negatively correlated with VCL (r = -.24, P = .021), linearity (r = -.24, 
P = .017) and sperm motility (r = -.31, P = .002). Sperm motility showed negative correlation with %TUNEL
ve (r = -.39, P < .001; Fig. 1). Negative correlations were observed between seminal ROS values with ROS-TAC score (r = -.71, P < .001). The ROS-TAC score was negatively correlated with levels of ROS in all patients (r = -.7, P < .001), group I patients with seminal ROS > 250 (r = -.39, P = .013), and in group II patients with seminal ROS < 250 (r = -.30, P = .03) (Fig. 2).

**DISCUSSION**

Our understanding of semen characteristics and patient characteristics associated with high seminal ROS generation is poor. Therefore, our study aimed to compare sperm DNA fragmentation, OS markers, and other routine infertility parameters in men with normal versus high seminal ROS generation. In this study, we only enrolled infertile patients who were seeking medical help for male factor related fertility issues.

The patients’ ages, infertility type/duration, and BMI had no effect on seminal ROS values. The two patient groups were comparable in terms of abstinence, semen pH, ejaculate volume, and other semen parameters.

**TABLE 1**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group I (ROS &lt; 250)a</th>
<th>Group II (ROS ≥ 250)b</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>36.53 ± 7.07</td>
<td>37.33 ± 8.25</td>
<td>.77</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>177.69 ± 8.73</td>
<td>179.08 ± 9.70</td>
<td>.49</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>90.98 ± 17.43</td>
<td>92.46 ± 16.79</td>
<td>.44</td>
</tr>
<tr>
<td>Body mass index (BMI)</td>
<td>28.81 ± 4.96</td>
<td>28.42 ± 3.94</td>
<td>.99</td>
</tr>
<tr>
<td>Infertility type</td>
<td></td>
<td></td>
<td>.51</td>
</tr>
<tr>
<td>Primary</td>
<td>33 (61.1%)</td>
<td>24 (54.5%)</td>
<td></td>
</tr>
<tr>
<td>Secondary</td>
<td>21 (38.9%)</td>
<td>20 (45.5%)</td>
<td></td>
</tr>
<tr>
<td>Infertility duration (y)</td>
<td>2.18 ± 2.04</td>
<td>2.38 ± 2.28</td>
<td>.68</td>
</tr>
<tr>
<td>Abstinence time (d)</td>
<td>3.45 ± 1.33</td>
<td>3.79 ± 1.68</td>
<td>.32</td>
</tr>
<tr>
<td>Semen pH</td>
<td>7.63 ± 0.25</td>
<td>7.65 ± 0.22</td>
<td>.71</td>
</tr>
<tr>
<td>Ejaculate volume (mL)</td>
<td>2.88 ± 1.45</td>
<td>3.26 ± 1.68</td>
<td>.35</td>
</tr>
<tr>
<td>Sperm Motility (%)</td>
<td>62.56 ± 22.87</td>
<td>45.21 ± 28.22</td>
<td>.001</td>
</tr>
<tr>
<td>Curvilinear velocity (VCL) µm/sec</td>
<td>42.00 ± 8.21</td>
<td>37.76 ± 12.28</td>
<td>.005</td>
</tr>
<tr>
<td>Linearity (%)</td>
<td>49.45 ± 9.24</td>
<td>46.15 ± 9.77</td>
<td>.026</td>
</tr>
<tr>
<td>WHO normal morphology (%)</td>
<td>15.33 ± 10.69</td>
<td>14.30 ± 10.99</td>
<td>.63</td>
</tr>
<tr>
<td>Strict criteria normal sperm (%)</td>
<td>2.82 ± 3.24</td>
<td>2.24 ± 2.54</td>
<td>.41</td>
</tr>
<tr>
<td>Total antioxidant capacity (TAC) (µmol trolox equivalent/mL)</td>
<td>2022.81 ± 696.68</td>
<td>1916.78 ± 565.55</td>
<td>.43</td>
</tr>
<tr>
<td>ROS-TAC score</td>
<td>53.96 ± 8.46</td>
<td>26.05 ± 10.02</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>
| Sperm TUNEL
tve %             | 22.97 ± 19.12        | 3.83 ± 25.80          | .055    |

Note: Results are presented as mean ± standard deviation (SD), frequency (%). Associations with categorical variables were assessed by chi-square tests. Associations with quantitative and ordinal variables were assessed by Wilcoxon rank sum tests.

a Group I: n = 57, 56.4%.

b Group II: n = 44, 43.6%.


**TABLE 2**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group I (ROS &lt; 250)a</th>
<th>Group II (ROS ≥ 250)b</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytospermia (ENDTZ test) (x 10⁶ wbc/mL of semen)</td>
<td>0.05 ± 0.15</td>
<td>0.64 ± 1.39</td>
<td>.001</td>
</tr>
<tr>
<td>Antibiotic prescription</td>
<td></td>
<td></td>
<td>.10</td>
</tr>
<tr>
<td>No</td>
<td>46 (80.7%)</td>
<td>29 (65.9%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (19.3%)</td>
<td>15 (34.1%)</td>
<td></td>
</tr>
<tr>
<td>Antioxidant supplementation</td>
<td></td>
<td></td>
<td>.041</td>
</tr>
<tr>
<td>No</td>
<td>40 (71.4%)</td>
<td>22 (51.2%)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>16 (28.6%)</td>
<td>21 (48.8%)</td>
<td></td>
</tr>
</tbody>
</table>

Note: Results are presented as mean ± standard deviation (SD), frequency (%). Associations with categorical variables were assessed by chi-square tests. Associations with quantitative and ordinal variables were assessed by Wilcoxon rank sum tests.

a Group I: n = 57, 56.4%.
b Group II: n = 44, 43.6%.

percentage of normal morphologically spermatozoa, and seminal TAC. These findings agree with previous findings suggesting that routine sperm parameters overlap between infertile and fertile men (24), indicating that seminal ROS is an independent parameter for male infertility (3). The patient groups had comparable levels of seminal plasma TAC. However, the ROS-TAC score demonstrated OS conditions in the infertile men with high seminal ROS levels. This agrees with our earlier reports recommending seminal ROS measurement as a routine test for infertile men (25) and the use of the ROS-TAC score over the use of just ROS or TAC alone in these patients (19, 26).

Infertile men with high seminal ROS levels had a lower percentage of motile sperm, VCL, and linearity (27). This can be explained by the disturbance of the energy supply that may occur following a disturbed mitochondrial membrane potential, which in turn, causes high levels of ROS to be released into the cytoplasm and depletion of the energy supply that affects both sperm motility and kinetics.

Our study provides evidence for OS-induced sperm DNA damage in infertile men with high seminal ROS levels. Infertile men may have high sperm DNA fragmentation that can be explained by the presence of additional risks factors with OS-associated conditions such as varicocele or other lifestyle issues as reported earlier (1, 28–30).

Although several recent reports have attempted to define levels of sperm DNA fragmentation in infertile men, additional studies are needed before the tests that measure this parameter can be recommended for routine clinical use in infertile men (31, 32). Seminal plasma TAC was comparable in both groups of our study, despite the difference in seminal ROS levels. This may be related to whether or not multivitamin/antioxidants supplements were prescribed. Furthermore, these findings also indicate that seminal TAC may vary during this compensatory phase in an attempt to scavenge the increasing levels of ROS. In an earlier study, we reported TAC cutoff values, in this study all patients had seminal plasma TAC levels within normal reference range (18). This finding confirms our above assumption indicating that infertile men with TAC levels below 1,420 μM trolox equivalent/mL may have depleted antioxidant capacity and are therefore more vulnerable to OS-induced damage. With seminal TAC below 1,420 μM trolox equivalent/mL, OS-induced sperm damage may occur even with low seminal ROS values. This again points to the importance of calculating the ROS-TAC score for these patients as it is more reflective of the actual OS status. Furthermore, our current study findings also point to the need to develop new OS markers and scores (such as ROS-TAC) that are more sensitive and account for more than one parameter of OS status. This also is in agreement with a previous report (8).

The high incidence of leukocytospermia in the infertile men with high seminal ROS levels may represent an additional cause of low sperm quality. Antibiotic supplementation may not be effective in normalizing ROS generation or protect against further deterioration in sperm quality. Doxycycline has been reported to induce sperm damage with in vitro treatment (33, 34). Moreover, the negative relationships of seminal ROS with sperm motility and its kinetics in all patients confirm that sperm damage occurred as a result of high ROS generation in these infertile men.

Our study showed a positive relationship between seminal ROS and leukocytospermia and sperm DNA fragmentation. We recommend examining infertile patients for leukocytospermia and for sperm DNA integrity to improve their clinical management.

Because our study was limited to diagnose the presence or absence of ROS, we recommend follow-up studies to develop new antibiotic/antioxidant strategies for these patients. Also, we did not further classify our patients based on their clinical diagnoses. We recommend more studies on specific clinical diagnosis and specific.
age groups to identify the seminal plasma reference values in those specific groups.

In conclusion, infertile men with high seminal ROS levels have high incidence of sperm DNA fragmentation. An increase of seminal ROS by 25% may be associated with a 10% increase in sperm DNA fragmentation. The percentage of total motile spermatozoa was negatively related to seminal ROS and sperm DNA fragmentation. The ROS-TAC score was better than ROS or TAC values alone thereby differentiating infertile men with OS from those without OS. Novel OS markers should be developed to help better identify infertile men with DNA damage. Newer strategies are required for male infertility oxidative stress management. We recommend establishing reference values for diagnostic/prognostic use of oxidative stress markers in andrology that may lead to more optimal treatments for infertile men with OS-induced sperm damage.

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REFERENCES


