Oxidative stress indices in seminal plasma, as measured by the thermochemiluminescence assay, correlate with sperm parameters

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Objective: To examine the effect of oxidation of proteins and lipids, as measured by a novel thermochemiluminescence (TCL) analyzer, and to evaluate the correlation between TCL indices in seminal plasma and sperm parameters.

Design: Experimental and prospective clinical studies.

Setting: An infertility unit.

Patient(s): One hundred forty-eight men undergoing semen analysis.

Intervention(s): Bovine serum albumin (BSA) and linolenic acid were oxidized and tested by TCL, protein carbonyls, and conjugated dienes assays. All participants underwent semen analysis. Seminal plasma was tested by TCL and conjugated dienes.

Main Outcome Measure(s): Thermochemiluminescence indices before and after oxidation of BSA and linolenic acid, compared with protein carbonyl and conjugated dienes indices. Correlation between semen parameters and TCL and conjugated dienes indices in seminal plasma.

Result(s): Oxidation of BSA and linolenic acid was marked by characteristic changes in their TCL curve pattern and an increase in the levels of protein carbonyls and conjugated dienes. Among 125 sperm-containing semen samples, the TCL curve exhibited two patterns: a positive relative ratio curve (group A, 87 patients) and a negative relative ratio curve (group B, 38 patients). Sperm concentration was lower and total motile sperm and rapid motile sperm were fewer in group B. A significant correlation was found between TCL indices, conjugated dienes, and sperm quality in group B.

Conclusion(s): Oxidation affects TCL curve pattern of proteins and lipids in a characteristic manner. Thermochemiluminescence indices in seminal plasma closely correlate with sperm characteristics among patients with sperm disturbances, and it might serve as a tool in the evaluation, treatment, and monitoring of subfertile men.

Key Words: Seminal plasma, sperm, oxidative stress, electronically excited species, thermochemiluminescence, protein carbonyls, conjugated dienes

Oxidative stress has been associated with various physiologic and pathologic processes, including aging, and might affect reproductive outcome (1). Recent data imply that, whereas high levels of oxidative stress might be detrimental, a certain amount of reactive oxygen species (ROS) is needed for normal sperm function (2, 3). Whereas free radicals synthesized by mammalian spermatozoa from oxygen (4) might be toxic to sperm function (5–7), seminal plasma has been shown to have some protective effect from oxidative damage (8, 9). Kolettis et al. (10) found that ROS combined with total antioxidant capacity could predict fertility in men after vasectomy reversal.

The principle of thermochemiluminescence (TCL) assay is based on the heat-induced oxidation of biological fluids (11), which leads to...
the formation of electronically excited species in the form of unstable carboxyls, which further decompose into stable carboxyls and light energy (low chemiluminescence) in the wavelength range of 300–650 nm.

During analysis, TCL reading indicates formation of unstable carbonyl fragments in the tested sample, which expresses its oxidizability. Thermochemiluminescence kinetic curve patterns reflect residual oxidative capacity due to prior in vivo molecular oxidation (i.e., a higher TCL curve slope represents a higher oxidative potential of the sample, which indicates lower oxidative activity before the test).

The aims of this study were to understand, in an experimental model, the possible effect of oxidation on proteins and lipids as measured by TCL assay and to evaluate possible relationships between TCL results and sperm parameters.

MATERIALS AND METHODS

Experimental Model Measurements

Bovine serum albumin (BSA), 10 mg/mL (Sigma, St. Louis, MO), was oxidized by Fe 3+ and vitamin C assay (12) and measured by TCL and protein carbonyl (13) before and after oxidation. In the protein carbonyl assay, BSA was added to 5 mL of 2.5 mmol/L dinitrophenylhydrazine, dissolved in 2 mol/L hydrochloric acid (HCl), and incubated for 1 hour. After the dinitrophenylhydrazine reaction, proteins were precipitated with an equal volume of 20% (wt/vol) trichloroacetic acid, and the pellets were washed once with 4 mL of 10% (wt/vol) trichloroacetic acid and three times with 4 mL of an ethanol/ethyl acetate mixture (1:1).

Washings were achieved by mechanical disruption of the pellets in the washing solution with a small spatula and repelleting by centrifugation at 6,000 × g for 5 minutes. Finally, the precipitates were dissolved in 6 mol/L guanidine–HCl solution, and the absorbance peak at 365 nm was determined by spectral scanning at the range of 320–400 nm.

Protein contents were determined on the HCl blank pellets with a BSA standard curve in guanidine–HCl and reading the absorbance at 280 nm. Linolenic acid, 0.5 mmol/L, was oxidized with an oxidation assay described by Kanner and Lapidot (14) and measured by TCL and conjugated dienes (spectrophotometric technique) before and after oxidation (15).

Clinical Study

One hundred forty-eight men undergoing semen analysis at the Carmel Medical Center during 2001–2002 were included in the study. The study was approved by the institutional review board, and upon entering the study every patient provided written informed consent. Fresh ejaculated semen was centrifuged for 10 minutes at 1,800 rpm to obtain seminal plasma, of which 50 μL was separated and tested for electronically excited species in the TCL analyzer and another sample was tested for conjugated dienes (15). After centrifugation, all semen samples were manually counted in a Mackler counting chamber (Sefi Medical Instruments, Rehovot, Israel) and analyzed with the use of prestained diagnostic slides (Testimplets; Roche Diagnostics, Lewes, United Kingdom) according to the criteria of the World Health Organization Laboratory Manual, 4th edition (2000).

Measurements of TCL

The TCL analyzer (Lumitest, Haifa, Israel) is managed and controlled by an embedded personal computer (Fig. 1). The TCL analyzer consists of two blocks: a sample preparation block and an analysis block. Tested fluid samples of 50 μL are distributed over the surface of a cylindrical aluminum tray (dimensions: diameter, 19 mm; height, 3 mm; thickness, 0.12 mm), which is positioned inside the sample preparation block, tightly sealed, and then vacuum-dried (U.S. patent number US 6,372,508 B1). The sample is then transferred to the analysis block, where the dish is mounted on a preheated oven with a constant temperature heater of 80°C ± 0.2°C.

Sequential photon counting in counts per second is performed by an electronic cathode tube (R6095P photo multiplier; Hamamatsu Photonics, Shizuoka, Japan) with spectral response in the wavelength range of 300–650 nm for 300 seconds. Total test time lasts <12 minutes. Photon readings
are depicted on a kinetic curve, which can be described mathematically by amplitude and slope. The thermochemiluminescence curve is a computerized trend line of individual points, each of which represents the mean photon count during 1 second (11).

The TCL relative ratio (RR) represents the relative change in TCL amplitude during the test. An RR >0 was defined as positive, whereas an RR <0 was defined as negative.

$$\text{TCL RR (\%) = } \frac{\Delta \text{(TCL amplitude}_{280 \text{ sec}} - \text{TCL amplitude}_{50 \text{ sec}}) \times 100}{\text{TCL amplitude}_{50 \text{ sec}}}$$

The rationale for selection of the 50- and 280-second amplitudes for the calculation of the RR was based on empiric observations. During the first 50 seconds, the heated sample underwent a “stabilization phase,” marked by a high variation (low reproducibility) in photon readings.

Once 50 seconds had elapsed, photon readings were marked by low variation. The period between 50 and 280 seconds produced the most significant changes in photon reading and were highly reproducible. Beyond 280 seconds, photon readings did not produce any additional meaningful information.

**Statistical Analysis**

Statistical analysis with linear regression analysis and Student’s t-test was carried out with a software package (SPSS, Chicago, IL). \(P < .05\) was considered significant.

**RESULTS**

**Experimental Model**

The basal TCL curve pattern of BSA showed a gradual increase in TCL amplitude from 50 to 280 seconds during heating (Fig. 2A-1). After oxidation, TCL amplitude at 50 seconds increased compared with the preoxidation level and then gradually decreased until 280 seconds (Fig. 2B-1). The basal TCL curve pattern of linolenic acid showed relatively stable TCL amplitude in the range of 50–280 seconds (Fig. 2A-2). After oxidation, the TCL curve pattern changed in a manner similar to that of BSA (Fig. 2B-2). Concomitantly, the levels of protein carbonyls and conjugated dienes increased significantly (\(P < .001\) and \(P < .001\), respectively)
after oxidation of BSA and linolenic acid, respectively (Table 1).

**Clinical Study**

Mean patient age was 32.2 years (SD = 6.65 years; range: 22–62 years). One hundred twenty-five semen samples (85.5%) contained spermatozoa. The TCL curve pattern of 87 samples (group A) was similar to that of BSA and linolenic acid before oxidation (positive TCL RR). A mean TCL curve of seminal plasma from group A patients is shown in Figure 2A–3. Thirty-eight samples (group B) had a TCL curve pattern similar to that of BSA and linolenic acid after oxidation (negative TCL RR). A mean TCL curve of seminal plasma from group B patients is shown in Figure 2B–3.

Mean sperm concentration was significantly lower and total motile sperm and rapid motile sperm were significantly fewer in group B (Table 2). Among the total population of patients (n = 125), the RR parameter correlated with total sperm motility (r = 0.27, P < .007) but not with sperm concentration. An inverse correlation was found between RR and TCL amplitude at 50 seconds.

No correlation was found between TCL amplitude at 50 seconds and sperm parameters. Among group B patients (negative RR, n = 38), RR levels correlated with sperm concentration (r = 0.45, P < .03) and total motility (r = 0.55, P < .007) parameters, whereas TCL amplitude at 50 seconds exhibited an inverse correlation to sperm concentration (r = −0.49, P < .01) and total motility (r = −0.56, P < .03) (Table 3). Among group B patients (negative RR), the levels of conjugated dienes in seminal plasma correlated with TCL amplitude at 50 seconds (r = 0.53, P < .04) and inversely correlated with RR levels (r = −0.61, P < .01).

White blood cells (WBC) were present in 61 (49%) of the samples (0.1–8 × 10⁶/mL). The prevalence of WBC in the samples among group A and B patients was 54% (47 of 87) and 36.8% (14 of 38), respectively. Mean WBC count was greater in group B (1.24 ± 0.9 × 10⁶) than in group A (0.77 ± 0.59 × 10⁶, P < .02). Among the total population (n = 125), an inverse correlation was found between RR and WBC count, (r = −0.63, P < .005), whereas TCL amplitude at 50 seconds correlated with WBC count (r = 0.485, P < .005) (Table 3).

**TABLE 1**

Changes in TCL indices and markers of oxidation in BSA and linolenic acid after oxidation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TCL amplitude at 50 sec (cps)</th>
<th>TCL amplitude at 280 sec, (cps)</th>
<th>TCL RR (%)</th>
<th>Mean protein carbonyls (nmol/mL) (n = 6)</th>
<th>Mean conjugate dienes (nmol/mL) (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA (10 mg/mL)</td>
<td>482 ± 26</td>
<td>544 ± 34</td>
<td>13 ± 1.1</td>
<td>1.31 ± 0.07</td>
<td>+33.5</td>
</tr>
<tr>
<td>BSA after oxidation</td>
<td>1211 ± 57</td>
<td>1040 ± 43</td>
<td>−14 ± 1.4</td>
<td>1.75 ± 0.09</td>
<td>+33.5</td>
</tr>
<tr>
<td>% change</td>
<td>+151</td>
<td>+91</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>P &lt; .001</td>
<td>P &lt; .001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linolenic acid (0.5 mmol/L)</td>
<td>616 ± 36</td>
<td>614 ± 32</td>
<td>0.32</td>
<td>21 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Linolenic acid after oxidation</td>
<td>798 ± 23</td>
<td>705 ± 18</td>
<td>−11 ± 1.3</td>
<td>29 ± 1.8</td>
<td>+38</td>
</tr>
<tr>
<td>% change</td>
<td>+29</td>
<td>+15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significance</td>
<td>P &lt; .01</td>
<td>P &lt; .01</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2**

Comparison of TCL amplitude at 50 seconds and sperm parameters between group A (positive TCL RR) and group B (negative TCL RR).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A (n = 87)</th>
<th>Group B (n = 38)</th>
<th>% change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean TCL amplitude at 50 sec (cps)</td>
<td>551 ± 101</td>
<td>831 ± 231</td>
<td>+50.8</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>Mean sperm concentration (10⁶/mL)</td>
<td>18.37 ± 27.88</td>
<td>3.930 ± 8.74</td>
<td>−78.7</td>
<td>P &lt; .02</td>
</tr>
<tr>
<td>Mean motile sperm (%)</td>
<td>32.92 ± 27.55</td>
<td>31.30 ± 23.40</td>
<td>−40.9</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>Mean rapid motile sperm (%)</td>
<td>25.6 ± 17.96</td>
<td>11.90 ± 11.60</td>
<td>−53.6</td>
<td>P &lt; .001</td>
</tr>
</tbody>
</table>

**Note:** Values are expressed as mean ± SD. cps = counts per second.

No correlation was found between TCL parameters (TCL amplitude at 50 seconds and TCL RR) and patient FSH levels ($r = -0.249$, $P = .116$ and $r = 0.173$, $P = .279$, respectively; mean FSH: $11.7 \pm 8.9$ IU/L) and between TCL parameters and the fraction of abnormal spermatozoa ($r = 0.041$, $P = .834$, $r = 0.109$, $P = .578$, respectively; mean fraction of abnormal spermatozoa: $91\% \pm 7\%$), either in the total population or in the selected groups.

## DISCUSSION

In this study we evaluated the oxidizability of seminal plasma by a novel technique, the TCL assay. Thermochemiluminescence results, in experimental models of BSA and linolenic acid as well as in seminal plasma, correlated with those of known oxidation assays—protein carbonyls and conjugate dienes. The TCL curve pattern of BSA before oxidation resembled that of seminal plasma samples of better sperm parameters (a positive ratio TCL curve).

Similarly, postoxidative TCL curve patterns of BSA and linolenic acid fitted with the curves of seminal plasma samples of sperm with lower parameters (a negative TCL ratio curve). These observations might indicate that a negative TCL curve pattern in seminal plasma reflects a reduced postoxidative capacity to undergo further oxidation during the TCL test.

Our finding that the highest correlation was between TCL indices and a subgroup of patients with lower sperm quality is supported by the existing literature regarding the adverse effects of oxidative stress on sperm function (10, 16). Additionally, the correlation that was found between TCL indices and WBC count in the samples is in concordance with other works (17), because granulocytes are major producers of ROS in semen. Only 36.8% of semen samples from group B patients contained WBC (although the mean WBC count was higher compared with group A patients).

Therefore, it is possible that oxidative stress, which is associated with sperm functional disturbances, might also stem from sources other than granulocytes. In a future clinical model that has yet to be validated, seminal plasma samples could undergo initial screening according to the TCL curve pattern. A negative TCL ratio curve should be interpreted as an indicator of oxidative stress. In these cases, the intensity of oxidative stress could be gained from the actual levels of TCL ratio and amplitude at 50 seconds. These data might serve as a monitor to evaluate the effects of occupational hazards on sperm quality and optimize antioxidant treatment. Future studies are needed to validate our results and correlate them with clinical outcome parameters, such as fertilization and pregnancy rates.

Oxidative stress plays a role in sperm physiology (5). The most prevalent ROS, hydrogen peroxide (H$_2$O$_2$) is synthesized from O$_2$ by mammalian spermatozoa (4) by a two-stage reduction of superoxide (O$_2^{-}$) by H$^+$ as an intermediate product (18). Oxidative stress might cause sperm functional disturbances by several mechanisms, such as [1] lipid peroxidation, which might cause a substantial loss of cell membrane unsaturated fatty acids, thus leading to reduced membrane fluidity and a functional defect in sperm-oocyte fusion and fertilization (19, 20), [2] reduced activity of membrane-bound Ca$^{2+}$ regulatory enzymes (21), which might impair sperm motility (19, 21–23), and [3] a loss of intracellular adenosine triphosphate, possibly via a direct toxicity on glycolytic enzymes (3), which might reduce sperm motility (24, 25). Free radical activity in seminal plasma was associated with antisperm antibodies (26, 27).

Nevertheless, ROS are essential to mammalian sperm function, including capacitation, acrosome reaction, sperm-oocyte fusion (2, 28), and the stimulation of hyperactivated sperm motility (29–31).

This emphasizes the great need for an applicable tool for rapid, easy, real-time evaluation of oxidative stress in seminal plasma. The existing methods for the measurement of oxidative stress (luminol-induced chemiluminescence, nitroblue tetrazolium cytochrome c-Fe$^{3+}$ complexes [32], electron spin resonance measurement [33, 34], protein carbonyl [13], and conjugated dienes [15]) require qualified

**TABLE 3**

<table>
<thead>
<tr>
<th>Population</th>
<th>TCL parameter</th>
<th>Sperm parameter</th>
<th>Regression coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients</td>
<td>RR</td>
<td>Total motility</td>
<td>0.27</td>
<td>$P &lt; .007$</td>
</tr>
<tr>
<td>Group B</td>
<td>RR</td>
<td>Concentration</td>
<td>0.45</td>
<td>$P &lt; .03$</td>
</tr>
<tr>
<td>Group B</td>
<td>RR</td>
<td>Total motility</td>
<td>0.55</td>
<td>$P &lt; .007$</td>
</tr>
<tr>
<td>Group B</td>
<td>50-sec amplitude</td>
<td>Concentration</td>
<td>-0.49</td>
<td>$P &lt; .01$</td>
</tr>
<tr>
<td>Group B</td>
<td>50-sec amplitude</td>
<td>Total motility</td>
<td>-0.56</td>
<td>$P &lt; .03$</td>
</tr>
<tr>
<td>All patients</td>
<td>RR</td>
<td>WBC count</td>
<td>-0.63</td>
<td>$P &lt; .005$</td>
</tr>
<tr>
<td>All patients</td>
<td>50-sec amplitude</td>
<td>WBC count</td>
<td>0.485</td>
<td>$P &lt; .005$</td>
</tr>
</tbody>
</table>

*Note: All patients, n = 125; group B, n = 38.
operating personnel, expensive instrumentation, are time-consuming, and most of them record a single measurement per sample and not a dynamic process.

In addition, the use of several methods to assess oxidative stress of plasma components of smokers compared with nonsmokers has shown that the TCL method was more sensitive than all other conventional methods (35).

Thus, the TCL technology provides another dimension of very sensitive and reproducible assay for measuring oxidative stress in biological samples. Because oxidative stress might play an etiologic role in a specific fraction of male factor cases, the advantages of TCL assay, in terms of its easy operation, speed, dynamic measurements, and high sensitivity, will enable it to become a viable tool for the understanding, evaluation, and monitoring of environmental hazards and future possible antioxidant treatment among patients with severe sperm disturbances.

References