EFFECTS OF TIME AND SPERM CONCENTRATION ON REACTIVE OXYGEN SPECIES FORMATION IN HUMAN SEMEN

M. SHEKARRIZ
A. J. THOMAS, JR.
A. AGARWAL

Andrology Laboratory, Department of Urology, Cleveland Clinic Foundation, Cleveland, Ohio, USA

Although generally accepted standards exist for routine semen analysis, recent methods of assessing reactive oxygen species (ROS) in human semen lack a standardized protocol. The purpose of this study was to investigate (1) the relationship between ROS level and the time interval between semen collection and analysis, and (2) the effect of sperm concentration on the level of ROS formation. Semen specimens from men (n = 40) consulting for infertility treatment were divided in two groups: in 20, routine semen analysis was performed and ROS formation evaluated at 1, 3, 5, and 24 h after semen collection; in the other 20, ROS formation was evaluated at four sperm concentrations (60, 30, 15, and 7.5 × 10^6/mL). White blood cell (WBC) concentration was assessed before ROS measurement using a myeloperoxidase staining technique (Endtz test). ROS level was measured by a chemiluminescence method. ROS formation decreased significantly over time. The mean ROS level 343.4 (1 h), 133.5 × 10^4 cpm (3 h, p = .004), 66.0 × 10^4 cpm (5 h, p ≤ .001), and 22.2 × 10^4 cpm (24 h, p ≤ .001), respectively. In the first group of 20 specimens, 14 were positive for ROS formation at 1 h after collection, and 4 of these were positive for the Endtz test (>1 × 10^4 WBC/mL). The number of ROS-positive specimens after 3, 5, and 24 h was eight, six, and two, respectively. In the second group, eight patients were positive for ROS formation at 1 h after collection. ROS levels were significantly lower at sperm concentrations of 15 × 10^6/mL and 7.5 × 10^6/mL as compared to 30 × 10^6/mL or 60 × 10^6/mL (p < .001). The results indicate that ROS level changes significantly at various sperm concentrations prepared from the same specimen. This change emphasizes the importance of the sperm concentration as a factor when comparing ROS levels between different positive specimens or between repeated measurements. Secondly, the ROS should be measured within 1 h after semen collection to evaluate the actual incidence of ROS formation.

Keywords spermatozoa, infertility, ROS, luminescence

Idiopathic infertility is the most common diagnosis of male infertility [4]. Advances in assessing the functional properties of spermatozoa have revealed that defective sperm function is common in these patients. Furthermore, excessive generation of reactive oxygen species (ROS) is associated with impaired sperm function and fertility potential in some infertile men.
The deleterious effect of ROS on spermatozoa is mediated by lipid peroxidation of the sperm membrane; the high content of polyunsaturated fatty acids in human sperm may make it susceptible to such peroxidation [11].

Currently, chemiluminescence is used most often to assess the ROS level [9]. However, various protocols have been used to determine the level of ROS formation. Sperm washing procedures using repeated centrifugation or Percoll washing before ROS assessment can increase ROS formation in semen [3, 5]. For clinical purposes, it is important to determine which factors may substantially affect the ROS level during analysis. For example, diluting semen with buffers before ROS measurement may change the ROS level by reducing cell (spermatozoa or leukocyte) concentration. Another important variable is the time interval between semen collection and ROS analysis.

The aim of this study was (1) to determine how the time interval between semen collection and determination of ROS activity affects the ROS level in human semen and whether an association exists between the changes in ROS level and other characteristics of semen analysis, and (2) to investigate the effect of sperm concentration on the level of ROS formation.

MATERIALS AND METHODS

Selection of Subjects. Forty semen samples were selected based on sperm concentration out of 70 specimens obtained from suspected subfertile patients visiting our center for treatment. Specimens were divided into two groups based on the semen analysis results and basal ROS response. In the first group, 20 semen specimens 14/20 (70%) with a sperm concentration of at least 20 × 10⁶/mL and with a positive ROS response) were used to study the effect of time on ROS formation. The second group, consisting of 20 semen specimens with a minimum sperm concentration of 60 × 10⁶/mL was used to evaluate the effect of concentration on ROS formation. Eight out of these 20 specimens (40%) were positive for ROS formation.

Semen Collection and Assessment of Semen Characteristics. Semen specimens were collected by masturbation after at least 2–5 days of sexual abstinence and liquefied at 37°C for 30 min. Five microliters of specimen were loaded on a 20-μL Microcell chamber (Conception Technologies, San Diego, CA, USA) and analyzed on a motility analyzer (Hamilton-Thorn, HTM version 10, model: IVOS; (Hamilton-Thorn Research, Beverly, MA, USA).

Quantitation of White Blood Cells. The presence of white blood cells (WBC) in the specimens was assessed by the Endtz test [8]. A 20-μL volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial; 20 μL of phosphate-buffered saline and 40 μL of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 min. Peroxidase-positive WBCs staining dark brown were counted in all 100 squares of the grid in a Makler chamber (Sefi Medical, Haifa, Israel) under the 20× bright-field objective. The results after correction for dilution were recorded as the number of WBCs counted × 10⁶/mL. The presence of peroxidase-positive WBCs in concentrations of more than 1 × 10⁶/mL in semen specimens was considered as abnormal [14].

Assessment of ROS Activity in Semen. ROS formation was measured in all specimens within 1 h of collection by the chemiluminescence assay using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione). A 100-mmol/L luminol stock solution was prepared by dissolving 100 mg powder of luminol (Bio Orbit, Turku, Finland) with 5.64 mL of dimethyl sulfoxide (DMSO). The working solution (5 mmol/L luminol) was 1:20 with DMSO before measurement. For the analysis, 20 μL of the working solution was then added to each semen aliquot. Chemiluminescence was measured using a Berthold (Autolumat LB 953,
Wallace Incorporated, Gaithersburg, MD, USA) luminometer 10 min after adding the luminol in the integration mode at 37°C. ROS production was expressed as counted photons × 10^4 per minute (cpm). One aliquot was used to measure the background luminescence for each specimen before adding luminol. The background readings were subtracted from the actual test value to obtain the ROS level.

The ROS level was considered abnormal (a positive test) when the luminescence curve peaked 1 to 4 mins after luminol was added (Figure 1). A positive response was always associated with a value of at least 10 × 10^4 cpm in the integration mode. Therefore, based on our observations, a chemiluminescence response of 10 × 10^4 cpm was considered as the upper limit of normal.

**Measurement of Semen Characteristics and ROS Activity After Various Time Intervals.** Sperm concentration in 20 specimens (group I) was adjusted to 20 × 10^6/mL for this study using modified HTF medium (human tubal fluid, Irvine Scientific, Santa Ana, CA, USA) with human serum albumin (5.0 mg/mL). Each specimen was then divided in five aliquots of 0.5 mL each using 17 × 120-mm polystyrene tubes (Falcon, Lincoln Park, NJ, USA). The first aliquot was used to determine the background luminescence. The four remaining aliquots were used to measure ROS at 1, 3, 5, and 24 h after collection. Specimens were kept in an incubator at 37°C during the experiment. Computer-assisted semen analysis was performed before ROS measurement.

**Measurement of ROS Activity at Different Sperm Concentrations.** Semen from 20 men with a minimum sperm concentration of 60 × 10^6/mL (group II) was used to measure ROS formation at different sperm concentrations (60, 30, 15 and 7.5 × 10^6/mL). Each specimen was divided into 5 aliquots. The sperm concentration was adjusted in each aliquot with HTF solution. To measure ROS, 0.5 mL of the diluted sperm aliquot was used. In addition, a sixth aliquot (0.5 mL) at a concentration of 60 × 10^6/mL from the eight specimens that were positive for ROS formation was centrifuged at 500g for 10 min to study ROS activity in seminal plasma. The seminal plasma was transferred to polystyrene tubes for assessment of ROS activity.

![Graph](image)

**FIGURE 1** A positive ROS curve in an Endtz-positive specimen. The chemiluminescence peaks 1 to 2 min after adding luminol, indicating a positive test. The baseline curve shows the background chemiluminescence.
TABLE 1 Change in Reactive Oxygen Species (ROS) Level and Sperm Motility over Time in 20 Specimens from Patients with Suspected Subfertility and a Positive ROS Level

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>ROS Level ($\times 10^4$ cpm) Median* (Interquartile Range)</th>
<th>Motility (%) Median (Interquartile Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>29 (14.5–222)</td>
<td>40 (23–44)</td>
</tr>
<tr>
<td>3</td>
<td>8 (4.6–51)</td>
<td>34 (17–40)</td>
</tr>
<tr>
<td>5</td>
<td>3.5 (1.5–20)</td>
<td>20 (6–34)</td>
</tr>
<tr>
<td>24</td>
<td>0 (0–1.6)</td>
<td>0 (0–0)</td>
</tr>
</tbody>
</table>

*Significant by the Wilcoxon ranked-sign test at $p < .05$.

Statistical Analysis. The Wilcoxon signed-rank test was used because data were found to be not normally distributed for comparison of ROS formation at various sperm concentration and after different time intervals. The results were reported as median and interquartile range. A $p$ value of less than .05 was considered significant. All statistical analysis was performed using the SAS statistical software package version.

RESULTS

Changes in ROS Activity With Time

Fourteen specimens were initially ROS positive and of these 14 specimens, 4 were also Endtz positive ($>1 \times 10^6$ WBCs/mL). ROS formation decreased significantly overtime in all ROS-positive specimens (Table 1, Figure 2).

FIGURE 2 ROS level decreases significantly as a function of time after semen collection in all ROS-positive specimens. Background luminescence was measured for each specimen. Although ROS formation assessed within 1 h after ejaculation is highly positive, the chemiluminescence response had dropped by more than 50% at 3 h after collection. Specimens were positive.
TABLE 2 Effect of Sperm Concentration on Reactive Oxygen Species Level (ROS) in Patients with Suspected Subfertility

<table>
<thead>
<tr>
<th>Sperm Concentration (×10⁶/mL)</th>
<th>All Patients (n = 20) Median* (Interquartile Range)</th>
<th>ROS-Positive Patients (n = 8) Median (Interquartile Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>3.62 (0–197.5)</td>
<td>297.3 (38.1–491.1)</td>
</tr>
<tr>
<td>30</td>
<td>2.05 (0.39–126.5)</td>
<td>165.6 (13.7–237.4)</td>
</tr>
<tr>
<td>15</td>
<td>1.23 (0–77.6)</td>
<td>77.6 (9.9–139.2)</td>
</tr>
<tr>
<td>7.5</td>
<td>0.12 (0–28.4)</td>
<td>37.0 (21.3–46.4)</td>
</tr>
</tbody>
</table>

*The differences between all sperm concentration groups were significant (p < .005).

Relationship Between Sperm Motility and ROS Formation

Sperm motility decreased significantly over time. No significant relationship was found between the decrease in ROS level and sperm motility. Sperm motility, however, showed the greatest decline between 5 and 24 h after semen collection (Table 1). A highly significant decline in ROS formation was seen 5 h after semen collection (p < .001).

Effect of Sperm Concentration on ROS Levels

In the second group of men, out of the 20 samples, 8 were ROS positive. The ROS level decreased in all specimens with the decrease in sperm concentration. The differences were highly significant in those specimens with an initial ROS-positive response (Table 2, Figure 3).

![Figure 3](image-url) Changes in chemiluminescence response at various sperm concentrations from the same specimens. The response decreased in all ROS-positive specimens. The ROS level fell significantly in semen specimens with a sperm concentration of 60 × 10⁶/mL as compared to 7.5 × 10⁶/mL (p < .001).
Two initially positive specimens with a sperm concentration of $60 \times 10^6$/mL did not show a positive response at the concentration of $7.5 \times 10^6$/mL.

**ROS Activity of Seminal Plasma**

None of the eight specimens initially positive for ROS formation showed ROS activity in seminal plasma.

**DISCUSSION**

The results of this study indicate the importance of time and sperm concentration as variables influencing ROS assessment. The ROS levels decreased significantly at lower sperm concentrations and after longer intervals of time as compared to the initial measurement. An explanation for this finding may be the decrease in sperm motion parameters seen with time, because immotile spermatozoa do not generate ROS (unpublished data). The maximum decrease in ROS occurred during the first 5 h after semen collection and may be related to the significant decline in sperm motility seen 5 h after specimen production. A gradual decline in sperm viability may also explain the decrease in ROS levels. The reason for the decrease in ROS level in the four Endtz-positive specimens is unclear. A change in the phagocytic activity of polymorphonuclear leukocytes may be responsible for this finding [6, 12].

Assessment of ROS in human semen by a chemiluminescence technique has been performed using two principal protocols: (1) measurement in a washed suspension of spermatozoa after the seminal plasma is removed, and (2) measurement in whole semen. In initial studies, the generation of ROS was determined after the seminal plasma was removed [4]. However, repeated centrifugation was used to separate spermatozoa from the plasma, which can result in a burst of ROS formation [1, 2, 5]. Furthermore, seminal plasma contains several natural antioxidants, such as catalase and superoxide dismutase [7]. Removal of seminal plasma before analysis per se may increase the level of ROS, because application of seminal plasma to a suspension of spermatozoa or leukocytes can decrease ROS activity [10, 13]. The measurement of ROS in unprocessed semen is therefore more accurate for clinical purposes.

The ROS level changes significantly at various sperm concentrations prepared from the same specimen. This change emphasizes the importance of the sperm concentration as a factor when comparing ROS levels between different positive specimens or between repeated measurements. ROS measurement by chemiluminescence needs to be performed in whole semen using a standard protocol within 1 h after semen collection when evaluating the impact of ROS in patients with suspected infertility.

**REFERENCES**


