We also examined the differences in levels of reactive oxygen species between antegrade and retrograde specimens from men with spinal cord injury. Antegrade samples are generally preferred over retrograde samples for assisted reproductive procedures because they have higher sperm motility. Often, however, men with spinal cord injury are unable to produce antegrade samples, so a retrograde sample must be used (19). Poor sperm quality in retrograde samples may result from the hostile and variable environment of the bladder. Our study showed that retrograde samples in men with spinal cord injury had lower motility. However, levels of reactive oxygen species did not differ between antegrade and retrograde samples for either unstimulated or stimulated samples. The lack of statistical differences despite the large difference in the median values of reactive oxygen species between the antegrade and retrograde specimens was because of the large range of reactive oxygen species generated.

Early reports describing levels of reactive oxygen species in men with spinal cord injury used only electroejaculation to obtain samples (10, 11). Vibratory stimulation was not used in controls because the purpose of our study was to determine whether the levels of reactive oxygen species were different in the two methods of ejaculation (vibratory versus electroejaculation). There are no previous reports on the generation of reactive oxygen species in specimens produced by vibratory stimulation, and to our knowledge, this is the first report that examines the levels of reactive oxygen species in ejaculates produced by two different methods. We found no statistically significant differences in levels of reactive oxygen species between the methods of ejaculation. Therefore, levels of reactive oxygen species may be independent of the method of ejaculation. The high levels in specimens produced by both electroejaculation and vibratory stimulation in our study cannot be attributed to electrical current alone, as suggested by Rajasekaran et al. (11), because they only used specimens obtained after electroejaculation.

The total antioxidant capacity of the semen is reduced in infertile able-bodied men (20). Biochemical constituents of semen (alkaline phosphatase, lactate dehydrogenase, glutamic oxaloacetic transaminase, fructose) are significantly lower in specimens produced by electroejaculation than in masturbated ejaculates of normal men (21). We also have observed that seminal plasma obtained from the ejaculates of men with spinal cord injury inhibited sperm motility in control specimens (22). Whether these differences contribute to high levels of reactive oxygen species warrants further study. If antioxidant levels are low in men with spinal cord injury, antioxidant supplementation may be beneficial in maximizing semen quality (6).

In conclusion, both spermatozoa and WBCs may be responsible for high levels of reactive oxygen species seen in the semen of men with spinal cord injury. Despite higher levels of reactive oxygen species, these specimens can be used successfully in assisted reproduction.

Acknowledgment. We thank Suzanne Kohn, M.T., Andrology Laboratory, Cleveland Clinic Foundation, for technical assistance.

REFERENCES


4. Sedor JF, Hirsch I. Evaluation of sperm morphology of elec-
Table 2  Semen Characteristics in Normal Controls and Men With Spinal Cord Injury

<table>
<thead>
<tr>
<th>Semen characteristics</th>
<th>Controls</th>
<th>Spinal cord injury</th>
<th>Pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm motility (%)</td>
<td>67.0 (12.5, 83.0)</td>
<td>12.5 (0.0, 66.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Sperm count (×10⁹)</td>
<td>60.0 (16, 164.2)</td>
<td>76.9 (3.3, 338.6)</td>
<td>0.35</td>
</tr>
<tr>
<td>Concentration of WBC (×10³/mL±)</td>
<td>0.0 (0.0, 0.4)</td>
<td>5.1 (1.7, 16.3)</td>
<td>0.001</td>
</tr>
<tr>
<td>Sperm morphology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>43.0 (25.0, 61.0)</td>
<td>18.0 (0.0, 49.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Round cells (%)</td>
<td>1.0 (0.0, 18.0)</td>
<td>4.0 (0.0, 77.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Head abnormalities (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amorphous head</td>
<td>26.0 (0.0, 50.0)</td>
<td>16.0 (2.0, 30.0)</td>
<td>0.01</td>
</tr>
<tr>
<td>Tapered</td>
<td>1.0 (0.0, 5.0)</td>
<td>2.0 (0.0, 6.0)</td>
<td>0.13</td>
</tr>
<tr>
<td>Small</td>
<td>0.5 (0.0, 2.5)</td>
<td>1.0 (0.0, 8.0)</td>
<td>0.29</td>
</tr>
<tr>
<td>Large</td>
<td>1.0 (0.0, 2.0)</td>
<td>1.0 (0.0, 12.0)</td>
<td>0.13</td>
</tr>
<tr>
<td>Duplicate</td>
<td>1.0 (0.0, 2.0)</td>
<td>0.0 (0.0, 8.0)</td>
<td>0.69</td>
</tr>
<tr>
<td>Midpiece abnormalities (%)</td>
<td>0.8 (0.0, 4.0)</td>
<td>0.0 (0.0, 1.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Vaculated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coiled</td>
<td>6.5 (1.0, 20.0)</td>
<td>26.0 (8.0, 52.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Multinucleate</td>
<td>1.0 (0.0, 4.0)</td>
<td>0.0 (0.0, 3.0)</td>
<td>0.22</td>
</tr>
<tr>
<td>Missing tail</td>
<td>1.0 (0.0, 7.0)</td>
<td>2.0 (0.0, 4.0)</td>
<td>0.55</td>
</tr>
<tr>
<td>Cytoplasmic droplet</td>
<td>3.0 (0.0, 14.0)</td>
<td>0.0 (0.0, 16.0)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

* Values are medians, with range in parentheses.
† P < 0.01 compared with controls.
‡ Quantified by Enuwit test.

head, midpiece, and tail defects (P < 0.001). The incidences of cytoplasmic droplets (P < 0.005) and coiled tails were significantly higher in specimens of men with spinal cord injury (P < 0.001). Specimens from controls contained a higher percentage of amorphous head forms (P < 0.01) and vacuoles in the midpiece region (P < 0.001) (Table 2). Specimens from subjects with spinal cord injury showed no significant correlation between various sperm abnormalities and the levels of reactive oxygen species in unstimulated and stimulated samples.

Semen from subjects with spinal cord injury showed a negative correlation between sperm motility and the levels of reactive oxygen species generated in unstimulated (r = −0.49, P = 0.02) and stimulated specimens (FMLP, r = −0.46, P = 0.02; PMA, r = −0.48, P = 0.02) (Fig. 1). Total sperm count did not differ between controls and subjects with spinal cord injury. However, levels of reactive oxygen species showed a negative but nonsignificant association with sperm concentration in the subjects with spinal cord injury. A significant positive correlation was seen between the WBC concentration and levels of reactive oxygen species in both unstimulated (r = 0.79, P < 0.001) and stimulated specimens (FMLP, r = 0.73, P < 0.001; PMA, r = 0.86, P < 0.001). An association was found between an excessive WBC concentration and total generation of reactive oxygen species. A concentration of WBCs >5 × 10⁹/mL correlated significantly with values of reactive oxygen species (P = 0.02).

Antegrade specimens had significantly higher sperm motility (P < 0.001) and WBC concentration (P < 0.03) than retrograde samples. However, sperm morphology and levels of reactive oxygen species did not differ significantly in this comparison (Table 3). No significant differences were seen in sperm motility, WBC concentration, sperm morphology, or levels of reactive oxygen species between ejaculates produced in subjects with spinal cord injury by the electroejaculation and vibratory stimulation methods (Table 3). When patients with spinal cord injury were grouped according to the level of injury (cervical or thoracic), no significant differences were seen in WBC concentration, sperm morphology, or levels of reactive oxygen species.

**DISCUSSION**

Our data support previous reports showing that men with spinal cord injury have decreased sperm motility (2, 3, 13), abnormal morphology (4), and increased WBC contamination of the ejaculate (4, 18). Our results also agree with the findings that levels of reactive oxygen species are higher in semen from men with spinal cord injury than in control subjects and able-bodied fertile men (10, 11). We found that the level of reactive oxygen species in specimens from men with spinal cord injury correlated negatively with sperm motility and correlated positively with the WBC concentration.

Abnormal sperm morphology, specifically midpiece abnormality, is associated with elevated levels of reactive oxygen species (7). The preponderance of abnormal tail forms, especially coiled tails, in the semen of subjects with spinal cord injury in our study is in conformity with the results reported by others (4). This defect may be caused by the high polyunsaturated fatty acid content in the midpiece sperm membrane (8). Additionally, antioxidant enzymes that protect against the detrimental effects of reactive oxygen species are located in the midpiece of the spermatozoon (7).

![Figure 1](image_url)  
**Figure 1** Chemiluminescence response in semen specimens from men with spinal cord injury. Correlation between sperm motility and levels of reactive oxygen species generated in (A) nonstimulated (r = −0.49, P = 0.02), (B) FMLP-stimulated (r = −0.46, P = 0.02), and (C) PMA-stimulated specimens (r = −0.48, P = 0.05).
burg, MD). Luminescence was recorded in the integration mode at room temperature using Luminol (5-amino-2,3-dehydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as the probe. The sperm suspension was divided into two 400-μL aliquots at 20 × 10^6 concentration. In the first aliquot, 25 μM Luminol were added, and luminescence was measured for 7 minutes (14).

Reactive oxygen species may be produced from the leukocytes (mainly neutrophils) as well as from the spermatozoon. N-Formyl-methionyl-leucylphenylalanine (FMLP) binds to the bacterial peptide receptors on polymorphonuclear leukocytes and stimulates a burst of reactive oxygen species generation. This reaction was used as a provocation test for selectively triggering the generation of reactive oxygen species by leukocytes. The capacity of the sperm population to generate reactive oxygen species was monitored by adding 12-myristate 13-acetate phorbol ester (PMA), the most powerful stimulus for oxidative generation by human spermatozoa (15).

Generation of reactive oxygen species was stimulated by adding 12.4 U of horseradish peroxidase (Sigma Chemical Co.) to the second aliquot of sperm suspension. We allowed 7 minutes to sensitize the assay for generation of extracellular hydrogen peroxide (16, 17). Sperm suspensions were stimulated with 25 μM luminol and 50 μM FMLP and were monitored for an additional 7 minutes to determine the magnitude of the peak chemiluminescence response and to allow the system to return to baseline. After this, 100 nM PMA was added and luminescence was monitored for an additional 15 minutes to assess the residual generation of reactive oxygen species. Measurements of reactive oxygen species were recorded in the integration mode. Levels of reactive oxygen species >10 × 10^6 counted photons per minute (cpm) were considered positive.

Statistical Analysis

Wilcoxon rank-sum test was used to compare the method of ejaculation and the type of ejaculate (ante
grade versus retrograde) in subjects with spinal cord injury and controls versus sperm motility, sperm
morphology, WBC concentration, and levels of reactive oxygen species. The frequency of positive results for reactive oxygen species between controls and subjects with spinal cord injury was compared by Fisher's exact test. Correlations of sperm motility with sperm morphology, WBC concentration, or levels of reactive oxygen species were estimated with Spearman's rank-order correlation. Because subjects with spinal cord injury had both antegrade and retrograde samples, we used a mixed-effects repeated-measures analysis to examine differences between the antegrade and retrograde specimens. The Wilcoxon signed-rank test was used to analyze differences in sperm motility, sperm morphology, WBC concentration, and levels of reactive oxygen species between ejaculates produced by vibratory stimulation and by electroejaculation in the same patients. All values are expressed as median and range (minimum, maximum) unless otherwise indicated. All statistical analyses were performed using the SAS statistical package (Cary, NC).

RESULTS

High levels of reactive oxygen species were observed in sperm suspensions from men with spinal cord injury. The median value in unstimulated controls was 0.079 × 10^6 cpm, compared with 160.3 × 10^6 cpm in patients with spinal cord injury. This latter value is 2,000 times higher than the levels of reactive oxygen species detected in specimens from normal men (controls). Abnormally high values in sperm suspensions also were observed between the groups after stimulation with FMLP and PMA.

Compared with control levels, levels of reactive oxygen species were significantly higher in unstimulated and stimulated specimens from men with spinal cord injury (P < 0.001) (Table 1). The incidence of positive specimens for reactive oxygen species in unstimulated controls and men with spinal cord injury was 47.3% (9/19) versus 100% (24/24), respectively (P < 0.001); for FMLP-stimulated specimens it was 26.3% (5/19) versus 91.7% (22/24) (P < 0.001); and in PMA-stimulated specimens it was 89.5% (17/19) versus 100% (24/24) (P = 0.19).

Comparing semen characteristics between controls and patients with spinal cord injury showed significant differences in sperm motility (P < 0.001) and seminal WBC concentration (P < 0.001) (Table 2). Sperm suspensions in the controls did not contain WBCs as determined by the Endtz staining test. However, semen specimens from the men with spinal cord injury contained elevated concentrations of WBCs. Compared with controls, men with spinal cord injury showed significant differences in sperm morphology when the specimens were grouped into

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Controls</th>
<th>Spinal cord injury</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>× 10^6 cpm</td>
<td>× 10^6 cpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonstimulated</td>
<td>0.079 (0.037, 0.65)</td>
<td>160.3 (0.15, 4.539)</td>
<td>0.001</td>
</tr>
<tr>
<td>Stimulated by FMLP</td>
<td>0.058 (0.001, 0.83)</td>
<td>141.7 (0.004, 3.740)</td>
<td>0.001</td>
</tr>
<tr>
<td>Stimulated by PMA</td>
<td>0.56 (0.001, 10.04)</td>
<td>144.3 (0.26, 2.86)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Values are medians, with range in parentheses.
† Counted photons per minute.

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still have poor semen quality (2, 3). The impaired semen is characterized by low motility (1–3), abnormal morphology, and an increased number of white blood cells (WBC) in the ejaculate (4).

Reactive oxygen species are found frequently in semen samples from infertile able-bodied men (5, 6). Reactive oxygen species have been linked to abnormal sperm morphology (5, 6) and reduced sperm motility (5, 7) and function (8) and are associated with WBC contamination of the ejaculate (9). Levels of reactive oxygen species in men with spinal cord injury are higher than those found in normal healthy men (10, 11). The purposes of this study were to determine the following: [1] whether the level and frequency of reactive oxygen species in men with spinal cord injury is higher than in normal controls, [2] whether the level of reactive oxygen species is affected by the method of ejaculation or specimen type, and [3] whether levels of reactive oxygen species are related to sperm motility, sperm morphology, or the level of spinal cord injury.

MATERIALS AND METHODS

Subjects

Twenty-four men with traumatic spinal cord injury participated in this study. Their mean age was 35 ± 1.5 years, and the mean time after injury was 4.7 ± 2.3 years. The levels of injury were cervical (n = 12) and thoracic (n = 12). Semen specimens were obtained from these men by vibratory stimulation (n = 15), electroejaculation (n = 8), or masturbation (n = 1). All subjects with spinal cord injury were in good general health and were volunteers.

Control subjects were 19 healthy men without spinal cord injury and with normal semen characteristics (12). Their mean age was 33 ± 1.5 years. All control subjects had a sperm concentration greater than 40 x 10⁹/mL and motility of at least 50%, and they abstained for 3 days before producing a sample by masturbation. This study was approved by the Institutional Review Board at both institutions, and informed consent was obtained from all men before participation.

Assisted Ejaculation Procedures

Immediately before ejaculation, the subject’s bladder was emptied using sterile catheterization technique, and 25 mL of warmed (37°C) sperm-washing medium (modified human tubal fluid medium, HTF; Irvine Scientific, Santa Ana, CA) was added to the subject’s bladder to provide a buffered medium for any retrograde ejaculate. In subjects with a history of autonomic dystreflexia, 10 to 20 mg of nifedipine was given sublingually 10 minutes before the procedure. For vibratory stimulation, the subjects were seated comfortably on an examination table and a vibrator (Sunbeam Home Comfort Wand Massager; Sunbeam, Hattisburg, MS; or Multiscept; Ferti Care Clinic, Copenhagen, Denmark) was applied to the penis for 5 minutes. If no ejaculation occurred, vibratory stimulation was applied again for 5 minutes after a 1-minute rest period. Electroejaculation was performed in the lateral decubitus position (13) using a Seager model 14 electroejaculation unit (National Rehabilitation Hospital, Washington, DC). All men with spinal cord injury were catheterized to check for retrograde ejaculate. Of the 24 men with spinal cord injury, 15 men produced only an antegrade specimen, 6 both antegrade and retrograde specimens, and 3 only a retrograde specimen. All retrograde samples contaminated with urine were collected by catheterization and had an average volume of 90 mL. They were centrifuged for 5 minutes at 300 x g and the pellet was reconstituted to a volume of 1 mL in HTF. Both antegrade and retrograde specimens were treated identically.

Semen Analysis and Sperm Preparation

Five microliters of fresh sample were loaded on a 20-μL sperm-counting chamber (Microcell; Concepcion Technologies, San Diego, CA), and 8 to 10 fields with a minimum of 200 sperm were analyzed manually for concentration and motility using a phase-contrast microscope (magnification x400).

The WBC concentration in semen was determined using the Endtz test, as described earlier (14). In brief, 20 μL of liquefied semen were placed in a 2.0-mL vial with 20 μL phosphate-buffered saline (pH 7.0) and 40 μL benzidine solution. The sample was vortexed and allowed to sit at room temperature for 5 minutes. Five microliters then were placed on a Makler chamber (Seif Medical, Haifa, Israel) and examined for cells that had stained dark brown, indicating cells positive for peroxidase. Specimens with more than 1 x 10⁶ WBC per milliliter were considered positive. Sperm morphology was scored according to World Health Organization criteria (12) using air-dried seminal smears stained with Diff-Quik (Baxter Scientific, Miami, FL).

After completion of the analysis, semen samples were mixed with an equal volume of HTF medium and centrifuged at 250 x g for 5 minutes. The seminal plasma was decanted and the sperm pellet was resuspended in sperm-washing medium at a final concentration of 20 x 10⁶ sperm per milliliter.

Determination of Reactive Oxygen Species

Formation of reactive oxygen species was measured using a luminometer (Wallac Inc., Gaithers-
Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury

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Rakesh K. Sharma, Ph.D.; Ashok Agarwal, Ph.D.

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Objective: To assess the generation of reactive oxygen species and its relation to semen characteristics in men with spinal cord injury.

Design: Cross-sectional study.

Setting: Andrology laboratory at a tertiary care facility and research laboratory at a major medical center.

Patient(s): Men with spinal cord injury and normal men.

Intervention(s): Collecting ejaculates from men with spinal cord injury by electroejaculation and vibratory stimulation and from normal men by masturbation.

Main Outcome Measure(s): Measurement of reactive oxygen species before and after stimulation with 50 µM N-formyl-methionyl-leucylphenylalanine (FMLP) and 100 nM 12-myristate 13-acetate phorbol ester (PMA), white blood cell (WBC) concentration, sperm motility and morphology, and ejaculation method.

Results: Compared with controls, levels of reactive oxygen species in men with spinal cord injury were significantly higher in unstimulated, FMLP-stimulated, and PMA-stimulated specimens. The WBC concentration was significantly elevated in patients with spinal cord injury. Sperm motility in men with spinal cord injury was inversely related to the level of reactive oxygen species. The percentage of morphologically normal spermatozoa was significantly lower in men with spinal cord injury. Levels of seminal reactive oxygen species did not differ when comparing specimen type (antegrade versus retrograde) or method of ejaculation in men with spinal cord injury.

Conclusion(s): Men with spinal cord injury had elevated levels of reactive oxygen species in their semen. Levels of reactive oxygen species were negatively correlated with sperm motility. Levels of reactive oxygen species were independent of the method of ejaculation or the type of specimen. (Fertil Steril® 1997;67:1115–20. © 1997 by American Society for Reproductive Medicine.)

Key Words: Sperm motility, spinal cord injuries, ejaculation, reactive oxygen species, chemiluminescence

Every year, 10,000 new spinal cord injuries occur in the United States (1). Most of these injuries occur in young men. Advances in rehabilitation medicine have facilitated their reintegration into society. Many of these men desire to father children, but they face the obstacles of asexual activity and poor semen quality (1–3). Assisted ejaculation methods can produce samples in 75% to 100% of patients with spinal cord injury. However, the majority of these patients have...