LEVELS OF REACTIVE OXYGEN SPECIES BEFORE AND AFTER SPERM PREPARATION: COMPARISON OF SWIM-UP AND L4 FILTRATION

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In previous studies, high levels of reactive oxygen species (ROS) have been associated with an adverse impact on the fertilizing ability of sperm. The present study evaluated ROS levels in unprocessed (raw) semen specimens and in specimens processed by the traditional swim-up method and by L4 filtration, thereby assessing the potential of these techniques to damage sperm. Semen specimens from 26 men (10 patients in whom subfertility was suspected and 16 donors with normal fertility) were randomly collected, and ROS levels were measured. For all specimens combined (those from patients and those from donors), ROS levels were significantly lower after L4 filtration (6.07 ± 1.97 mV/s 10⁶ sperm⁻¹, p < .01) and after swim-up (5.70 ± 0.96 mV/s 10⁶ sperm⁻¹, p < .001) than in unprocessed ejaculate (12.88 ± 2.32 mV/s 10⁶ sperm⁻¹). However, ROS levels in swim-up and L4 specimens were not statistically different from each other. It would appear that sperm processing by either swim-up or L4 filtration yields specimens with significantly lower levels of ROS than are found in unprocessed ejaculate.

Keywords L4 filter, spermatozoa, sperm processing, reactive oxygen species, swim up

Defective sperm function is the most frequent defined cause of idiopathic human infertility; of all couples attending infertility clinics, about 27% do so for this reason [12]. Despite its high incidence, little is known about the etiology of male infertility, and there are few (if any) rational effective therapies for this condition. Human spermatozoa are particularly susceptible to peroxidative damage because they contain an extremely high concentration of polyunsaturated fatty acids, exhibit no capacity for membrane repair, and possess a significant ability to generate reactive oxygen species (ROS), chiefly superoxide anion and hydrogen peroxide [2, 7, 15]. The higher levels of ROS produced by damaged or deficient spermatozoa have been associated with a loss of motility and a decreased capacity for sperm–oocyte fusion [2, 5, 6].

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Our understanding of the chemical nature of the damage to the sperm plasma membrane responsible for this refractory state has been advanced by numerous independent studies, suggesting a key role for lipid peroxidation in male infertility [2, 7, 10, 13, 14].

Aitken and Clarkson [3] investigated the mechanisms mediating the impact of sperm preparation protocols on human sperm function. They found that the detrimental effects of centrifugation were associated with a sudden burst of ROS production by a discrete subpopulation of cells that were characterized by significantly diminished motility and fertilizing capacity and that could be separated from normally functional spermatozoa on Percoll gradients. The objective of the present study was to determine whether high levels of ROS present in the semen of some men with suspected infertility could be reduced significantly by sperm-processing techniques, such as swim-up or L4 filtration.

MATERIALS AND METHODS

Semen Collection and Analysis. Twenty-six semen specimens—10 from men in whom subfertility was suspected and 16 from men with normal fertility—were collected by masturbation after at least 2 days of sexual abstinence. All specimens were tested for the presence of white blood cells (WBC) by the Endtz test [11] and only those specimens testing negative for WBCs (<1 × 10⁶/mL) were used in this study. The specimens were kept for liquefaction at 37°C in a CO₂ incubator for 30 min. Each semen specimen was analyzed on a Hamilton-Thorn Motility (HTM) analyzer, model IVOS (Hamilton-Thorn Research, Beverly, MA).

HTM Gate Settings. The main gate settings of the HTM analyzer were as follows: number of frames, 20; frame rate, 30 frames/s; minimum contrast, 7; minimum size, 5; Lo/Hi size gates, 0.5 to 1.8; Lo/Hi intensity gates, 0.5 to 1.8; nonmotile head size, 10; nonmotile intensity, 200; medium path velocity (VAP) value, 25; low VAP value, 10; slow cells motile, no; threshold straightness (STR), 80; VAP, 10 to 500; progressive velocity (VSL), 0 to 500; curvilinear velocity (VCL), 0 to 500; STR, 0 to 100; linearity (LIN), 0 to 100; and lateral head displacement (ALH), 0 to 0.

ROS Determination. The formation of ROS was measured with use of a computer-driven Luminometer, model 1251 (LKB-Wallac, Gaithersburg, MD). Luminescence was recorded after the addition of 20 μL of 4 mM luminol (5-amino-2,3-dihydro-1,4-phthalalazinedione) (Bio-Orbit, Turku, Finland) to 0.5 mL of washed spermatozoa in human tubal fluid (HTF) medium (Fertility Technologies, Inc., Natick, MA), and then diluted with 0.5 mL of HTF medium. Measurement of ROS activity was started 3 min after the addition of luminol and continued for 30 cycles. The readings were taken in the integration mode, with constant mixing of analyzed sample for 10 s. HTF medium alone acted as a control for each specimen analyzed for ROS activity. Mean ROS activity was expressed as the average value of 30 cycles (mV/s 10⁹ sperm⁻¹) with the mean ± SE of each ROS reading in each 30 cycles considered. The mean ROS was considered significant if comparison of experimental and control readings yielded a p value ≤.05; however, if the p value was >.05, then the mean ROS values were not considered statistically significant.

Swim-up Procedure. Semen specimens were processed by sperm washing and the swim-up technique [1]. In brief, these specimens were washed twice with HTF medium (Irvine Scientific, Santa Ana, CA) at a 1:4 ratio of semen to HTF and centrifuged at 600 g for 10 min in an IEC centrifuge (International Equipment Company, Needham, MA). The sperm pellets were resuspended in 2.0 mL of HTF, transferred in equal parts to two clean round-bottom test tubes, and centrifuged at 60 g for 5 min. Motile sperm were allowed to swim up during the incubation of test tubes at a 45° angle in 5% CO₂
at 37°C for 1 h. Supernatants were aspirated into a clean test tube and centrifuged at 600 g for 10 min. The final supernatants were aspirated and discarded. Sperm pellets were resuspended in 0.5 mL of HTF, and complete semen analysis was performed again.

Two L4 membrane disks (diameter, 16 mm) were placed at the bottom of a 12.5-mL Eppendorf pipette tip. Semen specimens were mixed with 5.0 mL of HTF medium supplemented with 10% heat-inactivated, filter-sterilized fetal bovine serum. The mixtures were gently vortexed and passed through the mounted disks by gravity. The pipette tip was rinsed with 4.0 mL of fresh medium for collection of the remaining motile sperm. The filtered specimens were centrifuged at 600 g for 5 min. The supernatant was carefully aspirated off and discarded. The sperm pellets were resuspended in 0.5 mL of HTF medium, and complete semen analysis was performed.

Statistical Analysis. Statistical differences among ROS levels in three groups (unprocessed, L4-filtered, and swim-up specimens) were analyzed by paired Wilcoxon rank sum test. ROS values for processed specimens from patients and donors were compared by unpaired Wilcoxon rank sum test.

RESULTS

The mean (±SE) levels of ROS formation in unprocessed, L4-filtered, and swim-up specimens from all subjects (patients and donors) were 12.88 ± 2.32, 6.07 ± 1.97, and 5.70 ± 0.96 mV/s 10⁹ sperm⁻¹, respectively. In all cases, ROS levels of swim-up and L4-filtered specimens were significantly lower than those in unprocessed ejaculate (p < .02 and p < .01, respectively; Figure 1). However, ROS levels in L4-filtered and swim-up specimens did not differ signifi-

![Bar chart](image)

**FIGURE 1** Levels of reactive oxygen species (ROS) in unprocessed, L4-filtered, and swim-up semen specimens from patients with suspected subfertility and from donors with normal fertility. Each bar shows the mean of actual ROS values; spz, spermatozoa; *p < .02 and *p < .01 vs. value for unprocessed specimens.
FIGURE 2  Levels of reactive oxygen species (ROS) in semen specimens from patients with suspected subfertility and from donors with normal fertility. Each bar shows the mean of actual ROS values; spz, spermatozoa; "p < .02 vs. value for unprocessed specimens.

significantly from each other. Results were similar when ROS levels in specimens from patients and donors were analyzed separately (Figure 1).

Comparison of ROS formation in unprocessed specimens (Figure 2) revealed significantly higher mean levels in specimens from patients than in those from donors (20.42 ± 4.28 vs. 8.17 ± 1.67 mV/s 10⁹ sperm⁻¹; p < .05). However, no such difference was found for L4-filtered specimens (patients vs. donors, 7.60 ± 2.59 vs. 6.31 ± 1.41 mV/s 10⁹ sperm⁻¹) or for swim-up specimens (patients vs. donors, 5.91 ± 1.87 vs. 5.58 ± 1.09 mV/s 10⁹ sperm⁻¹; Table 1).

DISCUSSION

In recent years, the growing interest in oxygen toxicity and free radical reactions in biology and medicine has led to the hypothesis that cell damage can be produced by increased free radical (OH⁻ and O⁻) generation [8]. In seminal plasma the presence of trace amounts of

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Patient ROS  (mV/s 10⁹ Sperm⁻¹)</th>
<th>Donor ROS  (mV/s 10⁹ Sperm⁻¹)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprocessed</td>
<td>20.42 ± 4.28</td>
<td>8.17 ± 1.67</td>
<td>p = .0125*</td>
</tr>
<tr>
<td>L4 filtered</td>
<td>7.60 ± 2.59</td>
<td>6.31 ± 1.41</td>
<td>NS</td>
</tr>
<tr>
<td>Swim-up</td>
<td>5.91 ± 1.87</td>
<td>5.58 ± 1.09</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note. Values are means ± SE. NS, not significant.
transition elements such as iron [16] lead to the initiation of ROS synthesis causing lipid peroxidation by hydroxyl radicals in the sperm plasma membrane [4]. Accumulation of lipid hydroperoxides in a membrane disrupts its structure and can cause it to collapse. In addition, lipid peroxides can decompose to yield a range of highly cytotoxic products. Lipid peroxidation, in turn, leads to a decrease in membrane fluidity, as a consequence of which the capacity of the sperm to participate in the membrane fusion events associated with fertilization is diminished.

The clinical significance of ROS formation in sperm from infertile patients remains to be evaluated. Nevertheless, there is firm evidence that ROS as well as lipid peroxides have serious deleterious effects on sperm motility [7, 14]. Other negative effects on sperm functions, such as inhibition of sperm–hamster zona-free oocyte interaction, have also been documented [2, 4]. Several researchers have reported that the formation of ROS in sperm increases by 20- to 50-fold after the repeated centrifugation and resuspension used in some sperm-washing techniques [4, 13]. Since oxygen radicals are rapidly exported from the germ cells, they can disrupt the plasma membranes of normal functional cells in the immediate vicinity [4]. It is therefore essential that this radical-generating subpopulation of spermatozoa be removed from the sperm suspension before any attempt is made to centrifuge the cells. This can be achieved by the swim-up from semen, L4 filter membrane, albumin column, and Percoll gradient techniques because the highly motile cells isolated by such procedures generate low levels of ROS. Our results indicate a significant reduction in levels of ROS formation in semen specimens obtained from subfertile patients and processed by swim-up and L4-filtration techniques. We postulate that the analysis of ROS formation is valuable in the assessment of sperm-processing techniques.

Previously reported studies [5, 13] have shown a direct relation of high ROS levels in infertile men to an adverse effect on sperm fertilizing ability. Thus, a high ROS level after sperm washing may indicate damage to the sperm during processing by a specific method.

REFERENCES


