Objective: To investigate sperm viability, incidence of apoptosis, and intracellular basal and induced reactive oxygen species (ROS) in sperm fractions.

Design: Prospective controlled study.

Setting: Center for Reproductive Medicine at a tertiary care hospital.

Method(s): Liquefied seminal ejaculates (n = 12) prepared by density gradient centrifugation were reconstituted to 2 mL with phosphate-buffered saline. Oxidative stress was induced by hydrogen peroxide (H2O2, 100 µM). Sperm viability, intracellular ROS, and incidence of apoptosis/necrosis in neat, immature, and mature sperm fractions were assessed.

Result(s): Before H2O2 exposure, mature spermatozoa fractions showed a significantly lower incidence of apoptotic sperm and intracellular O2−• levels but higher amounts of intracellular H2O2 compared with neat semen. Higher levels of intracellular H2O2 were demonstrated in immature sperm fractions compared with neat or mature fractions. In all sperm fractions, intracellular H2O2 levels correlated with the intracellular concentration of O2−•. After H2O2 exposure, neat semen showed a significantly higher percentage of apoptosis compared with the prepared mature spermatozoa. However, no differences were observed in the incidence of apoptosis between immature and mature sperm fractions.

Conclusion(s): There is a differential shift of both intracellular H2O2 and O2−• in each sperm fraction that may affect sperm quality. Sperm apoptosis is related to intracellular H2O2 levels, which in turn are affected by intracellular O•− levels. Oxidative stress was not associated with an increased incidence of apoptosis in immature or mature sperm fractions. (Fertil Steril 2010;93:814–21. ©2010 by American Society for Reproductive Medicine.)

Key Words: Reactive oxygen species, flow cytometry, apoptosis, human spermatozoa, intracellular staining

Oxidative stress has been implicated in male factor infertility (1–5). Superoxide (O2−•) and hydrogen peroxide (H2O2) are common reactive oxygen species (ROS), which are highly reactive and can interact with nearby molecules, inducing oxidative stress damage in cellular organelles and molecules (6–8).

Physiological levels of ROS are required for normal sperm functions such as hyperactivation, capacitation, and acrosome reaction (9–11). Oxidative stress occurs in spermatozoa when global levels of ROS (both extra- and intracellular) exceed the available total antioxidant capacity. Sperm have a limited amount of cellular cytoplasm in which scavenging enzymes are found, making sperm highly susceptible to ROS damage (10, 12, 13). As ROS are able to readily permeate the membranes, they can cause DNA, proteins, and lipid molecules peroxidative damage within the cell (7, 8, 14). Motile sperm have been shown to be activated by excessive ROS formation and undergo apoptosis-like changes (15–17). This insult has been linked to sperm apoptosis and male factor infertility (18, 19).

Sperm preparation plays an important role in a successful outcome in assisted reproductive techniques (ART). Double-density gradient centrifugation is a standard sperm selection method for ART to separate mature motile sperm with superior morphology. A mature sperm fraction shows less incidence of apoptotic sperm compared with ejaculated unprocessed sperm (20, 21). Oxidative sperm damage can occur during sperm preparation and processing for ART (22–24).

Oxidative stress–induced sperm damage and apoptosis-like changes may occur when the intracellular ROS levels are in excess of the cells’ scavenging capacity (20, 25). In addition, H2O2 has been recommended as a local vaginal contraceptive/spermicidal agent (26, 27). Measurement of the
intracellular ROS would, therefore, be more beneficial than global measurement of the seminal ROS (6, 8, 28). On the other hand, simultaneous selective measurement of intracellular H$_2$O$_2$ or O$_2$ $^.-$ levels may be important in understanding how sperm preparation affects ART success rates.

Dichlorofluorescin diacetate (DCFH-DA) and dihydroethidium (DHE) are used for measurement of intracellular H$_2$O$_2$ and O$_2$ $^.-$, respectively, by flow cytometry. The advantages of using flow cytometry for the measurement of intracellular ROS in ejaculated human spermatozoa have been reported recently (29–31).

In this study, oxidative stress was induced after exposure to exogenous hydrogen peroxide (H$_2$O$_2$). Sperm viability and intracellular ROS levels were evaluated in seminal ejaculates (unprocessed) and in immature and mature sperm fractions to determine the basal and stimulated intracellular ROS levels and examine their relationship with viability and apoptosis.

MATERIALS AND METHODS
Sample Collection and Preparation
This study was approved by the Cleveland Clinic Institutional Review Board. Semen samples were collected from 12 healthy male volunteers of unproven fertility status at the Cleveland Clinic andrology laboratory. All samples were collected by masturbation after 2–3 days of sexual abstinence.

After liquefaction, sperm count, percentage motility, viability, and presence of round cells were examined on an aliquot of the neat semen sample. The remaining aliquot was prepared for separating mature and immature fractions by double-density gradient centrifugation (PureCeption, SAGE BioPHARMA, Bedminster, NJ). Samples were centrifuged at 300 g for 20 minutes, and the resulting interface between the 40% and 80% layers (immature spermatozoa) was aspirated. Highly motile mature spermatozoa were obtained in an 80% pellet. Both fractions were resuspended in human tubal fluid media (HTF; Irvine Scientific, Santa Ana, CA).

Induction of Oxidative Stress
Oxidative stress was induced by exposing the sperm to H$_2$O$_2$. A 30% stock solution of H$_2$O$_2$ (Sigma Chemical Co., St Louis) was diluted to 100 µM and added to 1 mL of sperm suspension (stimulated ROS) and incubated for 15 minutes at 37°C. Another aliquot from the same fraction containing an equal volume of HTF served as a control (basal ROS).

Determination of ROS by Flow Cytometry
DCFH-DA, a specific probe for H$_2$O$_2$, and DHE, a specific probe for O$_2$ $^.-$, are cell-permeable stains. DCFH is oxidized selectively by the free intracellular H$_2$O$_2$ into DCF that binds to DNA and emits green fluorescence. DHE is oxidized by the free intracellular O$_2$ $^.-$ into ethidium bromide that binds to the DNA and emits red fluorescence (31–33). DCFH-DA (25 µM) and DHE (1.25 µM; Sigma) were added to the sperm suspension and incubated at 25°C for 40 minutes (DCFH-DA) and 20 minutes (DHE), respectively. Aliquots were subsequently analyzed using a flow cytometer. Green fluorescence (DCF) was evaluated between 500 and 530 nm, while red fluorescence (HE) was evaluated between 590 and 700 nm (excitation, 488 nm; emission, 525–625 nm in the FL-2 channel). Data were expressed as the percentage of fluorescent spermatozoa. Apoptotic spermatozoa were excluded by using counter nucleic acid stains. Propidium iodide (PI) was used as a counterstain dye for DCFH-DA; YO-PRO-1 was used as a counterstain dye for the HE (29).

Apoptosis Detection
To measure the apoptotic status of the spermatozoa, the Vybrant Apoptosis Assay (Invitrogen, Carlsbad, CA) was used. All samples were washed in cold phosphate-buffered saline (PBS), and the cell density was adjusted to ~1 × 10$^6$ cells/mL in PBS. One microliter of the YO-PRO-1 solution (10 µM) and 1 µL of the PI solution (50 µg/mL) were added to 1 mL of cell suspension and incubated for 20–30 minutes. Flow cytometric analysis of the stained cells was done within 1–2 hours using 488 nm excitation with green fluorescence emission for YO-PRO-1 (i.e., 530/30 band pass) and red fluorescence emission for PI (i.e., 610/20 band pass). Gating was performed to exclude any debris. Standard compensation was done using single-color stained controls. Three different populations can be identified by using this assay (Figure 1): viable sperm are negative for both PI and YO-Pro-1, apoptotic sperm are positive for YO-pro-1 but negative for PI, and dead sperm show positivity for both PI and YoPro-1.

Flow Cytometry Analysis
All fluorescence signals of labeled spermatozoa were analyzed by a Becton Dickinson flow cytometer FACScan (Becton Dickinson, San Jose, CA) equipped with a 488-nm argon laser as a light source. A minimum of 10,000 spermatozoa were examined for each assay at a flow rate of <100 cells/second.

The sperm population was gated using 90° and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm supplied by an argon laser at 15 mW. DCF/ YO-PRO-1 emitting green fluorescence and PI/HE emitting red fluorescence (580–630 nm) were recorded in the FL-1 and FL-2 channels, respectively. The percentage of HE-/PI-positive cells and the mean fluorescence were calculated on a 1023-channel scale and analyzed using the flow cytometer software FlowJo version 7.2.2 (FlowJo, Ashland, OR).

Statistical Methods
For all quantitative measured parameters, comparisons between stimulated and nonstimulated neat, mature, and immature spermatozoa were performed using the Wilcoxon
signed-rank test. Associations among quantitative variables were measured using Spearman’s correlation coefficients both within and across sample groups. \( P < .05 \) was considered statistically significant.

RESULTS

Neat semen samples showed a mean (±SD) semen volume of 2.7 (±1.3) mL, sperm concentration of 62.4 ± 53.1 \( \times 10^6 \) cells/mL, and motility of 66.6% ± 9.2%. Before \( \text{H}_2\text{O}_2 \)
exposure, mature sperm showed a significantly higher motility (72.7% ± 19.3%) when compared with the neat sample (48.9% ± 19.7%, \( P = .014 \)) and immature sperm (41.7% ± 12.3%; \( P < .001 \)), respectively.

**Sperm Population Identification**

When viable, apoptotic, and dead sperm were examined, three different sperm staining patterns were observed upon analyzing the sperm for apoptosis using the Yo-Pro-1/PI assay (Fig. 1A–F). The apoptotic sperm population stained only positive for Yo-Pro-1. Yo-Pro-1- and PI-positive cells represented the dead sperm population, while viable sperm showed negative Yo-Pro-1 and negative PI fluorescence. The percentages of each sperm population in neat, immature, and mature sperm fractions are shown in Figure 1 (A–F).

**Sperm Apoptosis and Intracellular ROS before \( \text{H}_2\text{O}_2 \) Exposure**

Density gradient centrifugation selected a population of mature cells with a significantly lower number of apoptotic cells than were present in neat semen (\( P = .006; \) Table 1). Mature sperm also displayed a higher percentage of DCF-\(^{+}\)ve (\( P < .001 \)) and a lower HE-\(^{+}\)ve (\( P = .004 \)) fluorescence compared with sperm in the neat semen. Immature sperm showed a significantly higher percentage of DCF-stained sperm when compared with both neat (\( P < .001 \)) and mature sperm (\( P = .05 \)).

**Sperm Viability, Apoptosis, and Intracellular ROS after \( \text{H}_2\text{O}_2 \) Exposure**

Exposure to \( \text{H}_2\text{O}_2 \) significantly reduced viability and increased the percentage of dead sperm in neat and immature and mature groups as shown in Figure 1. However, exposure to \( \text{H}_2\text{O}_2 \) was associated with an increase in the mean percentage of apoptotic sperm in neat semen and in the mature and immature sperm fractions, but the difference was not significant (Table 1). After \( \text{H}_2\text{O}_2 \) exposure, a significantly lower percentage of apoptosis was seen in mature versus neat sperm fractions (\( P = .024 \)). Other comparisons between immature versus mature and neat (stimulated or nonstimulated) showed no significant differences.

The percentage of sperm with DCF fluorescence (intracellular \( \text{H}_2\text{O}_2 \)) increased in both neat and mature sperm, but the difference was not significant in the immature sperm fractions (Table 1; Fig. 2A). Moreover, neat, immature, and mature sperm fractions exhibited a significant increase in the percentage of sperm showing HE fluorescence (increase in intracellular \( \text{O}_2^{2-} \); Table 1; Fig. 2B).

**Correlation of Intracellular ROS with Other Sperm Parameters**

Sperm viability was inversely related to the percentage of apoptotic spermatozoa (mature nonstimulated, \( r = -0.76, P = .006 \); mature stimulated, \( r = -0.58, P = .047 \); all fractions, \( r = -0.55, P < .001 \)). After \( \text{H}_2\text{O}_2 \) exposure, the percentage of viable spermatozoa was positively correlated with the intensity of DCF fluorescence in neat (\( r = 0.63, P = .031 \)) and in mature sperm fractions (\( r = 0.59, P = .04 \)).

The percentage of apoptotic sperm was positively correlated with DCF fluorescence (intracellular \( \text{H}_2\text{O}_2 \)) in neat nonexposed fractions (\( r = 0.60, P < .041 \)). We wanted to examine the relationship of apoptotic sperm with dead sperm. When the neat, immature, and mature fractions (overall) were examined before and after stimulation, the percentage of apoptotic sperm was positively correlated with the percentage of dead sperm in the overall group (\( r = 0.52, P < .001 \)) and in the mature sperm fraction (\( r = 0.78, P = .004 \)). After \( \text{H}_2\text{O}_2 \) exposure, the percentage of sperm positive for DCF showed a negative correlation with the percentage of dead sperm in neat (\( r = -0.68, P = .01 \)) and in mature sperm fractions (\( r = -0.59; P = .04 \)). Similarly, after exposure, the percentage of HE-\(^{+}\)ve sperm (intracellular \( \text{O}_2^{2-} \)) was significantly correlated with the percentage of DCF-\(^{+}\)ve sperm (intracellular \( \text{H}_2\text{O}_2 \)) in the overall (\( r = 0.52; P < .001 \)) and mature sperm fractions (\( r = 0.78; P = .004 \)).

**DISCUSSION**

Our study aim was to evaluate the basal and stimulated intracellular \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^{*} \) levels in different sperm fractions and to examine their relationship with sperm apoptosis. We have measured basal levels of both intracellular \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^{*} \) in neat, immature, and mature sperm fractions. Interestingly, both mature and immature sperm showed reduced intracellular levels of \( \text{O}_2^{*} \) compared with the neat sperm. Higher levels of intracellular \( \text{H}_2\text{O}_2 \) (as represented by DCF-\(^{+}\)ve fluorescence) were seen in immature compared with mature or neat sperm fractions (Table 1). We report for the first time the shift in intracellular \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^{*} \) levels. This shift may be explained by the fact that conventional centrifugation increases heat generation, which may affect sperm quality (17, 34, 35). Avoiding sperm centrifugation for longer times and/or higher speed(s) or its modification may be helpful in preserving sperm quality (36).

We hypothesize that conventional centrifugation may increase the activity of the superoxide dismutase enzyme that converts the generated superoxide ion during centrifugation into hydrogen peroxide, which may prove fatal to sperm cells (37). This conversion lowers the available intracellular superoxide levels in both immature and mature sperm when compared with the neat unprocessed spermatozoa. However, mature spermatozoa may have higher catalase activity/expression (38) when compared with immature spermatozoa, enabling them to scavenge the generated \( \text{H}_2\text{O}_2 \) more effectively. Interestingly, despite the higher levels of intracellular \( \text{H}_2\text{O}_2 \) seen in the mature sperm fraction, the percentage of dead sperm in this fraction did not increase significantly, even though a significant decrease was seen in the percentage of apoptotic sperm. This might be explained by the fact that \( \text{H}_2\text{O}_2 \) is not as lethal as \( \text{O}_2^{*} \) for cell viability. Another,
### TABLE 1

Comparison of the measured parameters in different sperm fractions before and after H2O2 exposure.

<table>
<thead>
<tr>
<th>Marker (ROS assessed)</th>
<th>Neat semen, mean ± SD (n = 12)</th>
<th>Mature sperm, mean ± SD (n = 12)</th>
<th>Immature sperm, mean ± SD (n = 12)</th>
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</thead>
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<tr>
<td></td>
<td>Nonstimulated</td>
<td>Stimulated</td>
<td>P</td>
</tr>
<tr>
<td>Viable sperm, %</td>
<td>59.4 ± 8.98</td>
<td>46.2 ± 11.9</td>
<td>.006</td>
</tr>
<tr>
<td>Apoptotic sperm, %</td>
<td>0.76 ± 0.77</td>
<td>1.7 ± 1.75</td>
<td>.2</td>
</tr>
<tr>
<td>Dead sperm, %</td>
<td>38.9 ± 10.5</td>
<td>50.3 ± 12.6</td>
<td>.035</td>
</tr>
<tr>
<td>DCFH-DA, % (H(_2)O(_2))</td>
<td>6.4 ± 8.7</td>
<td>38.2 ± 18.2</td>
<td>&lt;.001</td>
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<td></td>
<td>(&lt;&lt;.001)</td>
<td></td>
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<tr>
<td>DHE, % (O(_2)^-*))</td>
<td>3.5 ± 1.8</td>
<td>8.1 ± 5.7</td>
<td>.024</td>
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<td></td>
<td>(0.004)</td>
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**Note:** Statistical comparison between stimulated and nonstimulated samples within neat, mature, and immature sperm were performed using the Wilcoxon signed-rank test. Pairwise comparisons of neat, mature, and immature within stimulated and nonstimulated samples were also performed using the Wilcoxon signed-rank test.

\(^{a}\) Significant difference between nonstimulated mature and neat sperm fractions.

\(^{b}\) Significant difference between stimulated mature and neat sperm fractions.

\(^{c}\) Significant difference between nonstimulated immature and neat sperm fractions.

\(^{d}\) Significant difference between nonstimulated mature and immature sperm fractions.
likely, explanation is the fact that mature sperm have the ability to protect themselves against the harmful effects of H$_2$O$_2$ exposure (39, 40). On the other hand, our finding confirms the reports of Donnelly et al. (41) that supplementation with ascorbate and alpha-tocopherol in combination protects the sperm from H$_2$O$_2$-induced sperm DNA damage by scavenging the ROS generated during sperm preparation.

Our study data demonstrated that mature spermatozoa fractions exhibited lower levels of both apoptotic and dead sperm and displayed higher percentages of viable sperm compared with neat semen (Table 1). These findings were in agreement with earlier studies from our group (30, 42), as well as with other studies in which superoxide anions were shown to induce both caspase activation and apoptosis. From these results, it is also worth mentioning that acute exposure of spermatozoa to H$_2$O$_2$ results in decreased sperm viability and increased percentage of dead spermatozoa. Apoptosis and necrosis are different death pathways, and we examined their relations to sperm viability. This is in agreement with the findings of Conde de la Rosa et al. (6) who reported that H$_2$O$_2$ induced cell death. It also agrees with other investigators who evaluated the efficacy of H$_2$O$_2$ as a local vaginal contraceptive/spermicidal agent with a short time exposure (21, 43–45).

We have demonstrated that intracellular H$_2$O$_2$ levels are related to the intracellular O$_2^{•-}$ levels as both are end products of one reaction. Sperm motility is positively related to the percentage of viable sperm. When all the fractions were considered together, motility was inversely related to the percentage of dead or apoptotic sperm. This may explain why sperm motility decreases in pathologically high-ROS conditions in which there is an increase in the percentage of dead sperm. It may also explain why sperm preparation by density gradient separation shows higher motility due to reducing the percentage of apoptotic spermatozoa. As shown earlier, removal of apoptotic sperm improves the quality of the prepared sperm (42).

Our study findings show that the percentage of apoptotic sperm was positively related to the basal intracellular levels of H$_2$O$_2$ in the neat sperm fraction. However, under high-ROS conditions (pathological or induced), intracellular H$_2$O$_2$ level was positively related to the percentage of viable spermatozoa in stimulated as well as in neat sperm. This may be explained by the fact that induced high-ROS conditions will increase the percentage of apoptotic/dead sperm. In high-ROS conditions (pathological or induced), viable spermatozoa may have the ability to adapt to increased H$_2$O$_2$ as a result of their high defense status (Fig. 1). This observation may be important and is supported by the report that shows 100% efficacy (sperm immobilization and loss of viability) in mating studies not earlier than at 2 hours of H$_2$O$_2$ exposure (43). This may explain the poor efficacy of H$_2$O$_2$ as a local vaginal contraceptive for short-time usage (<1 hour) and is in accordance with a report (43) that 2 hours of exposure may be appropriate to improve the H$_2$O$_2$ contraceptive efficiency.

In conclusion, both immature and mature sperm produce higher intracellular H$_2$O$_2$ levels compared with neat spermatozoa. This probably is attributable to sperm processing with centrifugation. Mature spermatozoa may adapt to H$_2$O$_2$ generated during sperm preparation involving centrifugation. This may explain the presence of the higher percentage of

**FIGURE 2**

Representative flow cytometry marker histogram for stimulated neat, immature, and mature sperm fractions. (A) positive DCF fluorescence (represents intracellular H$_2$O$_2$); (B) positive fluorescence for HE fluorescence (represents intracellular O$_2^{•-}$). The histograms show the differential shift of the intracellular ROS in sperm fractions.

viable and the lower percentage of dead/apoptotic sperm in the
prepared mature spermatozoa fraction. Sperm preparation
may be associated with a differential shift of both intracellular
H₂O₂ and O₂⁻ that may affect the sperm quality. Apoptotic
changes in sperm are attributed largely to the intracellular
H₂O₂ levels, while dead sperm are related to intracellular
O₂⁻ levels. Finally, intracellular ROS levels may affect sperm
quality through their effects on sperm viability.

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