Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa

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Objective: To examine simultaneously the levels of hydrogen peroxide (H₂O₂) and superoxide (O₂⁻) using chemiluminescence and flow cytometry.

Design: Prospective laboratory study.

Setting: Reproductive research lab in a tertiary hospital.

Patient(s): Semen samples from 18 healthy male volunteers.

Intervention(s): Sperm preparation and measurement of reactive oxygen species (ROS) by chemiluminescence using luminol and lucigenin before and after H₂O₂ exposure and by flow cytometry using dichlorofluorescin diacetate (DCFH-DA) for H₂O₂ and dihydroethidium (DHE) for O₂⁻.

Main Outcome Measure(s): Sperm count, motility, viability, and ROS levels.

Result(s): Immature sperm fractions showed significantly higher levels of ROS measured by either luminol or lucigenin compared with the neat and mature fraction. ROS levels were detectable by flow cytometry in chemiluminescence-negative samples. Both immature and mature sperm fractions had a significantly higher percentage of cells positive for H₂O₂ compared with neat semen. On the other hand, the percentage of O₂⁻-positive cells in neat semen was significantly higher compared with the percentage found in mature fractions but significantly lower than that in the immature sperm fractions.

Conclusion(s): We recommend ROS measurement by flow cytometry on the basis that it requires a lower sperm count, is comparable to chemiluminescence, and has higher specificity for intracellular ROS in viable spermatozoa. Samples tested negative by chemiluminescence still may have high intracellular H₂O₂ generation that can be detected by flow cytometry. (Fertil Steril® 2009;92:819–27. ©2009 by American Society for Reproductive Medicine.)

Key Words: Superoxide, hydrogen peroxide, flow cytometry, chemiluminescence, semen analysis

The influence of oxidative stress (OS) on male fertility has been extensively studied in the last decade (1–3). Superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) are the common forms of reactive oxygen species (ROS). ROS are highly reactive and short-lived (half-life ranging from a few nanoseconds to milliseconds). They can interact with nearby molecules and play a key role in inducing sperm damage (1, 4–7). ROS damage affects the lipid and protein content of the spermatozoa. Sperm DNA damage by ROS occurs by induction of a high frequency of single or double DNA strand breaks (8–12). Both extracellular- and intracellular-generated ROS are highly diffusible and can produce significant sperm DNA damage beyond the repair capacity of the growing embryo after fertilization (13).

Chemiluminescence using luminol or lucigenin has been the common method to measure ROS (14, 15). Luminol probe can measure both intra- and extracellular ROS, especially O₂⁻, H₂O₂, and OH⁻ free radicals, while lucigenin can detect extracellular ROS, especially O₂⁻ and OH⁻ free radical. Consequently, both probes provide global ROS values but no detailed information on intracellular levels of H₂O₂ or O₂⁻ (14, 15). The chemiluminescence assay has several limitations. These include [1] the need for a relatively high volume and sperm concentration, [2] decline of ROS with time after ejaculation, [3] the interference of iron and copper in the culture media, [4] the inability to detect a specific type of ROS, and [5] the inability to differentiate viable from apoptotic spermatozoa. However, reference values for standard or clinically useful ROS levels are lacking (16). Recently, we reported (17) the cutoff values of ROS in clinical patients with and without leukocytospermia.

Recently, flow cytometry has gained popularity for semen analysis (18). Two main advantages of flow cytometry are the small number of spermatozoa it requires and its ability to...
measure multiple markers simultaneously (19, 20). These are particularly important features in patients presenting with poor sperm counts. Two specific dyes are available to measure intracellular ROS. Dichlorofluorescin (DCFH) can detect intracellular H$_2$O$_2$, while dihydroethidium (DHE) can detect intracellular O$_2$•$.^-$ (21–24).

The goals of this study were to [1] measure basal and stimulated ROS levels by the chemiluminescence method using luminol and lucigenin in different sperm fractions before and after exposure to hydrogen peroxide, [2] measure ROS by flow cytometry using DCFH for H$_2$O$_2$ and DHE for O$_2$•$.^-$, [3] examine the relationship of ROS measured by the two methods, and [4] examine the ability of flow cytometry to detect ROS in samples tested negative by the chemiluminescence method. ROS levels were measured in neat (liquefied seminal ejaculates without any processing), mature, and immature sperm fractions.

**MATERIALS AND METHODS**

**Sample Collection and Preparation**

This study was approved by the Cleveland Clinic Institutional Review Board. Semen samples were collected from 18 healthy donors by masturbation after sexual abstinence of at least 48 hours. Of these, three were proven fertile and had fathered a child in the last 2 years and 15 were of unproven fertility. Samples with leukocytospermia (> 1 x 10$^6$ white blood cells/mL) were excluded.

A portion of the neat semen samples (liquefied seminal ejaculates without any processing) was separated for performing routine semen analysis as per World Health Organization guidelines (25) and also for the assessment of basal and stimulated ROS levels. To separate mature and immature sperm, liquefied semen was subjected to double-density (40% and 80%) gradient centrifugation (PureCeption, SAGE BioPHARMA, Bedminster, NJ) and centrifuged at 300 g for 20 minutes (26). The resulting interface between the 40% and 80% layers (immature spermatozoa) was aspirated and resuspended in human tubal fluid media (HTF; Irvine Scientific, Santa Ana, CA). The resulting 80% pellet (mature, highly motile spermatozoa) was aspirated and resuspended in HTF. In addition, we also examined the effect of sperm concentration (range, 2.5, 5.0, 10.0, and 20 x 10$^6$ sperm/mL) on ROS measurement by chemiluminescence using both luminol and lucigenin.

**Induction of Artificial OS and Measurement of ROS**

Basal levels (unstimulated) of ROS were measured in the absence of an exogenous inducer of OS. Stimulated ROS levels were measured by inducing OS in the presence of 50 μL of freshly prepared H$_2$O$_2$ (100 μM) to each milliliter of semen specimen and incubating for 15 minutes at 37°C. ROS levels were measured by the conventional chemiluminescence assay using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis) and lucigenin (bis-N-methylacridinium nitrate, Sigma Chemical Co.) as probes. Test samples consisted of luminol (10 μL, 5 mM) or lucigenin (4 μL, 200 mM) and 400 μL of sperm suspension. Negative controls were prepared by replacing sperm suspension with phosphate-buffered saline. Chemiluminescence was measured for 15 minutes using a Berthold luminometer (Autolumat LB 953; Bad-Wildbad, Germany). Results were expressed as unadjusted ROS x 10$^6$ counted photon (cpm) or as adjusted ROS as x 10$^6$ cpm/20 x 10$^6$ spermatozoa (15).

**Determination of ROS by Flow Cytometry**

DCFH can detect intracellular H$_2$O$_2$, and DHE can detect intracellular O$_2$•$.^-$ (19). DCFH diacetate (DA; 25 μM) and Hydroethidium (HE) (1.25 μM) obtained from Sigma were added to the sperm suspension and incubated at 25°C for 20 minutes for DCFH-DA and for 40 minutes for HE. Each aliquot was analyzed using a flow cytometer equipped with a 488-nm argon laser as a light source (Becton Dickinson FACScan, San Jose, CA). Green fluorescence (DCFH) 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 stain dye for nucleic acid. We used propidium iodide (PI) as a counter stain dye for DCFH and Yo-Pro-1 as a counter stain dye for the HE.

**Flow Cytometry Analysis**

All fluorescence signals of labeled spermatozoa were analyzed by the flow cytometer FACScan (Becton Dickinson). 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. PI red fluorescence (580–630 nm) was analyzed in the FL-2 channel. The percentage of PI-positive cells and the mean fluorescence were calculated on a 1023-channel scale and analyzed using the flow cytometer software FlowJo, version 6.4.2 (FlowJo, LLC, Ashland, OR).

**Statistical Methods**

With respect to quantitative measures, comparisons between stimulated and nonstimulated samples within neat, mature, and immature were performed using the Wilcoxon signed rank test. Pairwise comparisons of neat, mature, and immature within stimulated and nonstimulated samples also were performed using the Wilcoxon signed rank test. Associations among quantitative variables were measured using Spearman correlation coefficients both within and across sample groups. Linear regression models were used to assess associations, with interaction terms used to test for differences in slopes of relationships for different levels of sample concentration (above and below 5), that is, effects of concentration on the relationships. Log transformation (log + 0.1) was...
used for regression analyses to avoid undefined values for negative samples (ROS = 0) showing skewed variables (including lucigenin, luminol, Dichlorofluorescin [DCF], and HE). Samples classified as positive or negative for adjusted luminol or lucigenin were compared with respect to quantitative sperm quality measures using linear models with generalized estimating equations to account for correlation among samples from the same donor. Analyses were performed using R (The R Foundation, www.R-project.org), version 2.3.1 (27).

RESULTS

The medians (25th, 75th percentile) for neat semen samples were volume 2.9 mL (1.5, 3.9); concentration 42.4 $\times$ 10^6/mL (34.4, 60.4); and percent motility 65.7% (59.3, 70.9). Medians and 25th and 75th percentiles of all other parameters are shown in Table 1.

**ROS by Chemiluminescence**

The ROS levels in unstimulated and stimulated matched (n = 13) as well as unstimulated samples (n = 18) showed significant differences in neat, immature, and mature sperm fractions by both luminol and lucigenin as well as by DCF and HE. Neat samples were assessed with HE for superoxide anion.

**Nonstimulated (basal; n = 18) ROS** Luminol and lucigenin did not show significant differences in basal levels of ROS (either adjusted or nonadjusted) in nonstimulated neat, mature, or immature sperm fractions (Table 1). Immature sperm fractions showed significantly higher levels of basal or adjusted ROS as measured by luminol compared with neat spermatozoa fractions ($P<.03$). A higher, although nonsignificant, level was seen in immature versus mature sperm ($P=.25$). No significant differences were seen in adjusted ROS levels by lucigenin in neat versus mature and immature sperm fractions ($P=.48$ and .67, respectively).

**Stimulated ROS (n = 13)** Significantly higher levels of ROS (both unadjusted and adjusted) as measured by luminol were seen in neat versus mature ($P=.003$ and .009, respectively) and in neat versus immature sperm fractions ($P=.005$ and .003, respectively). No significant differences in levels of ROS (unadjusted and adjusted ROS) with lucigenin were observed between neat versus mature ($P=.023$, and .21, respectively) and immature sperm fractions ($P=.05$, and .009, respectively)

**Intracellular ROS by Flow Cytometry**

ROS levels by flow cytometry in nonstimulated and stimulated neat, immature, and mature sperm by DCH and HE are shown in Figures 1 and 2.

**Nonstimulated (Basal; n = 18) ROS** The proportions of sperm cells stained for DCF ($H_2O_2$) and HE ($O_2^{-}$) were significantly higher in immature sperm fractions compared with mature fractions ($P=.021$ and .003 respectively). Both immature and mature sperm fractions had a significantly higher percentage of sperm positive for $H_2O_2$ compared with neat semen ($<.001$ and $P=.003$, respectively). On the other hand, the percentage of HE ($O_2^{-}$)-positive sperm in neat semen was significantly higher compared with mature fractions ($<.001$) but lower, although nonsignificant, in immature sperm fractions ($P=.08$; Figs. 1 and 2).

**Stimulated ROS (n = 13)** Both $H_2O_2$ (DCF$^{+ve}$) and $O_2^{-}$ (HE$^{+ve}$) levels were increased in neat ($<.001$ and .021, respectively), mature ($P=.009$ and .002, respectively), and immature ($P=.24$ and .002, respectively) sperm fractions. Also, mature sperm fraction showed higher poststimulation levels of both $H_2O_2$ (DCF$^{+ve}$) and $O_2^{-}$ (HE$^{+ve}$) compared with the neat ($P=.14$ and .2, respectively) and immature (969 and .26, respectively) sperm fractions (Figs. 1 and 2).

**Correlation of ROS by Flow Cytometry and Chemiluminescence**

The percentage of DCF$^{+ve}$ sperm showed significant correlation with unadjusted luminol ($r = .57$, $P<.001$), adjusted luminol ($r = .62$, $P<.001$), unadjusted lucigenin ($r = .51$, $P<.001$), and adjusted lucigenin ($r = .56$, $P<.001$). The percentage of HE$^{+ve}$ sperm had significant correlations with unadjusted luminol ($r = .40$, $P<.001$), adjusted luminol ($r = .32$, $P<.003$), unadjusted lucigenin ($r = .57$, $P<.001$), and adjusted lucigenin ($r = .49$, $P<.001$; Table 2).

Adjusted ROS values by chemiluminescence increase the correlation with the intracellular $H_2O_2$ level as measured by DCF. However, this correlation with the intracellular $O_2^{-}$ by HE decreases with adjusted ROS values in the chemiluminescence results for the same specimen. DCF$^{+ve}$ sperm is also correlated with percentage of HE$^{+ve}$ sperm in a given sample ($r = .38$, $P<.001$).

When we examined the effect of low and high sperm concentration after log transformation, regression analysis showed no significant effect of concentration on correlation of ROS by flow cytometry and chemiluminescence (Table 2).

**Evaluation of the Sperm Concentration on Chemiluminescence Results**

No effect of sperm concentration (2.5–20 $\times$ 10^6 sperm/mL) was seen in ROS levels by luminol (unadjusted $P=.15$; or adjusted $P=.07$) or lucigenin ($P=.53$). The Spearman correlation showed significant negative dependence of the adjusted ($r = -0.887$, $P<.001$) or log-adjusted lucigenin ($r = -0.887$, $P<.001$) on sperm concentration.

**Basal Intracellular ROS Level Measured by Flow Cytometry**

The basal levels of $H_2O_2$ and $O_2^{-}$ in neat semen of both proven and unproven donors (n = 18) were higher (7.9 ± 10.3 and 3.4 ± 2.5). Proven fertile men (n = 3) showed lower basal ROS levels in the neat samples with both DCF (5.0 ± 4.1) and HE (3.4 ± 1.6).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Neat semen</th>
<th>Mature sperm</th>
<th>Immature sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonstimulated</td>
<td>Stimulated</td>
<td>Nonstimulated</td>
</tr>
<tr>
<td>ROS: luminol, $10^6$ cpm</td>
<td>0.1 (0, 0.2) 0.4 (0.1, 5.7)</td>
<td>28</td>
<td>0.1 (0, 0.2) 58.3 (40.7, 74.8)</td>
</tr>
<tr>
<td>Adjusted ROS: luminol, $10^6$ cpm/20$10^6$ sperm</td>
<td>0.1 (0, 0.2) 1.3 (0.1, 6.8)</td>
<td>.037</td>
<td>0.2 (0.0, 0.7) 111.8 (61.5, 415.4)</td>
</tr>
<tr>
<td>ROS: lucigenin, $10^6$ cpm</td>
<td>0.0 (0, 0.2) 0.7 (0.4, 1.9)</td>
<td>.001</td>
<td>0.0 (0, 0) 3.0 (1.1, 4.6)</td>
</tr>
<tr>
<td>Adjusted ROS: lucigenin, $10^6$ cpm/20 $10^6$ sperm</td>
<td>0.0 (0, 0.2) 2.0 (0.7, 7.1)</td>
<td>.001</td>
<td>0.0 (0, 0) 8.2 (3.3, 14.5)</td>
</tr>
<tr>
<td>Hydrogen peroxide marker: DCF$^{+ve}$ sperm, %</td>
<td>3.2 (1.6, 9) 42.2 (35.7, 46.6) &lt; .001</td>
<td>20.4 (5.7, 36.1)</td>
<td>57.0 (32.4, 65.4)</td>
</tr>
<tr>
<td>Superoxide anion marker: HE$^{+ve}$ sperm, %</td>
<td>3.2 (1.5, 4.4) 7.2 (2.0, 10.3)</td>
<td>.06</td>
<td>0.9 (0.6, 1.9) 13.3 (3.0, 23.2)</td>
</tr>
</tbody>
</table>

Note: Data are given as median (25th, 75th percentile). $P < .05$ was considered significant using the Wilcoxon signed-rank test.

$^a$ Significant difference between stimulated mature and stimulated neat sperm fractions.

$^b$ Significant difference between stimulated immature and stimulated neat sperm fractions.

$^c$ Significant difference between mature and neat sperm fractions.

$^d$ Significant difference between immature and neat sperm fractions.

$^e$ Significant difference between mature and immature sperm fractions.

Comparison of ROS Negative Samples by Flow Cytometry

When all aliquots (stimulated and unstimulated) were analyzed, 13 aliquots tested negative by luminol (ROS = 0 cpm/20 × 10⁶ sperm), and 74 tested positive by luminol (ROS ≥ 0.2 cpm/20 × 10⁶ sperm; cutoff established by our lab). In contrast, both DCF and HE were able to detect ROS in all luminol-negative samples. Positive luminol samples showed significantly higher levels by DCF (33.9 ± 2.7; P = .0002) and HE (6.58 ± 0.98; P = .006) compared with luminol-negative aliquots (14.5 ± 4.6 for DCF and 3.02 ± 0.80 for HE-positive samples; Fig. 3).

Similarly, when we compared 44 lucigenin-negative and 43 lucigenin-positive aliquots by flow cytometry, both DCF and HE were able to detect ROS in lucigenin-negative samples. Lucigenin-positive aliquots showed significantly higher levels by DCF (41.7 ± 3.8; P < .0001) and HE (9.57 ± 1.50; Fertility and Sterility® 2009.)
DISCUSSION

In our study, we validated that flow cytometry is as useful and accurate as the chemiluminescence assay for the measurement of ROS in human semen. Furthermore, by using both DCF and HE probes, we can simultaneously measure the two main types of ROS generated intracellularly, namely, $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$. The increase in ROS values detected by both DCF and HE probes after exposure to $\text{H}_2\text{O}_2$ confirms their ability to specifically measure $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$. This is in agreement with Guthrie and Welch (19), who reported that these probes can selectively measure $\text{H}_2\text{O}_2$ and $\text{O}_2^{-}$. The significant relationships between DCF and luminol ($\text{H}_2\text{O}_2$) and HE and lucigenin ($\text{O}_2^{-}$) additionally validate these probes for sperm ROS measurements. Another important finding that we are reporting for the first time is that intracellular $\text{H}_2\text{O}_2$ can be detected using flow cytometry even in aliquots that test negative by chemiluminescence.

Flow cytometry is highly reproducible technology that allows simultaneous measurement of multiple parameters in a selected cell population. Recently, it was shown to accurately measure sperm count and other parameters such as antisperm antibodies as well as sperm DNA damage (28). Similar data have been reported on the assessment of sperm concentration, apoptosis, and leukocyte concentration using...
In our study, we demonstrated that specific intracellular ROS (O$_2$-$\cdot^\ast$ and H$_2$O$_2$) levels can be measured simultaneously in human spermatozoa with flow cytometry using DCF and HE probes. Our results showed that neat, mature, and immature spermatozoa had different levels of intracellular ROS. Oxidative sperm damage occurs when the intracellular ROS levels exceed the available antioxidant capacity of the cells. OS may cause damage to the sperm DNA, proteins, and lipids, leading to male factor infertility (32–34). ROS is produced both by leukocytes and abnormal spermatozoa (31). De Iuliis validated the use of HE as a probe together with flow cytometry to measure OS and compared this with other methods such as high pressure liquid chromatography (HPLC) and chemiluminescence for the investigation of intracellular O$_2$-$\cdot^\ast$ production (31). While there are reports on normal ROS levels detected by chemiluminescence in fertile men, similar data on intracellular ROS using HE as a probe together with flow cytometry are lacking (17).

In our study, we previously reported that chemiluminescence may be incor-rect for measuring intracellular ROS in semen samples from animals using both DCF and HE stains (19, 21, 29, 30) or HE only (31). De Iuliis validated the use of HE as a probe together with flow cytometry to measure OS and compared this with other methods such as high pressure liquid chromatography (HPLC) and chemiluminescence for the investigation of intracellular O$_2$-$\cdot^\ast$ production (31). While there are reports on normal ROS levels detected by chemiluminescence in fertile men, similar data on intracellular ROS using HE as a probe together with flow cytometry are lacking (17).

We are reporting for the first time the basal levels of the intracellular ROS for neat, immature, and mature sperm fractions. We observed a shift in the pattern of O$_2$-$\cdot^\ast$ and H$_2$O$_2$ levels in neat semen and in immature and mature sperm fractions prepared by double-density gradient centrifugation. This has not been reported earlier in the literature as largely global (intra- and extracellular) ROS levels were measured. Taking into account the advantages of flow cytometry, this might be a useful method for accurate studies on ROS in infertile men. In addition, it will provide more information on the specific ROS levels selectively in viable spermatozoa.

Measuring ROS levels using flow cytometry has many advantages such as [1] speed, [2] accuracy, and [3] reproducibility. In addition, our study demonstrated that flow cytometry detected intracellular ROS levels even in samples that tested negative by chemiluminescence. The clinical implication of our finding is that samples that would otherwise have tested negative for ROS chemiluminescence may still be compromised, with sperm damage resulting in impaired fertilization potential.

Another significant advantage of using flow cytometry to measure ROS levels is that semen samples with very low sperm count (oligozoospermia) can be accurately evaluated. There is evidence that sperm count is declining (37), and more men with very low sperm counts (with sperm concentration $<5 \times 10^6$/mL) are opting for assisted reproductive technologies such as IVF/Intracytoplasmic sperm injection (37, 38). Flow cytometry provides a reliable means of assessing ROS production at such low concentrations.

We previously reported that chemiluminescence may be accurate and reliable but only in samples with sperm concentration $>1$ million/mL (12, 39). Its sensitivity declines significantly even in specimen with sperm concentrations $<5$ million/mL (14, 15). In this current study, we performed measurements by luminometer using sperm samples with low concentration and found similar values for specimen with sperm concentrations of 2.5 and $>5 \times 10^6$/mL.

Our findings showed that using adjusted ROS values improves the relationships of lucigenin and luminol with the intracellular H$_2$O$_2$ level, but it may weaken such a relationship with the intracellular O$_2$-$\cdot^\ast$ level. This shows that adjusting

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**TABLE 2**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>DCF$^\ast$ve sperm, %</th>
<th>HE$^\ast$ve sperm, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$r$</td>
<td>$P$</td>
</tr>
<tr>
<td>Luminol, $\times 10^6$ cpmp</td>
<td>0.57</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Adjusted luminol</td>
<td>0.621</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Lucigenin, $\times 10^6$ cpmp</td>
<td>0.51</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>Adjusted lucigenin</td>
<td>0.56</td>
<td>$&lt;.001$</td>
</tr>
<tr>
<td>DCF$^\ast$ve sperm, %</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>HE$^\ast$ve sperm, %</td>
<td>0.38</td>
<td>$&lt;.001$</td>
</tr>
</tbody>
</table>

*Note: Adjusted ROS = $\times 10^6$ cpmp/20 $\times 10^6$ sperm. Parameters’ associations were assessed using Spearman correlation. $^a$Log values were used, and the effect was assessed using the interaction term in a linear model.*

sperm concentration is critical for intracellular H₂O₂, which is the main free radical detected by the luminometer (40). One of our study limitations was the relatively small sample size. We are planning a large-scale study using both fertile and infertile specimens, including those from oligozoospermic men, to compare both technologies in terms of sensitivity and specificity and to identify the best cutoff value for each probe.

In conclusion, we have standardized ROS measurement using the flow cytometry specific probes for simultaneous detection of both intracellular O₂⁻· and H₂O₂ selectively in viable human spermatozoa. We are reporting for the first time the basal levels of the intracellular ROS for neat, immature, and mature sperm fractions. Flow cytometry and chemiluminescence may be comparable in specimens producing high levels of ROS; however, flow cytometry is superior in samples generating low levels of ROS and in low-concentration (oligospermic) semen specimens. Use of adjusted ROS levels is useful in ROS measurement by chemiluminescence. Luminol and lucigenin results are related to intracellular H₂O₂ and O₂⁻· levels, confirming their global affinity to any free radical. Specimen testing negative by chemiluminescence assay may still have the ability to produce intracellular H₂O₂, which can be easily detected by flow cytometry.

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