Reactive oxygen species in human semen: validation and qualification of a chemiluminescence assay

Wayne Vessey, B.Sc.,a Ana Perez-Miranda, Ph.D.,a Rachel Macfarquhar, B.M.L.S.,a Ashok Agarwal, Ph.D.,b and Sheryl Homa, Ph.D.a,c

a Andrology Department, The Doctors Laboratory, London, United Kingdom; b Andrology Center, Cleveland Clinic, Cleveland, Ohio; and c Andrology Solutions, London, United Kingdom

Objective: To standardize and validate an assay for reactive oxygen species (ROS) in human semen.

Design: ROS levels assayed in blanks, negative and positive control samples (30% H2O2), and human semen, with the use of a luminol-based chemiluminescence assay measured in a single tube luminometer.

Setting: Andrology laboratory.

Patient(s): Semen samples from 19 men attending for routine semen analysis.

Intervention(s): None.

Main Outcome Measure(s): ROS levels reported in relative light units (RLU) per second, adjusted for sperm concentration.

Result(s): The ROS assay equipment performed according to expectations, generating a chemiluminescence signal for positive control samples and semen samples that decayed rapidly and was captured within 10 minutes. Blanks and negative control samples gave negligible readings. There was no significant intra- or interassay variation. Interference from extraneous factors was negligible. The assay distinguished changes in ROS over a wide range of concentrations and provided consistent results between reagent batches. Working reagents remained stable for 3 months. Acceptable levels for negative and positive control samples were established to set criteria for the test passing or failing on any given day. The assay was sensitive to ambient temperature >25°C. ROS declined significantly with time after ejaculation. Mechanical agitation doubled ROS production in semen.

Conclusion(s): These results validate the ROS assay and demonstrate that it is a highly reliable and accurate diagnostic test. (Fertil Steril 2014;102:1576-83. © 2014 by American Society for Reproductive Medicine.)

Key Words: Reactive oxygen species, oxidative stress, chemiluminescence, human semen, male infertility

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/vesseyw-reactive-oxygen-species-semen-chemiluminescence/

Oxidative stress is well established as a leading contributing factor to male infertility (1–3). The presence of excess levels of reactive oxygen species (ROS) has been associated with damage to the structural and functional integrity of sperm (1, 4–6) and interferes with capacitation and fertilization (6–10). Routine testing for male infertility is currently limited to semen analysis as the most widely accepted diagnostic test. A significant proportion of male infertility remains unexplained because there is a paucity of standardized tests available to distinguish molecular events that may cause damage to sperm integrity but are not necessarily evident in a semen analysis (11, 12). Several reports, including an extensive review of the literature with the use of a Medline search of studies published from 1980 to 2007, indicate that assays for oxidative stress may be promising for fertility testing (2, 11, 13, 14). The lack of standardization of protocols for ROS measurement has prevented it from being incorporated into routine clinical diagnostic practice. Because of the importance of oxidative stress in identifying potentially infertile patients and the role it plays in sperm damage, we established an assay for the detection of ROS in semen according to methods published by Agarwal et al. (15, 16) with a view to using this as a standard clinical diagnostic test. More important, we consider this test to be essential for providing an explanation for hitherto unexplained male infertility.

Over the past two decades, various assays have been proposed to determine the presence of free radicals in
biologic material [14]. The choice of sample to assess differs between laboratories, e.g., seminal plasma [17], whole semen or washed sperm suspensions [18–20], or blood [21, 22]. Furthermore, there are differences between methods for reporting ROS values regarding how units are expressed, i.e., relative light units (RLU) or counted photons per minute (cpm) [23], and whether they should be reported per million, 10 million, or 20 million sperm. The assay may be performed with the use of different methodology and equipment. Direct measurement of free radicals can be conducted with the use of electron paramagnetic resonance spectroscopy [24]. However, because the half-life of free radicals is short, the experiments must be performed at low temperatures. Other methods include quantification of nitroblue tetrazolium (NBT) activity [25, 26], measurement of cytochrome c reduction [25], and flow cytometry with the use of fluorescent probes [27, 28]. The most commonly used technique is the chemiluminescence assay, using luminol or lucigenin to detect oxidized end products [16, 23, 27, 29]. Lucigenin has the disadvantage that it can only detect extracellular free radicals, primarily superoxide, whereas luminol can detect both intracellular and extracellular deoxygenation, including hydrogen peroxide, superoxide, and hydroxyl ions. Free radicals combine with luminol to produce a light reaction. The photons are converted to an electrical signal in a luminometer. Chemiluminescence can be detected with the use of either a single-tube luminometer or a multiple-tube luminometer with different manufacturer’s specifications [23]. The single-tube luminometer is a relatively inexpensive piece of equipment compared with a multiple-tube luminometer or plate analyzer, and the chemiluminescence assay is uncomplicated to perform [16, 23]. It is very efficient for monitoring ROS levels and can accommodate ≥ 40 samples per day, which is more than sufficient for most specialized andrology laboratories. We therefore chose this method for standardizing our assay as we sought to validate it for use in measuring oxidative stress in human semen.

MATERIALS AND METHODS

Semen Samples

A total of 23 semen samples were obtained from 19 men attending the andrology laboratory for routine semen analysis. Each of the men had given informed consent to use the remainder of their sample before inclusion in the validation project, and the study was approved by the Institutional Quality Management Board. Institutional Review Board approval was deemed to be unnecessary. The sample group consisted of fertile and infertile men. Men were given instructions for sample production according to World Health Organization (WHO) 2010 guidelines [30]. Only compliant samples were accepted. Semen samples were produced on site after 2–5 days’ sexual abstinence. Samples were maintained for 10–30 minutes at 36 ± 1°C to liquefy and then assessed immediately. Semen analysis was performed as part of routine diagnostic testing according to the WHO 2010 criteria [30]. This included identification of polymorphonuclear leukocytes (PMN) with the use of a European Community (CE)–marked kit that differentiates round cells on the basis of their peroxidase content (Leucoscreen; Microm). All 23 samples used for ROS measurement had sperm concentrations > 1 million/mL, because ROS measurement may be unreliable when the sperm concentration falls below that value [23].

Measurement of Chemiluminescence

ROS were measured with the use of luminol, which is oxidized in the presence of ROS, resulting in chemiluminescence. The luminescence was measured with the use of a CE-marked single-tube luminometer (Turner Biosystems Instrument Modulus Model no. 9200-001). The general methodology for the test has been reviewed elsewhere [31]. A 100 mmol/L luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma-Aldrich) stock solution was prepared in advance in dimethylsulfoxide (DMSO). The solution was stored at room temperature in the dark in an aluminium foil–covered polystyrene Falcon tube. A luminol working solution (5 mmol/L luminol prepared in DMSO) was freshly prepared from the stock each day, covered in foil, and stored in the dark. All reagents were brought to room temperature before assaying. For blank tubes, 400 μL phosphate-buffered saline solution (PBS) was assayed in the absence of luminol. Negative control samples contained 400 μL PBS with 10 μL 5 mmol/L luminol working solution. Positive control samples contained 395 μL PBS, 5 μL 30% H₂O₂ (VWR), and 10 μL 5 mmol/L luminol working solution. All samples were mixed gently immediately before chemiluminescence reading in the luminometer. For measuring chemiluminescence in semen samples, liquefied whole semen was gently mixed with the use of a plastic pipette, and 400 μL was aliquoted with the use of a positive displacement pipette into a 1.5 mL microfuge tube. Ten microliters of luminol working solution was added and mixed gently immediately before reading in the luminometer. Care was taken to ensure no bubbles were present before measurement, because that can affect the reading. Because the reagent is light sensitive, the assay is performed with electrical lights switched off and the blinks drawn to reduce light exposure. Chemiluminescence is reported as RLU/s.

Reporting Results

RLU were generally assessed at 1-minute intervals over the course of 10 minutes for control and test samples, except where indicated, and the mean RLU/s calculated. It is important to correct for negative control values when reporting test results, to eliminate any background variation. The negative control mean value is subtracted from the test mean value to give the true value of the test sample.

Statistical Analysis

A Welch t test was used to analyze microtube variation and luminescence signal, ambient temperature, mechanical manipulation, patient time interval, and differences between reagent batches. This test is a modification of Student t test and was implemented to account for the unequal variance between samples [32]. A χ² test distribution was used to analyze luminol stock batches, the effects of environmental light
exposure, basal luminescence related to the photomultiplier cell, blank and negative control samples, positive and negative control samples, and semen samples, luminol half-life, and positive control samples run on the same day by three operators. A $P$ value of $<.05$ was considered to be statistically significant.

**RESULTS**

**Equipment Validation**

Figure 1A demonstrates that the equipment can detect a signal from the reagent standard and is able to distinguish changes in ROS over a wide range of concentrations. Average RLU levels increase with increasing concentrations of $\text{H}_2\text{O}_2$. Thirty percent $\text{H}_2\text{O}_2$ was selected as the stock concentration to use for the positive control in all further experiments, as it gives a suitable signal, in agreement with earlier studies (31). Chemiluminescence can also be detected in semen over a wide range (Fig. 1B). The signal decays rapidly after addition of luminol and shows a similar pattern for all samples. Samples with higher ROS activity show a maximum RLU/s value within the 1st minute of luminol addition. Thereafter, the signal decays but stabilizes by 10 minutes. Samples with lower initial signals do not decay as rapidly. Because the chemiluminescence of the luminol signal is relatively short lived, it is crucial to capture the signal measurements as soon as luminol is added. The RLU/s can be measured at 1-minute intervals until the signal reaches a plateau and then averaged to give a value for each sample. Because the signal is generally captured within 10 minutes, RLU values should ideally be calculated by averaging values reported for this period of time. To determine any effects of the equipment on the assay, RLU values were measured in seven replicates of tubes filled with either air or water. A similar low-level background signal was detected from both tube types and remained consistent whether tubes were assayed on the same or on different days, confirming that there was no significant variability of the basal luminescence signal related to the photomultiplier cell (Supplemental Table 1, available online at [www.fertstert.org](http://www.fertstert.org)).

**Internal Quality Control Procedures and Criteria**

**Interassay and intra-assay variability.** For the test to work efficiently, RLU values should be relatively low for blanks and control samples, whereas a positive control should give a relatively high reading. Blanks provide background luminescence readings from the luminometer, whereas control samples show background luminescence and any impurities in reagents. These values should be similar. Blanks and negative control samples produce minimal chemiluminescence (Fig. 2A and B), and positive control samples show a marked chemiluminescence signal (Fig. 2C) following luminol addition for each of 3 replicates assayed on the same day. The pattern of the signal was almost identical for each triplicate with no significant difference ($P>.1$), demonstrating intra-assay consistency. Blanks, negative control samples, and positive control samples were additionally measured in duplicate on 20 different days. Blank and negative control samples consistently demonstrated relatively low mean RLU values ($61.4 \pm 1.8$ and $62.5 \pm 1.2$, respectively), and the positive control consistently demonstrated high mean RLU values ($83,169 \pm 1,864$). There was no significant difference in RLU values for control samples or blanks observed on different days ($P>.1$), demonstrating negligible interassay variability. There was no significant difference in assays performed by 3 different operators (Fig. 2D; $P>.1$).

**Establishing acceptable ranges for positive and negative control samples.** To validate the ROS test, acceptable levels for negative and positive control samples must be established to set criteria for the test passing or failing on any given day. A negative control sample and a positive control sample are run with every assay to ensure reliability of results. Each control sample should give consistent readings for each assay.

---

**FIGURE 1**

Generation of a chemiluminescence signal generated in a single tube luminometer. (A) Standard curve showing chemiluminescence of luminol with increasing concentrations of $\text{H}_2\text{O}_2$. To determine whether the equipment can detect changes in concentration of reactive oxygen species (ROS), a standard curve was generated with the use of hydrogen peroxide ($\text{H}_2\text{O}_2$) as an internal standard control. The test was calibrated with the use of increasing percentages of $\text{H}_2\text{O}_2$ between 0 and 30% diluted in AnalaR water. Phosphate-buffered saline solution (PBS) was used as a negative control. Five $\mu$L of each concentration was added to 395 $\mu$L PBS and mixed well. At time 0, 10 $\mu$L luminol (5 mmol/L) was added to the first sample and ROS was assessed in the luminometer every minute for 10 minutes, as this was determined to be the optimum time to capture the signal. Values were averaged. This was repeated for each dilution. (B) Decay of chemiluminescence signal over time following addition of luminol to six random semen samples. Ten $\mu$L luminol (5 mmol/L) was added to 400 $\mu$L liquefied semen. ROS were immediately assessed in the luminometer every minute for 10 minutes. RLU = relative light units.

However, the values will not be exactly the same each time. It is therefore important to establish the highest and lowest values that would be considered to be within range for each control. If the control value falls outside of this range, the assay fails. In other words, it is important to establish action limits for positive and negative control values.

RLU values for every negative and positive control sample were recorded over a 3-year period. The mean and standard deviation for 219 negative control samples was 66.7 ± 17.8 RLU/s (range 43.3–125.2 RLU/s). For 207 positive control samples it was 93,111 ± 24,237 RLU/s (range 51,336–211,170 RLU/s). Acceptable lower and upper limits were established as 2 standard deviations outside the mean value for each control. This was determined to be 31.1–102.3 RLU/s for the negative control, and 44,637–141,586 RLU/s for the positive control. If values for these control samples fall outside of these limits, the test fails. The individual RLU/s values were relatively consistent between 2 standard deviations for both negative and positive control samples. For the negative control data, 15 samples exceeded the upper threshold limit, whereas 8 positive control samples fell outside the accepted limits. Tests run on these days would be considered to be unreliable. None of the negative or positive control samples fell below the acceptable limits during this time.

Reproducibility of ROS levels assessed with different luminol stock solutions. If the ROS test is robust, ROS values should be similar regardless of the batch of luminol stock solution used for the assay. Three assays were set up in duplicate using a different batch of luminol for each replicate (Supplemental Table 2, available online at www.fertstert.org). There was no significant difference in RLU/s between batches for positive control or semen samples (P > .1): (all values in RLU/s) assay 1: 73,914 and 69,445 for positive control, 15,741 and 15,201 for semen sample batches 1 and 2, respectively; assay 2: 86,984 and 90,646 for positive control, 2,756 and 2,654 for semen sample batches 3 and 4, respectively; assay 3: 76,310 and 74,032 for positive control, 1,432 and 1,414 for semen sample batches 5 and 6, respectively.

Determination of the stability of luminol stock solution. Luminol is light sensitive and is stored in a dark environment; however, it may oxidize with time, decreasing its performance in the test. To determine its stability, positive control values were monitored over several months using three separate luminol stock solutions. The average RLU/s for positive control samples using the same batch of luminol stock solution and other reagents remained relatively constant during the first 3 months, regardless of the stock solution (Supplemental Fig. 1, available online at www.fertstert.org). Although stock 3 appeared to have slightly less stability and lower values of ROS than stocks 1 and 2, the values were still within range of 2 standard deviations from the mean values for all samples (see section on “Establishing acceptable ranges for positive and negative control samples”). Average RLU/s for positive control samples declined after ~15 weeks for all three stock solutions tested. Therefore, fresh stocks are prepared every 2–3 months to ensure assay reliability.
Effect of External Factors on the Chemiluminescence Signal

Environmental light exposure. The chemiluminescence signal can be affected by extraneous light. Phosphorescence can occur in almost any material, such as glass or polypropylene tubes, following exposure to room light, which may interfere with the assay. Assay tubes kept in light for 7 hours in dark conditions to keep background readings to a minimum.

Effects of temperature on the luminescence signal. Some variability in the luminescence signal was observed in both negative and positive controls as ambient temperature increased up to 25°C (Supplemental Fig. 2, available online at www.fertster.org). This may reflect daily changes unrelated to temperature as it did not become significant (P>0.99). Nevertheless, the assay should ideally be performed with the use of tubes previously kept in dark conditions to keep background readings to a minimum.

Measurement of ROS in Semen Samples

Determination of the appropriate time after ejaculation to assess ROS levels. ROS were assayed in semen at increasing time points after ejaculation. Figure 3 shows that ROS levels in semen do not remain constant, even within 60 minutes after ejaculation and that they decline significantly with time after ejaculation (P<.001). This decline in ROS levels is observed regardless of the amount of the initial measured ROS level in the semen sample.

Reproducibility of the ROS test in semen. To test the reproducibility of the ROS assay within individual patients, 4 patients were tested for seminal ROS levels and again 2–3 days later. Results are presented in Table 1. For patients 1, 2, and 3, ROS values were similar when they were reassessed 2–3 days later. However, ROS values for one individual (patient 4) were considerably increased in the second assay.

Effect of mechanical agitation on production of ROS in semen. Mechanical agitation of semen samples can generate ROS. Two semen samples were split after liquefaction and one half vigorously vortexed for 20 seconds before and 10 seconds after addition of luminol. The other half was not vortexed at all. The mean RLU/s for the vortexed samples were approximately twice that of the samples that were not vortexed (625 vs. 300 RLU/s, sample 1; 466 vs. 279 RLU/s, sample 2, and 3, ROS values were similar when they were reassessed 2–3 days later. However, ROS values for one individual (patient 4) were considerably increased in the second assay.

DISCUSSION

The purpose of this study was to validate a chemiluminescent assay for determination of ROS in human semen. We have

| TABLE 1 |

<table>
<thead>
<tr>
<th>Patient Abstinence (d)</th>
<th>Sperm count (10^6/mL)</th>
<th>PMN count (10^6/mL)</th>
<th>RLU/s/10^6 sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay no.</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Patient 1</td>
<td>2</td>
<td>2</td>
<td>186</td>
</tr>
<tr>
<td>Patient 2</td>
<td>3</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>Patient 3</td>
<td>2</td>
<td>2.5</td>
<td>11.1</td>
</tr>
<tr>
<td>Patient 4</td>
<td>5</td>
<td>3</td>
<td>15</td>
</tr>
</tbody>
</table>

Note: ROS levels were measured in each patient after 2–5 days’ sexual abstinence. ROS levels were assessed again in a second sample produced 2–3 days later. The data were adjusted for sperm count to account for the influence of sperm on ROS production. PMN = polymorphonuclear leukocytes.
demonstrated that this assay is a valid test that is reliable and reproducible. This report validates and qualifies the equipment used for the test, the Modulus single-tube luminometer, and the method used for ROS measurement in human semen. The method is similar to that reported previously [15, 16]. The test is robust, the equipment performing as expected, detecting chemiluminescence signals over a broad range of values with the use of the methods described, for both positive and negative control samples as well as semen samples. The assay can distinguish between blanks and negative and positive control samples, consistently producing low values for blanks and negative control samples with no significant difference. It is important to subtract negative control values from sample readings to eliminate any possible variation. The assay can detect changes in concentration of ROS, as shown in the standard curve where RLU/s values are positively correlated with increasing ROS concentration.

For all samples, the rate of decay of the chemiluminescence signal differs between samples but usually plateaus within 10 minutes after addition of luminol. By reporting the value for the first minute only, the remainder of the signal would be lost. On the other hand, because the decay of the signal tends to be gradual, it would be almost impossible to select a time point that represents the beginning of the plateau. We therefore decided to measure the signal over the entire period of the decay and take an average reading, which is more reliable than a single measurement. Other studies have averaged the chemiluminescence signal over a 15-minute period [15, 19, 20, 33], but those assays were performed with the use of luminometers different from the brand used in our study and with different specifications.

Our assay shows a high degree of reliability and accuracy, with consistent results between replicates for negative and positive control samples (intra-assay) and between assays conducted on different days (interassay). This confirms earlier observations by Kobayashi et al. [34] who assessed quality control for the luminol-based ROS test. Furthermore, there was no significant intra- or interassay variation due to the photomultiplier cell. For the test to be reproducible, it is important that operators trained to perform the test produce similar data and that similar results are obtained with different batches of stock solution, which we have demonstrated. Stock solutions remain stable for up to 15 weeks, suggesting that they should be newly prepared at least every 3 months to ensure reliability of results. Regardless, results should be continually monitored in case there is an unexpected change in the positive control reading.

Although the test is reliable, the accuracy of the assay can be affected by several factors [15, 16, 23, 29]. Strict quality control is essential to ensure that the volumes and concentrations of reagents are kept constant [23], because this can affect the performance of the assay. We observed some variability in results with the use of chemical reagents from different sources, indicating that the assay may be sensitive to contamination of trace metals and other impurities (data not shown). Heavy metals, such as copper and to a lesser extent cadmium, iron, silver, and lead, quench peroxidase-mediated luminol-enhanced chemiluminescence, whereas zinc enhances the signal at high concentrations [35]. The mechanism of action of this observation is likely due to interaction with the catalytic subunit of the peroxidase itself, which is an important factor when measuring ROS production in neat semen owing to the presence of peroxidase-positive cells. Nonenzymatic luminol-induced chemiluminescence in the presence or absence of H2O2 can be catalyzed by trace amounts of metal ions, such as cobalt, iron, chromium, copper, and manganese [36, 37], and in combination they are either stimulatory or inhibitory depending on the metal ions present [38]. Metal ions and H2O2 concentration also affect chemiluminescence intensity [36, 37]. Other contaminants also have quenching or enhancing effects on luminol-generated chemiluminescence, such as phenolic compounds and amino acids [39]. Nonspecific interference from NADPH- or thiol-containing compounds can artificially generate chemiluminescence [29] whereas low-molecular-weight free radical scavengers, such as uric acid or ascorbate, quench the signal [40]. The chemical composition of the buffer used in the assay and the pH have a considerable impact on chemiluminescence [38–40], which is also influenced by chelating agents [38].

Temperature is a critical factor for rates of reactions and affects the rate of light emission from chemiluminescent reactions. Schinde [41] demonstrated the effect of temperature on the chemiluminescence of luminol in aqueous aliphatic amines, where the signal increases with increasing temperature then decreases once it reaches an optimum value. We have shown a similar effect of changes in ambient temperature on the luminescence signal and have confirmed earlier findings that exposure to extraneous light should be kept to a minimum. Samples should ideally be read with the use of tubes previously kept in dark conditions, because exposure to light may affect the chemiluminescence.

To control for impurities, a blank reading provides the background luminescence of the equipment, whereas a negative control provides additional information about luminescence caused by any impurities in the reagent. Our experiments showed minimal reagent impurities with similar chemiluminescent values for blanks and negative control samples. We have also shown that ROS are generated artificially in semen through the action of mechanical agitation; therefore, vigorous mixing of the sample should be avoided. This supports earlier evidence that shearing forces in multiple centrifugation, resuspension, and vortexing during sperm preparation for assisted conception procedures result in ROS production [20, 40, 42–44].

We measured seminal ROS at different times after ejaculation and have shown that ROS levels decline with time. A decrease in ROS with semen age was demonstrated previously [34], where the signal diminished significantly 60 minutes after ejaculation. That and other studies recommended assessing samples at 30 minutes after ejaculation and certainly within 60 minutes after ejaculation [15, 16]. Time points before 60 minutes were not measured in any of these studies. We examined shorter time intervals up to 60 minutes after ejaculation to determine the stability of ROS levels in semen. Our data show ROS levels declining significantly even after 20 minutes, and the rate of decline is not consistent between
samples and do not stabilize. The trend suggests that ROS may decline even immediately after ejaculation. Given that seminal fluid is a mixture of secretions from the testes and the male accessory glands that combine only at ejaculation, and that seminal fluid contains antioxidants and antioxidant pathways, it is not surprising that any free radicals released into the semen are short lived. The half-life of ROS also depends on the activity of the antioxidant pathways that they are exposed to. It is therefore very important that the age of the sample at the time of assay is consistent. We have chosen to assess ROS in semen 20 minutes after ejaculation, because this usually gives sufficient time for sample liquefaction and to still obtain measurable levels. This requires men to be able to produce their samples on site to avoid delay and to record the time of ejaculation.

Our preliminary studies indicate that seminal ROS values are relatively consistent from one assay to the next when measured 2–3 days later, although one individual showed an increased value. It is not clear whether the period of abstinence affects ROS production, but it is unlikely to have affected the difference in ROS levels in samples from this patient, because the period of abstinence was lower for the second sample. It is more likely explained by the exceptionally high levels of PMN that were observed in the second assay, because PMNs are a major source of ROS (25, 29, 33). Although the effects of mechanical agitation, time from ejaculation, and reproducibility of seminal ROS values are noteworthy, the sample size in this study was relatively small and may warrant further investigation.

In conclusion, the ROS assay equipment performed as expected, providing a high degree of assurance that this method for ROS testing is robust and will produce a result that is consistent with specifications and quality attributes published in the scientific literature (15, 16). The ROS assay is not currently offered in the U.K. as a routine test for infertility. It is envisaged that it will be implemented as a simple, reliable, and accurate clinical diagnostic test to aid in the diagnosis of male infertility.

REFERENCES


32. Ruxton G. The unequal variance t test is an underused alternative to Student t test and the Mann-Whitney U test. Behav Ecol 2006;17:688–90.


Stability of luminol stock solutions. The 100 mmol/L stock solutions of luminol were prepared as described in Materials and Methods. RLU/s for positive control samples using each luminol stock solution were recorded over several months from 2010 to 2012. RLU = relative light units.

SUPPLEMENTAL FIGURE 2

Effect of temperature on the chemiluminescence signal. To determine whether ambient temperature could affect the assay, blank and negative controls (A) and positive controls (B) were run on different days at ambient temperatures ranging from 22°C to 29°C. On each day, the room temperature was recorded and blank, negative and positive control samples prepared as described in Materials and Methods. RLU = relative light units.

### SUPPLEMENTAL TABLE 1

Descriptive parameters for the seven air-filled (A) and seven water-filled (W) tubes assayed on three separate days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>W</td>
<td>A</td>
</tr>
<tr>
<td>n</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Min</td>
<td>56.3</td>
<td>60.4</td>
<td>63.2</td>
</tr>
<tr>
<td>Max</td>
<td>64.4</td>
<td>69.4</td>
<td>69.5</td>
</tr>
<tr>
<td>Mean</td>
<td>61.2</td>
<td>64.2</td>
<td>65.5</td>
</tr>
<tr>
<td>Standard error</td>
<td>1.0</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Variance</td>
<td>6.3</td>
<td>10.7</td>
<td>6.8</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.5</td>
<td>3.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Median</td>
<td>61.5</td>
<td>62.8</td>
<td>64.7</td>
</tr>
</tbody>
</table>

Note: To determine whether the photomultiplier cell contributes to variability of the signal, the background and luminescence changes due to the air-water phase transition was measured. This was achieved by measuring the basal luminescence signal in air- and water-filled tubes on three separate days. The basal luminescence signal was measured in seven air-filled tubes and seven tubes filled with 400 μL deionized water. The signal for each tube was monitored at 1-minute intervals and averaged. This experiment was repeated on three separate days.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Assay 1</th>
<th>Assay 2</th>
<th>Assay 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminol batch</td>
<td>73,914</td>
<td>69,945</td>
<td>86,984</td>
</tr>
<tr>
<td>Positive control</td>
<td>86,984</td>
<td>90,646</td>
<td>76,310</td>
</tr>
<tr>
<td>Semen sample</td>
<td>15,741</td>
<td>15,201</td>
<td>2,756</td>
</tr>
<tr>
<td></td>
<td>2,654</td>
<td>1,432</td>
<td>1,414</td>
</tr>
</tbody>
</table>

Note: To test whether different batches of stock luminol solutions produce consistent results, duplicate assays were carried out on three separate days using a different batch of luminol for each replicate. For each assay, a positive control and a semen sample were set up in duplicate as described in Materials and Methods. A stock solution batch was added to each of one replicate positive control and one replicate semen sample, and the chemiluminescence was read. The assay was repeated with the second replicate positive control and semen sample, to which a different stock solution batch was added. The chemiluminescence was read every minute for 10 minutes for the positive control and every second for 10 seconds for the semen sample, to avoid time delay between the duplicate semen assays that could affect the results. The mean values were calculated. RLU = relative light units.