

Physiologic and pathologic levels of reactive oxygen species in neat semen of infertile men

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Objective: To define physiologic levels of reactive oxygen species in infertile men and establish a cutoff value of reactive oxygen species level in neat semen with a high sensitivity and specificity to differentiate infertile men from fertile donors (controls).

Design: Reactive oxygen species levels were measured in the neat semen samples ($n = 51$) from fertile donors and infertile patients ($n = 54$).

Setting: Reproductive research laboratory at a tertiary care hospital.

Patient(s): Infertile patients from male infertility clinic.

Intervention(s): Reactive oxygen species measurement in neat semen sample using luminol-based chemiluminescence method, receiver operating characteristic curves.

Main Outcome Measure(s): Seminal reactive oxygen species levels, cutoff value, sensitivity and specificity, positive and negative predictive values.

Result(s): The best cutoff value to distinguish between healthy fertile donors and infertile men was 0.0185×10^6 counted photons per minute/ 20×10^6 sperm. At this threshold, the specificity was 82% and the sensitivity was 78%. This value can be defined as basal reactive oxygen species level in infertile men.

Conclusion(s): Reactive oxygen species levels in neat semen samples as measured by luminol-based chemiluminescence are a highly specific and sensitive test in the diagnosis of infertility. This test also may help clinicians treat patients with seminal oxidative stress. (*Fertil Steril*® 2009;92:1626–31. ©2009 by American Society for Reproductive Medicine.)

Key Words: Sperm, reactive oxygen species, chemiluminescence, sensitivity, specificity, receiver operating characteristic (ROC) curve

Free radicals are a group of highly reactive chemical molecules consisting of one or more unpaired electrons. Free radicals derived from oxygen metabolism are designated as reactive oxygen species (ROS). Reactive oxygen species generated by spermatozoa play an important role in normal physiologic processes such as sperm capacitation, acrosome reaction, oocyte fusion, and stabilization of the mitochondrial capsule in the midpiece (1–5). Reactive oxygen species produced by spermatozoa and leukocytes are scavenged by various antioxidants in the seminal plasma (6, 7). However, uncontrolled production of ROS that exceeds the antioxidant capacity of the seminal plasma leads to oxidative stress, which is harmful to spermatozoa through a variety of mechanisms (2, 6–8).

Spermatozoa are particularly vulnerable to oxidative stress because their plasma membrane is rich in polyunsaturated fatty acids (9, 10), and the cytoplasm contains low amounts of antioxidants (2, 11, 12). High levels of ROS generated by human spermatozoa damage DNA and adversely affect fertilizing potential and pregnancy rates (13–20). Between 25% and 88% of infertile men have elevated ROS levels in their semen (21). To minimize ROS levels, clinicians should identify and treat the cause of ROS (e.g., varicocele, smoking, genital tract infections) (22–27). After treatment, antioxidant supplementation may be prescribed to augment the scavenging capacity of the seminal plasma. However, treatment should be based on the patient's oxidative stress status because physiologic levels of ROS are important in spermatogenesis, and excessive antioxidant supplementation in infertile patients with normal ROS levels may impair sperm function by lowering their physiologic levels (28–30).

In infertile patients, the basal levels of ROS needed for physiologic function of spermatozoa have yet to be defined with a high specificity and sensitivity. On the other hand, studies have demonstrated that high ROS levels are an independent marker of male factor infertility (31). This “high” or “abnormal” level of ROS that is positively predictive of infertility also remains to be accurately defined (32–34). We have reported previously that ROS measurement using luminol-based chemiluminescence is both reproducible and

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reliable when samples are analyzed within the first hour of specimen collection (35).

The aim of our study was [1] to define the cutoff value of ROS in ejaculated (neat) semen samples with maximum specificity and sensitivity to detect infertile men and [2] to define an accurate reference value of ROS that documents a patient's seminal oxidative stress status to aid in the success of any therapeutic intervention.

MATERIALS AND METHODS

Sample Collection and Preparation

This study was approved by the Cleveland Clinic Institutional Review Board. Semen samples were collected from a group of men ($n = 54$) with a history of infertility who came to Cleveland Clinic for infertility treatment. They were evaluated by a single male-infertility specialist (E.S.) between October 2006 and January 2008 and referred for measurement of ROS at the Andrology Laboratory. All female partners of these men had undergone gynecologic evaluation and had normal results on a fertility workup. Normal healthy proved-fertile male volunteers ($n = 51$) provided semen samples and served as controls. All samples were collected by masturbation after sexual abstinence of at least 48 hours.

Semen Analysis

After complete liquefaction at 37°C for 20 minutes, 5 μL of each specimen was loaded on a 20- μL Microcell chamber (Conception Technologies, San Diego, CA) and analyzed for sperm concentration and motility according to World Health Organization (WHO) guidelines (36). Seminal smears were stained with Diff-Quik stain (Baxter Healthcare, McGaw Park, IL), and sperm morphology was assessed with use of WHO criteria (36).

White Blood Cells

The presence of white blood cells (WBCs) in the specimen was detected by the Endtz test (34). A 20 μL volume of liquefied specimen was placed in a Corning 2.0 mL cryogenic vial (Corning Life Sciences, Lowell, MA); 20 μL of phosphate-buffered saline solution and 40 μL of benzidine solution were added. The mixture was vortexed and allowed to sit at room temperature for 5 minutes. Peroxidase-positive WBCs, which stain dark brown, were counted in all 100 squares of the grid in a Makler chamber under the $\times 20$ bright-field objective. The results after correction for dilution were recorded as the number of WBCs counted times $10^6/\text{mL}$. Leukocytospermia was defined as the presence of $>1 \times 10^6$ WBCs/mL of semen.

Assessment of ROS Activity in Semen

Seminal ejaculates that had not undergone any additional processing (neat samples) were used for ROS measurement by chemiluminescence assay with use of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St.

Louis, MO). A 100 mmol/L stock solution of luminol was prepared in dimethyl sulfoxide. For the analysis, 10 μL of the working solution (5 mmol) was added to 400 μL of neat sperm sample. Chemiluminescence was measured for 15 minutes with a Berthold luminometer (Autolumat LB 953; Bad-Wildbad, Germany). Results were expressed as $\times 10^6$ counted photons per minute (cpm)/ 20×10^6 sperm (35, 37).

Statistical Analysis

The Wilcoxon rank sum test was used to compare semen parameters and ROS levels. Within patients, groups defined by ROS values (high and low) were compared with use of the Wilcoxon test. Associations between quantitative measures (sperm concentration, motility, and morphology) were assessed with use of Spearman correlation coefficients.

The difference in the distribution of ROS levels between infertile patients and fertile donors was assessed; the summaries of these distributions are described with use of mean, SD, and median (25th and 75th percentile). A receiver operating characteristic curve (ROC), which is a graphical plot of sensitivity versus 1 minus specificity for all possible ROS cutoffs, was used to assess the ability of ROS as a means of distinguishing patient and fertile donor values. A cutoff value was chosen that maximized the sum of estimated sensitivity and specificity. A SE and confidence interval for this optimal cutoff was obtained with use of the bootstrap resampling method (38). Bootstrap samples are samples taken with replacement from the original data and are of the same size as the original dataset. Standard deviations across bootstrap samples estimate the sampling SEs of characteristics of the original sample. All analyses were performed with use of R version 2.3.1 (The R Foundation, www.R-project.org). P values of $<.05$ were considered statistically significant.

RESULTS

Fertile Donors Versus Patients

Comparisons of overall, patient, and fertile donor groups with respect to study variables are shown in Table 1. The fertile donors had significantly higher sperm concentration and percent motility but lower ROS levels ($P < .001$).

Receiver Operating Characteristic Curve Analyses

We examined ROS levels in the study subjects. The mean \pm SD and median 25th to 75th percentiles were 0.35 ± 0.67 and 0.06 (0.02 – 0.33) $\times 10^6$ cpm/ 20×10^6 for the infertile patients and $0.01 \pm 0.02 \times 10^6$ cpm/ 20×10^6 and 0.009 (0.004 – 0.014) $\times 10^6$ cpm/ 20×10^6 for the proved-fertile donors (Table 1).

The ROS cutoff value that maximized the sum of estimated sensitivity and specificity was 0.0185. At this cutoff value (≥ 0.0185), the estimated sensitivity was 77.8% and the estimated specificity was 82.4%. The estimated sensitivity and specificity values for all possible cutoffs are shown in Table 2. The area under the ROC curve for ROS values of neat semen samples was 0.82 (Fig. 1).

TABLE 1**Summary of quantitative variables in infertile patients and healthy donors with unproved fertility.**

Variable	Overall (n = 105)	Patients (n = 54)	Donors (n = 51)
Concentration ($\times 10^6$ /mL)	83.66 \pm 65.20 ^a	65.59 \pm 82.37	102.79 \pm 30.41
Motility (%)	78 (40.2, 113) ^b	43.50 (22.45, 65.67)	100.70 (87.80, 120.50)
	62.80 \pm 20.26	54.85 \pm 24.09	71.22 \pm 9.91
ROS ($\times 10^6$ cpm/ 20 $\times 10^6$ sperm)	68 (54, 78)	58.50 (36.50, 74.00)	73.00 (64.50, 78.50)
	0.19 \pm 0.51	0.35 \pm 0.67	0.01 \pm 0.02
	0.015 (0.008, 0.078)	0.0605 (0.02, 0.3265)	0.009 (0.004, 0.014)

$P < .001$ for all comparisons. $P < .05$ was significant by Wilcoxon rank-sum test between patients and donors.

^a Mean \pm SD.

^b Median (25th and 75th percentiles).

Desai. Reference values of ROS in ejaculated (neat) semen. *Fertil Steril* 2009.

With use of a cutoff of 0.0185, the unadjusted positive predictive value was 82.4% and the negative predictive value was 77.8%. Using bootstrapping as means of estimating the SE for the optimal ROS cutoff determined by the ROC curve, we obtained an estimated SE of approximately 0.005, and the 95% confidence interval for the optimal cutoff was 0.0086 to 0.0284.

Patients With High Versus Low ROS Value

Table 3 summarizes the study variables for the patients with an ROS value below and above the derived cutoff of 0.0185. Of the 54 infertile patients, 12 (22.2%) had an ROS value < 0.0185 and 42 (77.8%) had an ROS value ≥ 0.0185 . On the basis of comparisons with respect to these groups, concentration, motility, and morphology (WHO) were significantly lower for the patients with a higher ROS value

compared with the infertile patients with a lower ROS value (Table 2) ($P < .05$ for these variables). Sperm concentration was correlated negatively with the ROS level ($r = -0.32$; $P = .02$). Higher Endtz values were seen in the patients with higher ROS levels and were positively correlated with the ROS level ($r = 0.46$; $P = .001$). All patients with ROS values < 0.0185 tested negative with use of the Endtz test.

DISCUSSION

Previous studies from our center have demonstrated that ROS is an independent marker of male factor infertility and that a log (ROS +1) value of 1.48 is predictive of male factor infertility with an accuracy of $\leq 80\%$ (31). We have also reported various ROS cutoff values for washed semen samples (21, 39, 40). Using washed semen to measure oxidative stress in vivo (33) will not produce accurate results because various

TABLE 2**Sensitivity, specificity, and unadjusted positive and negative predictive values for various ROS cutoff values.**

ROS cutoff (10^6 cpm/ 20 $\times 10^6$ sperm)	Sensitivity (%)	Specificity (%)	Unadjusted positive predictive value (%)	Unadjusted negative predictive value (%)	Accuracy (%)
0.0000	100	0	51.4	NA	51.4
0.0060	92.6	39.2	61.7	83.3	66.7
0.0100	85.2	52.9	65.7	77.1	69.5
0.0185 ^a	77.8	82.4	82.4	77.8	80
0.0200	75.9	82.4	82	76.4	79
0.0335	59.3	92.2	88.9	68.1	75.2
0.0350	57.4	92.2	88.6	67.1	74.3
0.0600	50	94.1	90	64	71.4
0.0720	50	100	100	65.4	74.3

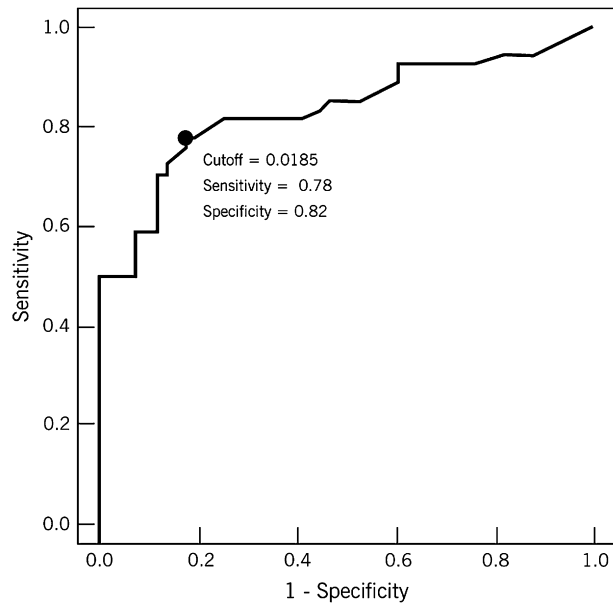
Note: NA = not applicable.

^a ROS cutoff value (of neat semen) to identify infertile patients with maximum accuracy.

Desai. Reference values of ROS in ejaculated (neat) semen. *Fertil Steril* 2009.

FIGURE 1

Receiver operating characteristic curve for various ROS values of fertile donors and infertile patients.



Desai. Reference values of ROS in ejaculated (neat) semen. *Fertil Steril* 2009.

processing steps such as multiple centrifugations, resuspension, and vortexing during sperm washing may cause artifactual increases in ROS levels. Reactive oxygen species levels also may increase after sperm washing because the procedure removes seminal plasma containing endogenous antioxidants (39). Other studies on neat semen have reported ROS cutoff values as well, but they either had a small number of infertile

patients or did not include a group of infertile patients and the cutoff had either low sensitivity or low specificity (33, 34).

For any test to be recommended clinically, the test must be reliable, must have low variability, and should be highly sensitive, specific, and accurate. We demonstrated earlier that ROS measurement using luminol-dependent chemiluminescence is a reliable test that has the least variability and most reproducible results (35). In the present study, we report a cutoff value in infertile patients with a higher specificity and sensitivity than values reported in previous studies (21, 33, 34, 40). With use of a cutoff value of 0.0185×10^6 cpm/ 20×10^6 sperm, semen samples from infertile men can be classified as ROS negative or ROS positive. This cutoff can be used as a diagnostic or screening tool in general (to diagnose male factor infertility), as a prognostic tool in assisted reproduction, or for therapeutic interventions. Reactive oxygen species-positive values can diagnose male factor infertility with a sensitivity of 77.8% and 82.4% specificity. Clinicians also can predict infertility by using an ROS cutoff value of 0.0185.

One can determine the positive (PPV) and negative predictive values (NPV) with the help of the following equation and prevalence of infertility in a particular population:

$$\text{Estimated PPV} = \frac{(0.778 \times \text{Prevalence})}{\{(0.778 \times \text{Prevalence}) + [0.176 \times (1 - \text{Prevalence})]\}}$$

$$\text{Estimated NPV} = \frac{[0.824 \times (1 - \text{Prevalence})]}{\{(0.222 \times \text{Prevalence}) + [0.824 \times (1 - \text{Prevalence})]\}}$$

TABLE 3

Reactive oxygen species and sperm parameters in infertile patients with high versus low ROS cutoff values.

Variable	ROS ($\times 10^6$ cpm/ 20×10^6 sperm)		P value
	< 0.0185 (n = 12)	≥ 0.0185 (n = 42)	
ROS ($\times 10^6$ cpm/ 20×10^6 sperm)	0.0100 \pm 0.01 ^a	0.4500 \pm 0.74	
Concentration ($\times 10^6$ /mL)	0.0070 (0.0023, 0.0102) ^b	0.1150 (0.0345, 0.3890)	.021
Motility units (%)	96.36 \pm 92.30	56.80 \pm 78.28	
	77.45 (49.40, 105.65)	37.45 (21.62, 56.95)	.039
Morphology (WHO normal) (%)	67.75 \pm 22.46	51.17 \pm 23.50	
	70.50 (61, 82.25)	47.5 (33.25, 73)	.021
	26.58 \pm 11.37	17.59 \pm 11.21	
	28.5 (18.75, 34.25)	17 (8, 24)	

Note: $P < .05$ was significant by Wilcoxon rank sum test.

^a Mean \pm SD.

^b Median (25th and 75th percentiles).

Desai. Reference values of ROS in ejaculated (neat) semen. *Fertil Steril* 2009.

There is some potential for favorable bias in the estimates, because the sensitivity and specificity estimates from the ROC analyses are derived from the same data used to create the curve. To provide evidence that the current study does not have any such bias, we performed a leave-one-out cross-validation technique. For each patient, we performed the ROC analysis again with the individual patient left out of the analysis and then used the derived cutoff to try and correctly identify the status of the left-out patient. The failure or success for each patient then was used to estimate sensitivity and specificity in an unbiased fashion. The result of the cross-validation process was an estimated sensitivity of 70% (38 of 54) and an estimated specificity of 80% (41 of 51).

When ROS is used as a screening test, the test must have a high sensitivity to identify a maximum number of infertile patients who have elevated levels of ROS. To balance both specificity and sensitivity according to the need of the physician, we derived sensitivity and specificity values for all possible ROS level cutoffs (Table 2). We have shown that when using ROS measurement as a screening test, physicians can use a cutoff value of $0.01 \times 10^6 \text{ cpm}/10^6 \times 20 \text{ sperm}$ with a sensitivity of 85% and specificity of 53.2%. On the other hand, a cutoff value of $0.072 \times 10^6 \text{ cpm}/10^6 \times 20 \text{ sperm}$ leads to 100% specificity and 50% sensitivity.

Similar to earlier reports (34, 41), our study also showed that sperm concentration, motility, and normal morphology were significantly poorer in the infertile patients who also had higher ROS values than in the infertile patients with lower ROS values. Seminal oxidative stress measurement is also important as a predictive tool in assisted reproductive technology clinics (18, 42). The ROS concentration in seminal plasma has a significant deleterious effect on fertilization rates after IVF–intracytoplasmic sperm injection (32, 43). Therefore, measuring ROS levels before assisted reproductive technology may be beneficial in predicting various end points of assisted reproductive technology such as the fertilization rate and the fertilizing capacity of spermatozoa (32, 44). Taking oral antioxidant supplements and adding antioxidants to the sperm preparation media and assisted reproductive technology media have improved sperm parameters and pregnancy outcome (45–51). Thus, antioxidant treatment should be considered when high levels of ROS are detected.

Basal levels of ROS are needed for physiologic functions of spermatozoa. From our data, an ROS level $<0.0185 \times 10^6 \text{ cpm}/20 \times 10^6 \text{ sperm}$ should be considered physiologic. Because in infertile patients with a ROS level $<0.0185 \times 10^6 \text{ cpm}/20 \times 10^6$, ROS is not the cause of the infertility, only those patients who have ROS levels above this cutoff should be considered for antioxidant supplementation.

In conclusion, we have demonstrated that ROS measurement using the luminol-dependent chemiluminescence is a reproducible test with high specificity and sensitivity. An ROS level $>0.0185 \times 10^6 \text{ cpm}/20 \times 10^6 \text{ sperm}$ is highly predictive of infertility. This cutoff can be used to select patients in large

clinical trials of antioxidant treatment and may be beneficial in improving semen parameters.

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