

Characterization of oxidative stress status by evaluation of reactive oxygen species levels in whole semen and isolated spermatozoa

We defined the basal levels of reactive oxygen species (ROS) in normal donors in neat (whole unprocessed) semen specimens, and in mature and immature spermatozoa isolated by a double-density gradient technique. In addition, we demonstrated that the ROS levels were significantly lower in neat semen compared with washed spermatozoa. The reference values of ROS in neat semen and mature spermatozoa can be used to define the pathologic levels of ROS in infertile men and may guide in therapeutic interventions. (*Fertil Steril*® 2005;83:800–3. ©2005 by American Society for Reproductive Medicine.)

Seminal oxidative stress status is emerging as a significant diagnostic and prognostic tool in infertility/assisted reproductive technology (ART) clinics (1, 2). This is because reactive oxygen species (ROS) plays an important role in the pathophysiology of damage to human spermatozoa (3–5). High levels of ROS generated by human spermatozoa negatively affect the fertilizing potential of human spermatozoa and pregnancy rates after IVF (6) and in vivo (7). However, “high ROS” is an unclear concept because pathological levels of ROS in infertile semen samples have not been defined accurately.

Generally, ROS levels can be monitored in neat (whole unprocessed) semen (8–10), washed sperm suspensions (11–16), and after sperm preparation techniques (17–19). Multiple centrifugation, resuspension, and vortexing steps involved in sperm washing artificially elevate ROS levels (9, 20). The antioxidant activity of seminal plasma is removed during sperm washing steps, which also results in elevated ROS levels (9, 21).

Developing a proper diagnostic and prognostic test to document seminal oxidative stress status may help in the success of any therapeutic intervention. Because sperm washing inflates levels of ROS, we postulated that the ROS levels in washed spermatozoa may inaccurately reflect the in vivo oxidative stress status of an individual. The first objective of our study was to define the basal levels of ROS in normal healthy donors in neat semen and to compare them with ROS levels after a simple wash and resuspension.

Studies have demonstrated that ROS levels in mature spermatozoa correlate significantly with the fertilizing potential of spermatozoa, and their estimation may help in

predicting the success of ART procedures (21–23). Furthermore, characterizing the normal levels of ROS in mature spermatozoa may help avoid the use of specimens that present with elevated ROS levels and potentially higher incidence of DNA damage for use in ART. Therefore, the second objective of our study was to define the basal levels of ROS in mature and immature spermatozoa of normal healthy donors after density gradient separation.

The institutional review board approved the study. Semen specimens were collected from 66 healthy donors (average age 33.2 ± 4.4 years) after 48 to 72 hours of abstinence. Specimens with normal standard semen parameters according to the World Health Organization (WHO, 1992) criteria were included in the study (24). We performed two experiments. In the first experiment, ROS levels in the ejaculates from 34 donors were measured in both neat semen (i.e., unprocessed, whole ejaculate) and after a simple wash and resuspension of spermatozoa. In the second experiment, ROS levels were measured in mature and immature fractions of semen specimens of 32 healthy donors after double-density gradient separation. Semen specimens from infertile patients with abnormal semen parameters with ($n = 17$) and without ($n = 41$) leukocytospermia ($>1 \times 10^6$ white blood cells/mL of seminal ejaculate) served as positive controls.

ISOLATION OF MATURE SPERM POPULATION

Aliquot of the liquefied semen was loaded onto a 47% and 90% discontinuous ISolate gradient (Irvine Scientific, Santa Ana, CA) and centrifuged at $500 \times g$ for 20 minutes at room temperature. The resulting interfaces between the 47% and 90% gradient (immature spermatozoa) and the 90% pellet (mature spermatozoa) were collected and transferred to separate test tubes (25). Sperm suspensions were washed in 1 volume of human tubal fluid and centrifuged at $500 \times g$ for 7 minutes. The pellet was then resuspended in 1 mL of human tubal fluid and an aliquot from each fraction was examined for ROS production.

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TABLE 1

Semen parameters and levels of ROS in neat semen.

Variable	Normal donors	Positive controls (neat semen)		<i>P</i> ^a	<i>P</i> ^b
	Neat semen (n = 34)	Patients with abnormal semen parameters (n = 31)	Patients with leukocytospermia (n = 13)		
Sperm concentration ($\times 10^6$ /mL)	75.9 (60, 96.75)	35.0 (18.5, 55.5)	40.0 (29, 70)	<.0001 ^c	<.009 ^c
Motility (%)	71.2 \pm 10.6	45.1 \pm 15.1	46.8 \pm 19.6	<.0001 ^d	<.0001 ^d
Morphology (%)	38.8 \pm 6.1	18.9 \pm 6.5	19.7 \pm 8.8	<.0001 ^d	<.0001 ^d
ROS levels ($\times 10^6$ cpm/20 million sperm)	0.029 (0.008, 0.13)	0.168 (0.022, 0.546)	0.29 (0.058, 1.43)	.031 ^c	.007 ^c

Note: Values are presented as median (25th, 75th percentile) and as mean \pm SD.

^a*P* value between normal donors and patients with abnormal semen parameters.

^b*P* value between normal donors and patients with leukocytospermia.

^c*P* value of <.05 was considered significant by Mann–Whitney *U* test.

^d*P* value of <.05 was considered significant by Student's unpaired *t*-test.

Allamaneni. Assessment of oxidative stress in semen. *Fertil Steril* 2005.

MEASUREMENT OF ROS

An aliquot (400 μ L) of liquefied semen (neat semen) was used for the measurement of ROS. For determination of ROS levels in the washed specimens, liquefied semen specimens were centrifuged at $300 \times g$ for 7 minutes, and seminal plasma was removed. The sperm pellet was washed and resuspended to 1 mL volume in phosphate-buffered saline (PBS). A 400- μ L aliquot was used for assessment of ROS levels; 400- μ L aliquots each of mature and immature fractions were used for the measurement of ROS after density gradient separation. Then, 10 μ L of luminol (5-amino-2, 3, -dihydro-1, 4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) prepared as 5-mM stock in dimethyl sulfoxide, was added for each evaluation as a probe. Levels of ROS were assessed by measuring chemiluminescence with an Autolumat LB 953 luminometer (Berthold Technologies, Bad-Wildbad, Germany) in the integrated mode for 15 minutes. The results were expressed as $\times 10^6$ counted photons per minute (cpm) per 20×10^6 sperm (26).

STATISTICAL ANALYSIS

The Wilcoxon matched-pairs signed-ranks test was used to compare the ROS levels with in the donor specimens. The Mann-Whitney *U* test was used to compare the ROS levels between donor and patient groups. Unpaired *t*-test was used where data was normally distributed. A *P* value of <.05 was considered statistically significant. GraphPad InStat version 3.00 statistical software (GraphPad Software, Inc., San Diego, CA) was used for such comparisons. Receiver operating curve (ROC) analysis was performed by Stats-

Direct program (StatsDirect Ltd., Gresham Way, United Kingdom).

Semen characteristics of the donors (n = 34) and patients (n = 44) used in the first experiment are listed in Table 1. The median, 25th and 75th percentile, of ROS levels in healthy donors were 0.029 (0.008, 0.13) $\times 10^6$ cpm/20 $\times 10^6$ sperm in neat semen specimens and 0.111 (0.075, 0.41) $\times 10^6$ cpm/20 $\times 10^6$ sperm in washed sperm suspensions. The levels of ROS were significantly lower in neat semen than in washed sperm suspensions (*P*<.0001). The positive ROS control levels measured in neat semen of patients with abnormal semen parameters, and patients with leukocytospermia were significantly higher than in healthy donors (*P*=.031, *P*=.007, respectively) (Table 1).

Semen characteristics of donors used in the second experiment were as follows: sperm concentration ($\times 10^6$ /mL): 83.6 (58.8, 88.6); motility (%): 73.1 \pm 11.4; and morphology (% normal by WHO): 39.7 \pm 5.6. Levels of ROS in mature spermatozoa of normal donors, 0.021 (0.004, 0.075), were significantly lower than ROS levels in immature spermatozoa (0.066 [0.015, 0.156]) (*P*=.01).

The positive control values measured in mature spermatozoa of patients with abnormal semen parameters compared with healthy donors (0.104 [0.018, 0.362] vs. 0.021 [0.004, 0.075]; *P*=.037) and in patients with leukocytospermia compared with healthy donors (0.66 [0.365, 2.682] vs. 0.021 [0.004, 0.075]; *P*=.003) were significantly higher.

ROC Analysis to Identify Subjects with Oxidative Stress

The area under curve for neat semen ROS levels was 0.68 (0.57–0.80). The ROC analysis identified 0.185×10^6 cpm per 20×10^6 sperm as optimum cut-off value for identifying patients with oxidative stress after giving equal weight to both sensitivity and specificity. The sensitivity and specificity with confidence intervals at this cut-off were 50% (34%–65%) and 82% (65%–93%), respectively.

ROC Analysis to Identify Mature Sperm Suspensions with Oxidative Stress

The ROC analysis identified mature sperm ROS levels of 0.145×10^6 cpm per 20×10^6 sperm as optimum cut-off value. The sensitivity and specificity with confidence intervals at this cut-off were 57% (29%–82%) and 87% (71%–96%), respectively. The area under curve was 0.77 (0.63–0.92).

We have defined the levels of ROS in neat semen in a group of normal healthy donors. A significant difference was noted between ROS levels measured in neat semen compared with washed spermatozoa. Measurement of ROS levels in neat semen after liquefaction in the presence of seminal antioxidant protection should accurately represent the true in vivo oxidative stress status of an individual and overcome the drawbacks of previous methods.

Defining the normal levels of a diagnostic test is very important in the development of a standardized protocol, and its application in research and in clinical practice. Lack of a well-established testing tool and criteria for abnormal oxidative stress might be a reason for the inability to develop effective therapeutic intervention against oxidative stress.

Sperm preparation is necessary to enhance and maintain sperm quality and function after ejaculation before the semen specimen can be used for ART procedures (27). ROS levels in mature spermatozoa may have both diagnostic and prognostic importance. Elevated ROS levels in mature spermatozoa may reflect the oxidative stress status of the semen sample to be used for ART (diagnostic), and may also be used to predict the fertilizing potential of the spermatozoa (prognostic) (21–23). The reference values of ROS may be used to characterize the semen specimens with oxidative stress and in selecting the semen specimens intended for ART use.

In summary, accurate assessment of seminal levels of oxidative stress may help in the diagnosis of infertility, in which oxidative stress plays a major role. For this, a consensus on normal levels of ROS in semen is very essential. Semen specimens from infertile men can be classified as oxidative stress-positive or oxidative stress-negative for therapeutic interventions using normal reference values. Other laboratories, using their own group of normal donors, can easily validate these reference ranges. In vivo supple-

mentation of antioxidants to infertile men as well as in vitro addition of antioxidants to the sperm preparation media and ART media to scavenge the oxidants should be considered when high levels of ROS are detected.

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