Published-Ahead-of-Print April 4, 2007, DOI:10.2164/jandrol.106.001966
Journal of Andrology, Vol. 28, No. 4, July/August 2007

DOI: 10.2164/jandrol.106.001966

Development of Normal Reference Values for Seminal Reactive Oxygen Species and Their Correlation With Leukocytes and Semen Parameters in a Fertile Population

KELLY S. ATHAYDE*,†, MARCELLO COCUZZA*,†, ASHOK AGARWAL†, NATALIE KRAJCIR*, ANTONIO M. LUCON*, MIGUEL SROUGI* AND JORGE HALLAK*

*From the * Department of Urology, University of Sao Paulo, Sao Paulo, Brazil; and † Reproductive Research Center, Glickman Urological Institute and Department of Obstetrics-Gynecology, Cleveland Clinic, Cleveland, Ohio.*

Correspondence to: Dr Jorge Hallak, Rua Dr Eneas de Carvalho Aguiar, 255 7º andar - Sala 7123, ICHC - Pós-Graduação Divisão de Urologia, CEP: 05422-970 Brazil – Sao Paulo (e-mail: hallakj{at}androscience.com.br).

Received for publication October 11, 2006; accepted for publication March 22, 2007.

Abstract

Although reactive oxygen species (ROSs) are clearly implicated in the pathogenesis of male infertility, few studies have attempted to define the basal levels of ROSs in fertile men. Levels of ROSs are highly influenced by the presence of leukocytes and are associated with decreased seminal parameters. The objective of our study was to determine the normal ROS reference values in neat and washed semen of a fertile population and to correlate the leukocyte concentrations with seminal parameters. We evaluated 114 fertile men seeking vasectomy and 47 subfertile patients as a positive control. All samples were subjected to semen analysis and Endtz testing; chemiluminescence assay was used to determine ROS levels. All seminal parameters were significantly higher in the fertile men than in the subfertile patients. In nonleukocytospermic samples, ROS levels were lower in the fertile men than in the subfertile patients in neat (0.29 [0.18, 0.54] vs 0.94 [0.38, 1.51]) (P = .001) and washed semen (5.73 [1.90, 14.71] vs 23.4 [9.46, 115.55]) (P = .001). Similarly, in samples with leukocytes (Entdz, less than 1 x 10^6/mL), ROS levels were lower in the fertile men in neat (0.75 [0.27, 1.71] vs 2.0 [0.97, 27.41]) (P = .001) and washed semen (15.85 [4.18, 62.16] vs 239.83 [33.4, 1193.75]) (P < .0001). As expected, samples with leukocytes had significantly higher ROS values in washed and neat semen. In the fertile population, ROSs were

positively correlated with leukocytes and negatively correlated with sperm count and motility. In semen samples without leukocytes, the normality cutoff of ROSs was 0.55 x 10^4 counted photons per minute with 76.4% area under the curve (AUC) in the neat samples and 10.0 x 10^4 counted photons per minute with 77% AUC in the washed samples. In semen samples with leukocytes, the cutoff for ROSs in neat samples was 1.25 with 72.7% AUC and 51.5 with 81% AUC in the washed samples. We defined the cutoff levels of ROSs in a fertile population. Seminal leukocyte levels below 1 x 10^6/mL were associated with increased ROSs. ROS levels were positively correlated with leukocytes and negatively correlated with sperm motility and concentration. Patients with normal seminal parameters and lower seminal leukocyte levels may benefit from therapeutic interventions that improve semen quality.

Key words: Oxidative stress, male infertility, Endtz test, spermatozoa, standardization

---

Infertility affects about 15% of couples during their reproductive years, and male-related factors play a role in approximately half of these cases (Sharlip et al, 2002). In some couples, there is no clear cause, leading to a diagnosis of unexplained or idiopathic infertility (Hendin et al, 1999). Semen analysis is the backbone of male infertility evaluation. However, standard seminal parameters do not detect abnormalities in up to 20% of subfertile males (Romeo et al, 2001). The routine measurements do not reveal seminal defects at molecular levels that might be induced by reactive oxygen species (ROSs), which are associated with male infertility (Agarwal and Said, 2005).

The World Health Organization (WHO) considers leukocytospermia to be a condition in which leukocyte levels are equal to or exceed 1 x 10^6/mL. Studies have reported oxidative stress in sperm cells from subfertile men even when the leukocyte concentration is low. However, the minimum leukocyte level that can cause oxidative stress in semen has yet to be determined (Sharma et al, 2001).

Other possible sources of ROSs include xenobiotics, pollutants, and abnormal spermatozoa (Aitken and Krausz, 2001). High levels of ROSs generated by abnormal spermatozoa negatively affect fertilization as well as pregnancy rates after in vitro fertilization and are associated with increased sperm DNA damage (Aitken et al, 1991; Zorn et al, 2003). In addition, clinical studies have found that urogenital inflammation significantly influences acrosomal functionality by the presence of inflammatory mediators such as ROSs (Henkel et al, 2006).

Many methods are available to measure ROS levels in semen, but chemiluminescence is the most widely used in both neat and washed semen samples (Agarwal et al, 2004). Sperm-washing techniques include many steps such as multiple centrifugations, resuspension, and vortexing. These steps may artificially increase ROS levels and produce inaccurate test results (Agarwal et al, 1994; Twigg et al, 1998). When seminal plasma is extracted during the washing procedure, so too are protective antioxidants. As a result, levels of ROSs increase. Determining ROS levels in washed semen may reflect an inaccurate measure of the in vivo oxidative stress status (Allamaneni et al, 2005).

The aims of this study were to 1) determine the normal reference levels of ROSs in neat and washed semen of a proven fertile population and 2) evaluate the correlation between ROSs, seminal parameters, and low leukocyte levels (Endtz test, less than 1 x 10^6/mL).

Materials and Methods

The University of São Paulo Institutional Review Board approved this study. All subjects provided informed consent prior to their first appointment.

▲ Top
▲ Abstract
▪ Materials and Methods

Results
Discussion
References

Study Population

In this cross-sectional study, the population consisted of 114 fertile and 47 subfertile males. The fertile group was composed of men seeking voluntary sterilization by vasectomy at the Division of Urology, Medical School, University of São Paulo between May 2004 and January 2006. The fertile group included healthy males with no history of fertility problems and whose partners were pregnant or had delivered a child in the past. These patients constituted the proven fertile population. The fertile population was divided into 2 groups based on the presence of leukocytes in semen: 39 fertile men with leukocytes (Endtz, more than 0 and less than 1 x 10^6/mL) and 75 fertile men without leukocytes (Endtz, 0). A group of 47 subfertile patients from couples who had failed to achieve pregnancy after more than 1 year of regular unprotected intercourse served as the positive control. All female partners of these men had undergone gynecologic evaluation and had normal results.

Both groups were evaluated by 2 male infertility specialists. Patients were excluded from the study when there was history of illicit drug use; exposure to any environmental or occupational toxicants; use of medication with proven toxicity on fertility; exposure to radiation or heat; mumps with orchitis; sexually transmitted or systemic diseases; cryptorchidism regardless of treatment; testicular torsion; genitourinary anomalies; epydidimal or vas deferens alterations; and/or scrotal or inguinal surgery. The same exclusion criteria were applied to the fertile and infertile groups.

All samples were subjected to semen analysis, leukocytospermia testing (Endtz), and chemiluminescence to determinate ROS levels in neat and washed semen.

Semen Analysis

Semen was collected by masturbation after 48 to 72 hours of sexual abstinence. After liquefaction, all semen analyses were performed manually. Macroscopic and microscopic parameters were performed according to WHO guidelines (World Health Organization, 1999). For each fresh measurement, a 5-µL aliquot from patient samples was loaded into a Makler chamber (Sefi Medical, Haifa, Israel). The following variables were determined: sperm concentration; total motility; percentage of spermatozoa grade A, grade B, grade C; total motile sperm; and total sperm.

Sperm morphology was assessed using the methodology described by Kruger (Kruger et al, 1988) and by the WHO (World Health Organization, 1992). During morphology evaluation, seminal smears were stained with Giemsa stain (Diff-Quick; Baxter Healthcare, McGraw Park, Ill). Semen smears were performed in duplicate and stained at the clinical sites. Stained slides were air dried, and coverslips were applied with 2 drops of Cytoseal mounting medium (Richard-Allan Scientific, Kalamazoo, Mich). Manual morphologic assessment was performed at immersion magnification.

Leukocytospermia (Endtz) Test

Leukocyte concentrations in semen were quantified using a myeloperoxidase-staining test (Shekarriz et al, 1995a). A 20-µL volume of liquefied specimen was placed in a 1.8-mL microtube. Twenty microliters of phosphate-buffered saline ([PBS] pH 7.0) and 40 µL of benzidine solution were added. The mixture was vortexed and allowed to sit for 5 minutes. Five microliters of this mixture was placed in a Makler chamber and examined for brown stained cells, indicating the presence of peroxidase. These brown stained cells were therefore identified as granulocytes (Shekarriz et al, 1995a).

Measurement of ROSs

ROSs in Washed Semen— Aliquots of liquefied semen were centrifuged at 300 x *g* for 7 minutes. The sperm pellet was washed twice with human tubal fluid, HEPES buffered (Irvine Scientific, Santa Ana, Calif), and resuspended in the same medium at a concentration of 20 x 10^6 sperm per milliliter (Shekarriz et al, 1995a). ROS production was measured by the chemiluminescence assay method using luminol (5-amino-2,3-dihydro 1,4-phthalazinedione; Sigma Chemical Co, St Louis, Mo) as the probe. Ten microliters of 5-mM luminol prepared in dimethyl sulfoxide (DMSO) (Sigma) was added to 400 µL of the washed sperm suspension.

ROSs in Neat Semen— Ten microliters of 5-mM luminol prepared in DMSO was added to 400 µL of liquefied semen.

For both neat and washed semen, ROS levels were determined by measuring chemiluminescence via a luminometer (MicroBeta TriLux, version 4.7; Perkin Elmer Life Sciences, Turku, Finland) for 15 minutes. The results were expressed as 10^4 counted photons per minute (cpm) per 20 x 10^6 sperm.

Statistical Analysis

The median and interquartile range (IQR) (25%, 75%) were used to describe the populations. The Mann-Whitney *U* test, a nonparametric test, was used to compare the groups. Correlation between variables was calculated using the Spearman correlation coefficient for nonparametric data. The sensitivity, specificity, and accuracy were calculated for different cutoffs to determine reference values. Receiver operating characteristic (ROC) curves and the area under the curve (AUC) were calculated to identify which cutoff value would be superior in differentiating between the subfertile and fertile subjects. Accordingly, different cutoffs were defined and evaluated. All analyses were performed with MINITAB (version 14.2; State College, Pa), SAS (version 8.1; Cary, NC), and SPSS (version 14.0; Chicago, Ill). A *P* value of less than .05 was considered statistically significant.

Results

- Top
- Abstract
- Materials and Methods
- Results
- Discussion
- References

Fertile Men and Subfertile Patients

No statistically significant differences in the median (IQR) value in age were found between the fertile men (34.3 years [31, 38 years]) and subfertile patients (34.2 years [28, 40 years]) (*P* = .15) (Table 1). Semen parameters of the fertile men were significantly higher than those of the subfertile patients. The fertile men had significantly lower ROS levels in neat and washed semen than the subfertile patients. The levels of ROSs were significantly lower in neat semen than in washed sperm suspensions.

View this table: [in this window] [in a new window]

Table 1. *Comparison of seminal parameters, leukocyte levels, and reactive oxygen species (ROS) levels in neat and washed semen between fertile men and subfertile patients**

Fertile men with leukocytes had higher sperm counts (10^6/mL) than the subfertile men with leukocytes (99.3 [59, 150] vs 41 [18, 81]) (*P* = .0001). In the same population, ROS levels (x 10^4 cpm /20 x 10^6 sperm) in the fertile men were lower in neat (0.75 [0.27, 1.71] vs 2.0 [0.97, 27.41]) (*P* = .001) and

washed semen (15.85 [4.18, 62.16] vs 239.83 [33.4, 1193.75]) ($P < .0001$).

Similarly, the fertile men without leukocytes had a higher sperm count (10^6/mL) than the subfertile men without leukocytes (74 [50.5, 142] vs 23 [10.8, 41.5]) ($P < .0001$). In the same population, ROS levels (x 10^4 cpm/20 x 10^6 sperm) in the fertile men were lower in neat (0.29 [0.18, 0.54] vs 0.94 [0.38, 1.51]) ($P = .001$) and washed semen (5.73 [1.90, 14.71] vs 23.4 [9.46, 115.55]) ($P = .001$).

Fertile Men With Lower Seminal Levels of Leukocytes (Endtz, More Than 0 and Less Than 1 x 10^6/mL)

Correlations between the 2 measurements of ROSs (neat and washed) and sperm concentration; total motility; percentage of grade A, B, and C spermatozoa; and morphology (strict criteria and WHO guidelines) were performed considering ROSs as the sole predictor in each model (Table 2). This analysis was performed in all fertile men with leukocyte levels below 1 x 10^6/mL.

View this table: [in this window] [in a new window]

Table 2. *Correlation of reactive oxygen species (ROS) levels (neat and washed semen) with sperm parameters in fertile men with leukocytes below 1 x 10^6/mL**

In neat and washed semen, ROSs were negatively correlated with sperm concentration, total motility, and grade A and B motility. A negative correlation was found between ROSs, grade C motility, and morphology using both WHO and strict criteria. A significant positive correlation between ROSs in neat and washed semen ($r = .62$; $P < .0001$) and leukocyte levels was determined.

Fertile Men With Leukocytes (Endtz, Less Than 1 x 10^6/mL) and Fertile Men Without Leukocytes (Endtz, 0)

No statistically significant differences were found in the median (IQR) value in age between fertile men with leukocytes (34.7 years [32, 40 years]) and fertile men without leukocytes (34.1 years [31, 37 years]) ($P = .29$) (Table 3). Fertile men with seminal leukocytes had significantly higher levels of ROSs in washed and neat samples than the fertile men without seminal leukocytes. There were no significant differences in sperm concentration, total motility, and grade A, B, and C motility when the 2 populations of fertile men were compared. Although sperm morphology according to the WHO criteria was higher in the men with seminal leukocytes, no significant difference was found when using strict criteria. ROS levels in neat and washed semen were significantly lower in the fertile men without leukocytes than in the fertile men with leukocytes.

View this table: [in this window] [in a new window]

Table 3. *Comparison of seminal parameters and reactive oxygen species (ROS) levels in neat and washed semen between fertile men with leukocytes (Endtz, more than 0 and less than 1 x 10^6/mL) and fertile men without leukocytes (Endtz, 0)**

ROC Curves

To determine whether the ROS test on neat and washed semen could better differentiate fertile patients from subfertile patients, we examined various cutoff values to determine one with a high sensitivity (Table 4). This evaluation was performed by generating ROC curves and the AUC, as shown in the Figure.

View this table: [in this window] [in a new window]

Table 4. *Areas under the curve (AUC) for seminal reactive oxygen species (ROS) levels in neat and washed semen in fertile patients with (Endtz, more than 0 and less than 1 x 10^6/mL) and without (Endtz, 0) leukocytes in semen**

ROSs in Neat and Washed Semen Without Leukocytes (Endtz, 0)

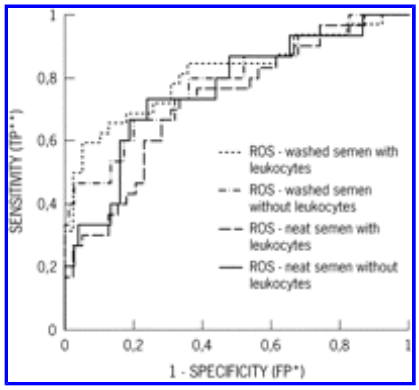
With a cutoff value of 0.2 (x 10^4 cpm/20 x 10^6 sperm) for ROSs in neat semen without leukocytes, the sensitivity was high at 93.3% and the specificity was low at 34.7%. The accuracy was 44.4% (defined as the positively diagnosed subfertile patients and correctly excluded fertile patients). With a cutoff value of 0.4, the sensitivity was 80%, but the specificity was 56%. With this cutoff value, levels of ROSs could correctly identify 80% of the subfertile patients as subfertile, but the accuracy to identify fertile patients from the subfertile sample was only 60%.

An increase in specificity implies a corresponding decrease in sensitivity. One option to combat this was to give equal weight to both variables and evaluate accuracy. By giving equal weight to sensitivity and specificity, the cutoff value was 0.5 with 75.6% accuracy. The AUC for this test was 76.4% with a 95% confidence interval (CI) of 62.6% to 90.2%. Sixty-eight patients of 90 could be correctly classified using the ROS test in neat semen without leukocytes (true positive and true negative).

In the same group of patients, performing the test on washed samples with a cutoff of 3.0 yielded 93.3% sensitivity and 32% specificity and only 42.2% accuracy. Using 5.0 as a cutoff, the sensitivity was 86.7% and the specificity was 48%. The accuracy was 54.4%. By giving equal weight to sensitivity and specificity, the cutoff value was 10.0 with 68.9% accuracy. The AUC for this test was 77% with a 95% CI of 62.6% to 91.7%. Sixty-two patients of 90 could be correctly classified with the ROS test on washed semen without leukocytes (Table 4).

ROS in Neat and in Washed Semen with Leukocytes (Endtz, More than 0 and Less Than 1x 10^6/mL)

For patients with leukocytes in neat semen, a cutoff of 0.36 (x 10^4 cpm/20 x 10^6 sperm) yielded 90% sensitivity but a very poor specificity of 33.3% and accuracy of 58.0%. Using the cutoff of 0.6, the sensitivity was 80% and specificity was 61%. By giving equal weight to sensitivity and specificity, the cutoff value was 1.25 with 69.6% accuracy. The AUC for this test was 72.7% with a 95% CI of 60.8% to 84.7%. Forty-nine patients of 71 could be correctly classified with the ROS test on neat semen with leukocytes.



View larger version (16K):
[in this window]
[in a new window]

Receiver operating characteristic curves showing the area under the curve in male factor infertility patients and fertile men using reactive oxygen species level on neat and washed semen with or without leukocytes. *FP indicates false positive; **TP, true positive.

Using the ROS test on washed samples with leukocytes and a cutoff of 8.0, the sensitivity was 93.8% with 33.3% specificity and 60.6% accuracy. When this cutoff was increased to 12.0, the sensitivity was 87.5% but the specificity and accuracy were both 38.5%. By giving equal weight to sensitivity and specificity, the best cutoff value determined was 51.5 with 73.2% accuracy. The AUC for this test was 81% with a 95% CI of 70.3% to 91.6%. Fifty-two patients of 71 could be correctly classified with the ROS test on washed semen with leukocytes. There was no difference between the AUC of these tests, because their 95% CIs overlapped on at least 1 range/period.

Discussion

- Top
- Abstract
- Materials and Methods
- Results
- Discussion
- References

Limited amounts of ROSs are physiologically involved in the regulation of sperm function, but ROSs at higher concentrations can potentially cause toxic effects on sperm (de Lamirande et al, 1995; Padron et al, 1997). Human ejaculate is contaminated with potential sources of ROSs such as peroxidase-positive leukocytes and abnormal spermatozoa, both of which produce free radicals continuously (Aitken et al, 1995).

Studies imply that oxidative sperm DNA damage is closely associated with impaired sperm function and male infertility. Strong evidence suggests that DNA fragmentation—commonly observed in the spermatozoa of subfertile men—is mediated by high levels of ROSs (Sun et al, 1997).

In approximately 25% of males, the cause of infertility cannot be determined (Alkan et al, 1997). Studies have shown that 40% to 88% of nonselected subfertile patients have high levels of ROSs (Padron et al, 1997). Developing a proper diagnostic and prognostic test to evaluate the oxidative stress status may contribute to a major understanding of idiopathic infertility. Therefore, defining the normal levels of a diagnostic test is crucial to allow future clinical practice interventions.

A test used as a diagnostic tool must identify a maximum number of patients during routine evaluation. It must be sensitive at a given cutoff to accurately differentiate fertile from subfertile patients. In this study, we have defined the levels of ROSs in neat and washed semen in a fertile population. We determined the sensitivity, specificity, and accuracy of different cutoff values. To determine reference values for normality, we considered different cutoffs statistically

determined by ROC curves.

Shekarriz et al (1995b) described all ROS values of 10 x 10^4 cpm or more as abnormal. According to Sharma et al (1999), the mean level of ROSs was 1.39 ± 0.73 (Log ROS + 1) for fertile men, 2.1 ± 1.21 (Log ROS + 1) for subfertile men with varicocele, and 3.25 ± 0.89 (Log ROS + 1) for subfertile men with seminal infection. Allamaneni et al (2005) reported that the mean ROS level of washed semen was 11.1 x 10^4 cpm (0.075 x 10^4 – 0.41 x 10^4) (20 x 10^6 sperm) in donors, 16.8 x 10^4 cpm (0.022 – 0.546) in subfertile patients, and 29.0 x 10^4 cpm (0.058 – 1.43) in leukocytospermic patients. In the same study, the value for ROSs in neat semen for the fertile population was 2.9 x 10^4 cpm (0.008 – 0.13). Nallella et al (2005) found that in washed semen, a cutoff of 1.25 using Log ROS + 1 had 74.6% accuracy.

For washed semen, we found an optimal cutoff of 10.0 (accuracy, 69.0%) for samples without leukocytes and 51.5 (accuracy, 73.2%) for semen with leukocytes but without leukocytospermia. Although our results are different than those of Nallella's study, they are more similar to the reference values described by Shekarriz et al (abnormal values, 10.0 x 10^4 cpm or more) (Nallella et al, 2005; Shekarriz et al, 1995b). Nevertheless, in these studies, there are no considerations regarding the presence of low levels of leukocytes. In the present study, the established cutoff was much higher for samples with white cells (51.5). The AUC for the ROS test on washed semen was 77% (range, 62.2% – 91.7%) for the samples without leukocytes and 80.9% (range, 70.3% – 91.6%) for the samples with leukocytes. In the studies of Nallella et al (2005) and Sharma et al (2001), the AUC was 78.92% (range, 72.60% – 85.23%) and 68.9% (range, 57.0% – 80.7%), respectively. For washed samples, ROS test showed a sensitivity of 71.9% and 73.3% for samples with and without leukocytes, respectively, compared with 83.5% from Nallella's study (Nallella et al, 2005).

For neat semen, we identified an optimal cutoff of 0.56 (0.5575) for samples without leukocytes and 1.25 (1.2480) for semen with leukocytes (Endtz, more than 0 and less than 1 x 10^6/mL) whereas Allamaneni et al showed 18.5 as an optimum cutoff value independent of leukocyte presence (Allamaneni et al, 2005). When evaluating the AUC for the ROS tests on neat semen, our findings were 76.4% (range, 62.6% – 90.2%) and 72.7% (range, 60.8% – 84.7%) for samples without and with leukocytes, respectively, compared with 68% (range, 57% – 80%) from Nallella et al (2005). In the same study, the authors showed a 50% sensitivity for the test. Our findings reported 73.3% for both samples with and without leukocytes in neat semen.

The identification of potential sources of ROSs is extremely important for guiding any clinical intervention. As reported previously, leukocytes are the major source of ROS production (Lemkecher et al, 2005). This strongly suggests the need for a more specific evaluation of their interference with ROS levels. When we compared the fertile groups with and without leukocytes (excluding samples with leukocytospermia), a significant difference was identified. In neat and washed semen, fertile men with the presence of leukocytes (less than 1 x 10^6/mL) had significantly higher levels of ROSs. Other parameters showed no statistical differences.

Like previous studies (Lemkecher et al, 2005; Saleh et al, 2002), we found that leukocytes interfere greatly with ROS levels. Novotny et al (2003) and Sharma et al (2001) reported that lower leukocyte levels were associated with significant ROS levels in semen. However, these 2 previous studies were based on subfertile patients whereas we showed that lower levels of leukocytes also affect ROS levels in a fertile population. Recently, Henkel et al reported that leukocyte counts of less than 1 x 10^6/mL were significantly correlated with ROS production as well as decreased DNA integrity (Henkel et al, 2003, 2005). These findings suggest that the established cutoff for leukocytospermia given by the WHO should be reevaluated.

The influence of ROSs on sperm motility can be explained by lipid peroxidation. This process results in a loss of plasmatic membrane solubility that decreases the phosphorylation of axonemal proteins and causes sperm immobilization (Agarwal et al, 2003). Decreased sperm count could be attributed to extended exposure of the seminiferous epithelium to high levels of ROSs produced by immature or abnormal spermatozoa. ROSs damage these tubules, leading to testicular atrophy, reduction of gamete production, motility loss, and DNA damage to mature sperm.

Previous studies have shown that ROS production is negatively correlated with the percentage of normal sperm, concentration, and motility and is positively correlated with increased percentages of abnormal spermatozoa (Aziz et al, 2004). Similarly, our study showed a negative correlation of ROS and sperm concentration and motility as well as positive correlation with leukocyte levels. In addition, we were able to show these results in a fertile population and in those with lower levels of leukocytes.

An important implication of our study is that the normal reference values were determined in a proven fertile population. There is a strong recommendation from the WHO to individualize the references for normal semen parameters in each laboratory. However, due to the difficulty of recruiting a fertile population, most studies have used normal healthy donors as controls. Because the ultimate evaluation of fertility is pregnancy, donors presenting with normal semen parameters cannot be considered fertile unless their fertility status is known. In addition, although most published studies have assessed ROSs in washed semen, our study measured ROS levels in both neat and washed semen. It is crucial to measure ROSs in washed semen because removing the seminal plasma is the first step in processing samples for assisted reproductive techniques. However, measuring ROSs in processed samples would be interesting because this is also an essential step during assisted reproductive procedures.

The most significant drawbacks of our study include the limited number of patients and the cross-sectional design. However, the data were collected prospectively, and the same exclusion criteria were applied to all patients. Patients selected for the subfertile group included men with idiopathic infertility, which may partially explain why the seminal parameter values in the subfertile patients were not severely abnormal. Although samples were adjusted for concentration (20 x 10^6 sperm per milliliter), increased seminal parameters, especially molitity, might have interfered in seminal ROS levels. Additional participants would improve our efficacy in identifying the cutoffs of normality in this population.

In conclusion, the present study determined normal reference values for ROSs in neat and washed semen in a population of men with proven fertility. These values can help to identify the pathologic levels of ROSs in infertile men and may guide in clinical therapeutic interventions. In addition, increased ROS levels were associated with leukocyte counts below 1 x 10^6/mL, and ROSs showed a significant negative correlation with sperm motility and count. Our findings suggest that patients with normal seminal parameters and lower seminal leukocyte levels may benefit from treatment to improve spontaneous pregnancy rates as well as assisted reproduction outcomes, especially in couples with unknown causes of infertility.

References

Agarwal A, Allamaneni SS, Said TM. Chemiluminescence technique for measuring reactive oxygen species. *Reprod Biomed Online*. 2004;9: 466 –468.[Medline]

Top
Abstract
Materials and Methods
Results

▲ Discussion
▪ References

Agarwal A, Ikemoto I, Loughlin KR. Effect of sperm washing on levels of reactive oxygen species in semen. *Arch Androl*. 1994;33: 157 –162.[Medline]

Agarwal A, Said TM. Oxidative stress, DNA damage and apoptosis in male infertility: a clinical approach. *BJU Int*. 2005; 95: 503 –507.[CrossRef][Medline]

Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril*. 2003;79: 829 –843.[CrossRef][Medline]

Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol*. 1991; 164: 542 –551. [Medline]

Aitken RJ, Krausz C. Oxidative stress, DNA damage and the Y chromosome. *Reproduction*. 2001; 122: 497 –506.[Abstract]

Aitken RJ, Paterson M, Fisher H, Buckingham DW, van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci*. 1995; 108(pt 5): 2017 –2025.[Abstract]

Alkan I, Simsek F, Haklar G, Kervancioglu E, Ozveri H, Yalcin S, Akdas A. Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. *J Urol*. 1997;157: 140 –143.[CrossRef][Medline]

Allamaneni SS, Agarwal A, Nallella KP, Sharma RK, Thomas AJ Jr, Sikka SC. Characterization of oxidative stress status by evaluation of reactive oxygen species levels in whole semen and isolated spermatozoa. *Fertil Steril*. 2005; 83: 800 –803.[CrossRef][Medline]

Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, Thomas AJ Jr, Agarwal A. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril*. 2004; 81: 349 –354.[CrossRef][Medline]

de Lamirande E, Leduc BE, Iwasaki A, Hassouna M, Gagnon C. Increased reactive oxygen species formation in semen of patients with spinal cord injury. *Fertil Steril*. 1995; 63: 637 –642.[Medline]

Hendin BN, Kolettis PN, Sharma RK, Thomas AJ Jr, Agarwal A. Varicocele is associated with elevated spermatozoal reactive oxygen species production and diminished seminal plasma antioxidant capacity. *J Urol*. 1999;161: 1831 –1834.[CrossRef][Medline]

Henkel R, Kierspel E, Stalf T, Mehnert C, Menkveld R, Tinneberg HR, Schill WB, Kruger TF. Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertil Steril*. 2005; 83: 635 –642.[CrossRef][Medline]

Henkel R, Ludwig M, Schuppe HC, Diemer T, Schill WB, Weidner W. Chronic pelvic pain syndrome/chronic prostatitis affect the acrosome reaction in human spermatozoa. *World J Urol*. 2006; 24: 39 –44. [CrossRef][Medline]

Henkel R, Maass G, Hajimohammad M, Menkveld R, Stalf T, Villegas J, Sanchez R, Kruger TF, Schill WB. Urogenital inflammation: changes of leucocytes and ROS. *Andrologia*. 2003; 35: 309 –313.[CrossRef] [Medline]

Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril*. 1988; 49: 112 –117.[Medline]

Lemkecher T, Dartigues S, Vaysse J, Kulski O, Barraud-Lange V, Gattegno L, Wolf JP. Leucocytospermia, oxidative stress and male fertility: facts and hypotheses [in French]. *Gynecol Obstet Fertil*. 2005;33: 2 –10.[CrossRef][Medline]

Nallella KP, Sharma RK, Allamaneni SS, Agarwal A. Identification of male factor infertility using a novel semen quality score and reactive oxygen species levels. *Clinics*. 2005; 60: 317 –324.[Medline]

Novotny J, Oborna II, Brezinova J, Svobodova M, Hrbac J, Fingerova H. The occurrence of reactive oxygen species in the semen of males from infertile couples. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2003;147: 173 –176.[Medline]

Padron OF, Brackett NL, Sharma RK, Lynne CM, Thomas AJ Jr, Agarwal A. Seminal reactive oxygen species and sperm motility and morphology in men with spinal cord injury. *Fertil Steril*. 1997; 67: 1115 –1120.[CrossRef][Medline]

Romeo C, Ientile R, Santoro G, Impellizzeri P, Turiaco N, Impala P, Cifala S, Cutroneo G, Trimarchi F, Gentile C. Nitric oxide production is increased in the spermatic veins of adolescents with left idiophatic varicocele. *J Pediatr Surg*. 2001; 36: 389 –393.[CrossRef][Medline]

Saleh RA, Agarwal A, Kandirali E, Sharma RK, Thomas AJ, Nada EA, Evenson DP, Alvarez JG. Leukocytospermia is associated with increased reactive oxygen species production by human spermatozoa. *Fertil Steril*. 2002;78: 1215 –1224.[CrossRef][Medline]

Sharlip ID, Jarow JP, Belker AM, Lipshultz LI, Sigman M, Thomas AJ, Schlegel PN, Howards SS, Nehra A, Damewood MD, Overstreet JW, Sadovsky R. Best practice policies for male infertility. *Fertil Steril*. 2002; 77: 873 –882.[CrossRef][Medline]

Sharma RK, Pasqualotto AE, Nelson DR, Thomas AJ Jr, Agarwal A. Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl*. 2001; 22: 575 –583.[Abstract]

Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ Jr, Agarwal A. The reactive oxygen species-total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod*. 1999;14: 2801 –2807.[Abstract/Free Full Text]

Shekarriz M, Sharma RK, Thomas AJ Jr, Agarwal A. Positive myeloperoxidase staining (Endtz test) as an indicator of excessive reactive oxygen species formation in semen. *J Assist Reprod Genet*. 1995a;12: 70 –74.[CrossRef][Medline]

Shekarriz M, Thomas AJ Jr, Agarwal A. Incidence and level of seminal reactive oxygen species in normal men. *Urology*. 1995b;45: 103 –107.[CrossRef][Medline]

Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol Reprod*. 1997; 56: 602 –607.[Abstract]

Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod*. 1998;4: 439 –445.[Abstract/Free Full Text]

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction*. Cambridge, United Kingdom: Cambridge University Press; 1992 .

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. 3rd ed*. Cambridge, United Kingdom: Cambridge University Press; 1999 .

Zorn B, Vidmar G, Meden-Vrtovec H. Seminal reactive oxygen species as predictors of fertilization, embryo quality and pregnancy rates after conventional in vitro fertilization and intracytoplasmic sperm injection. *Int J Androl*. 2003; 26: 279 –285.[CrossRef][Medline]

This article has been cited by other articles:

HUMAN REPRODUCTION UPDATE HOME

K. Tremellen
Oxidative stress and male infertility--a clinical perspective
Hum. Reprod. Update, May 1, 2008; 14(3): 243 - 258.
[Abstract] [Full Text] [PDF]