

# Reactive oxygen species as an independent marker of male factor infertility

Ashok Agarwal, Ph.D.,<sup>a</sup> Rakesh K. Sharma, Ph.D.,<sup>a</sup> Kiran P. Nallella, M.D.,<sup>a</sup> Anthony J. Thomas, Jr., M.D.,<sup>a</sup> Juan G. Alvarez, M.D.,<sup>b,c</sup> and Suresh C. Sikka, Ph.D.<sup>d</sup>

<sup>a</sup>Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Glickman Urological Institute and Department of Obstetrics-Gynecology, Cleveland Clinic Foundation, Cleveland, Ohio; <sup>b</sup>Centro de Infertilidad Masculina ANDROGEN, La Coruña, Spain; <sup>c</sup>Harvard Medical School, Boston, Massachusetts; and <sup>d</sup>Department of Urology, Tulane University Health Sciences Center, New Orleans, Louisiana

**Objective:** To determine the abnormal patterns of reactive oxygen species (ROS) production in male factor infertility (MFI) patients and to define the ROS reference values in such patients.

**Design:** A retrospective study.

**Setting:** Male infertility clinic at a tertiary healthcare center.

**Patient(s):** We examined 132 MFI patients (all normal sperm parameters,  $n = 24$ , and all abnormal sperm parameters,  $n = 38$ ) and 34 healthy donors.

**Intervention(s):** Routine semen analysis, measurement of ROS.

**Main Outcome Measure(s):** Sperm parameters, ROS levels ( $10^4$  cpm/ $20 \times 10^6$  sperm).

**Result(s):** Normal, healthy donors had significantly higher ( $P < .0001$ ) sperm concentration, motility, and morphology compared with all MFI patients. Univariate analysis indicated a significant association between MFI and log (ROS + 1) (odds ratio [OR] = 3.84), besides sperm parameters and age. A multivariate model using logistic regression analysis also indicated an independent association of log ROS with MFI (OR = 4.25). The ROS cutoff values of 1.2–1.4 had a sensitivity of 0.70–0.78 with a corresponding specificity of 0.82. However, at a cutoff point of 1.2, the OR was 68.6, which increased with an increase in the cutoff.

**Conclusion(s):** High ROS is an independent marker of MFI, irrespective of whether these patients have normal or abnormal semen parameters. We suggest the inclusion of ROS measurement as part of idiopathic infertility evaluation. Treatment with antioxidants may be beneficial in such patients. (Fertil Steril® 2006; 86:878–85. ©2006 by American Society for Reproductive Medicine.)

**Key Words:** Human spermatozoa, male factor infertility, oxidative stress, reactive oxygen species, antioxidants, sperm parameters

Semen analysis constitutes the first biological step in the evaluation of male factor infertility (MFI) in an infertile couple (1). However, there is a considerable debate concerning the diagnostic and prognostic significance of semen parameters in the evaluation of MFI, except in conditions such as azoospermia or severe oligospermia (2–4). A basic semen analysis does not adequately reflect all the parameters of semen quality and function that are required for an optimum fertility status (5). This is much more relevant in cases of idiopathic MFI.

Sperm function tests are highly specialized and mostly subjective. These tests also require experienced laboratory personnel and are generally reserved for those patients where these results will affect treatment plan (6). In some cases, routine evaluations of an infertile couple reveal no detectable

abnormalities that result in the diagnosis of idiopathic or unexplained infertility.

It is known that human spermatozoa generate reactive oxygen species (ROS) in physiologic amounts, which play a role in sperm functions during sperm capacitation, acrosome reaction, and oocyte fusion (7, 8). However, uncontrolled and excessive production of ROS, when it overwhelms the limited antioxidant defenses in semen, results in seminal oxidative stress (9, 10). Recently, a substantial body of growing evidence suggests that such seminal oxidative stress is involved in many cases of idiopathic MFI (9–13).

Seminal oxidative stress causes impairment of semen quality by multiple mechanisms, including damage of the sperm membrane and DNA. Studies have demonstrated that 25%–88% of nonselected infertile patients have high levels of seminal ROS (14, 15). Infertile males generating high levels of ROS are seven times less likely to initiate a pregnancy compared with those who have low levels of ROS (15, 16). A recent meta-analysis by our group demonstrated that ROS levels were significantly correlated with the fertilization rate in couples undergoing IVF (estimated overall correlation,  $-0.37$ ; 95% confidence interval [CI],  $-0.52$ ,  $-0.20$ ) (17). However, there is no consensus concerning the

Received November 17, 2005; revised and accepted February 13, 2006. Supported by the Glickman Urological Institute and the Cleveland Clinic Research Programs Committee, The Cleveland Clinic Foundation, Cleveland, Ohio.

Reprint requests: Ashok Agarwal, Ph.D., Professor, Lerner College of Medicine of Case Western Reserve University, Glickman Urological Institute, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Desk A19.1, Cleveland, Ohio 44195 (FAX: 216-445-6049; E-mail: agarwaa@ccf.org).

inclusion of ROS measurement as part of the routine diagnostic workup of an infertile male. This may be due to the lack of standardization on what constitutes the abnormal or pathological levels of ROS in semen. There is also a paucity of literature examining the patterns of ROS generation in semen samples of infertile men who exhibit normal and abnormal sperm characteristics.

The purpose of this study was to [1] determine the relationship between ROS levels and presence or absence of abnormal semen parameters within the MFI patients, and [2] define the reference values of ROS in normal healthy donors compared with infertile group.

## MATERIALS AND METHODS

The Institutional Review Board of the Cleveland Clinic Foundation approved the study. Patients attending our infertility clinic provided a semen sample for routine semen analysis as the first step in the evaluation of their infertility.

### Study Population

The study population included normal healthy donors ( $n = 34$ , provided 72 samples) and MFI patients ( $n = 132$ ). Male factor infertility is defined as the inability of a couple to conceive a child after 1 year of unprotected sexual intercourse with a normal female partner or spouse with a normal reproductive history, normal ovulation (by follicular ultrasound scan, luteal phase progesterone levels, and endometrial biopsy), and tubal patency (hysterosalpingogram).

The MFI patients consisted of three subgroups: [1] patients with normal sperm parameters ( $n = 24$ ) according to World Health Organization (WHO) (18) criteria, [2] patients with all abnormal sperm parameters ( $n = 35$ ), and [3] patients with  $\geq 1$  abnormal sperm parameter ( $n = 73$ ). Healthy donors had normal semen characteristics according to the WHO guidelines (18). All patients were evaluated with a complete medical history, age, physical examination, and semen analyses. Patients and donors with positive leukocytospermia (white blood cell counts  $\geq 1 \times 10^6/\text{mL}$  ejaculate) were excluded in this study.

### Semen Analysis

Semen samples were obtained by masturbation after 48–72 hours of abstinence. Samples were collected into sterile containers and allowed to liquefy at  $37^\circ\text{C}$  for 20 minutes. A  $5\text{-}\mu\text{L}$  aliquot of well-mixed specimen was loaded onto a  $20\text{-}\mu\text{L}$  Microcell chamber (Conception Technologies, San Diego, CA). Sperm concentration and percent motility were assessed according to WHO criteria (18).

A minimum of 200 spermatozoa were counted per sample. All manual and computer-aided sperm analysis (CASA) measurements were performed by a well-trained and licensed medical technologist. The CASA results were used when the difference between the manual and CASA values

was  $<20\%$ . In cases where the difference was  $>20\%$ , the manual results were considered for further evaluations. For morphological evaluation, seminal smears were stained with Giemsa stain (Diff-Quik; Baxter Healthcare Corporation, Deerfield, IL), and the percent sperm with normal morphology was assessed by WHO guidelines (18) and Tygerberg's strict criteria (19).

### Measurement of Seminal ROS

Aliquots of liquefied semen were centrifuged at  $300 \times g$  for 7 minutes. The sperm pellet was washed twice with phosphate-buffered saline (PBS), pH 7.4, and resuspended in the same medium at a concentration of  $20 \times 10^6$  sperm/mL. The ROS production was measured in the suspension by the chemiluminescence assay 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 Chemical Co.) was added to  $400 \mu\text{L}$  of the washed sperm suspension. The ROS levels were determined by measuring chemiluminescence using a luminometer (Model LKB 953, Berthold Technologies, Bad Wildbad, Germany) in the integrated mode for 15 minutes, and results were expressed as  $\times 10^4$  number of photons counted per minute (cpm)/ $20 \times 10^6$  sperm.

### Statistical Analysis

The ROS values were log transformed into  $\log(\text{ROS} + 1)$  to obtain a normal distribution and were used for further analysis. Sperm parameters and ROS levels were compared among the groups of study population using the unpaired Student's *t*-test. Statistical significance was assessed at a value of  $P < .05$ .

Univariate models on only one predictor variable at a time were examined in addition to a multivariate model with all predictors including age (in years), leukocyte count ( $\times 10^6/\text{mL}$ ), ROS levels ( $\times 10^4$  cpm/ $20 \times 10^6$  sperm), percent motility, concentration ( $\times 10^6/\text{mL}$ ), and morphology (%) by both WHO and Tygerberg's criteria (17, 18). Because the variables examined in the univariate logistic model were continuous variables, interpretation of ORs is for a unit increase of the predictor variable.

A receiver operating characteristic (ROC) analysis was also performed to determine an optimum cutoff point for log ROS. This cutoff was then used to dichotomize the log ROS variable in the multivariate model. Correlation between predictor variables was used to assess the possibility of confounding of the relationship between MFI and established parameters for a diagnosis of infertility. Statistical analysis was performed using SAS statistical software, Version 9.1 (SAS Institute Inc., Cary, NC).

## RESULTS

Normal healthy donors had significantly higher ( $P < .0001$ ) sperm concentration ( $\times 10^6/\text{mL}$ ), motility (%), and morphol-

ogy (%) (WHO and Tygerberg's) compared with all MFI patients (Table 1). However, sperm concentration ( $P=.07$ ), motility ( $P=.57$ ), and Tygerberg's morphology ( $P=.76$ ) parameters were comparable between normal donors and MFI patients with normal sperm parameters (Table 1). Overall, MFI patients and all the subgroups had significantly higher mean ROS levels compared with normal donors (Table 1). Among the MFI patient groups, mean ROS levels were significantly higher in the subgroup of those who had all abnormal sperm parameters compared with MFI patients with all normal sperm parameters.

Univariate analysis was performed to examine the association between age and MFI. Age was significantly associated with MFI (OR = 1.08,  $P<.0094$ ). Similarly, log (ROS + 1) levels were significantly associated with MFI (OR = 3.84,  $P<.0001$ ). Furthermore, sperm concentration, motility, and the morphology (WHO and Tygerberg's criteria) were also significantly associated with MFI, when taken as the sole predictor in each model (Table 2). The extent of the presence of white blood cells (WBCs) in the samples was very high (95% CI: 0.73, 276.42); therefore, although the OR was very large (14.23), it was not significant.

A multivariate model using logistic regression analysis was used to examine predictors of MFI, adjusting for other potential predictors and confounders, such as age, seminal leukocyte count, and morphology. This model included all continuous variables, such as age; seminal WBC count, sperm concentration, and motility; Tygerberg's and WHO morphology; and ROS production. In this model, log ROS values remained statistically significant with the observed OR between log ROS and MFI (OR = 4.25,  $P=.0034$ ) (Table 2). However, the WBC parameter was not significant even though the OR was 27.32, and this was largely because of the wide range of the 95% CI (0.48, <99).

The ROC analysis indicated optimum sensitivity and specificity at a cutoff value between 1.2 and 1.48, with a sensitivity between 0.70 and 0.78 and a corresponding specificity of 0.82. This information was then applied to the logistic models.

To examine the effect of dichotomizing the log ROS values in a model containing all other predictors as continuous variables, we selected two cutoff values between 1.2 and 1.4 that optimized sensitivity and specificity (Table 3). An additional cutoff point of 1.8 was also examined. All cutoff points were chosen such that each corresponded to the range of optimum sensitivity and specificity.

With a cutoff value of 1.2 or an ROS level of 14.8, an OR of 68.64 ( $P=.0003$ ) was observed. In other words, an individual with a log ROS value above the cutoff of 1.2 was 68.64 times more likely to be categorized as MFI when compared with an individual with an ROS level below this cutoff. Similarly, with a cutoff of 1.4, or an ROS level of 24.1, the OR was 105.36 ( $P<.0004$ ). This increased further with a cutoff of 1.8 or an ROS value of 62.1, OR >999.99 ( $P<.0089$ ).

TABLE 1 Comparison of sperm parameters and ROS levels in normal donors and MFI patients.									
Sperm variable	Donors (n = 34)	MFI patients			P value <sup>a</sup>				
		Overall (n = 132)	Normal semen parameters (n = 25)	All abnormal semen parameters (n = 35)	A	B	C	D	
Concentration ( $\times 10^6$ /mL)	71.50 (46.27, 4.31)	22.03 (8.22, 46.55)	48.55 (39.45, 7.95)	6.1 (4.3, 9.8)	<.0001	.07	<.0001	<.0001	<.0001
Motility (%)	62.00 $\pm$ 13.74	35.92 $\pm$ 21.13	63.59 $\pm$ 9.72	21.82 $\pm$ 11.93	<.0001	.57	<.0001	<.0001	<.0001
Morphology (%)									
WHO	35.37 $\pm$ 6.66	29.72 $\pm$ 11.80	40 $\pm$ 9.23	20.47 $\pm$ 6.20	<.0001	<.02	<.0001	<.0001	<.0001
Tygerberg's strict criteria	10.60 $\pm$ 3.33	7.61 $\pm$ 4.42	11.25 $\pm$ 4.22	5.54 $\pm$ 2.99	<.001	<.76	<.0001	<.0001	<.0001
ROS	0.98 (0.60, 1.30)	2.02 (1.34, 2.98)	1.70 (0.75, 2.13)	2.43 (1.76, 3.66)	<.001	<.03	<.0001	<.0001	.012

Note: MFI = male factor infertility; WHO = World Health Organization; ROS = reactive oxygen species  $\times 10^4$  counted photons per minute (cpm)/20  $\times 10^6$  sperm; log<sub>10</sub> (ROS + 1); A = comparison between normal donors and all MFI patients; B = comparison between normal donors and MFI normal semen parameters; C = comparison between normal donors and MFI-abnormal semen parameters; D = comparison between MFI-normal semen parameters and MFI-abnormal semen parameters.  
<sup>a</sup> Compared using Student's unpaired t-test.

Agarwal. ROS and MFI. Fertil Steril 2006.

**TABLE 2****Univariate and multivariate analysis—association between leukocyte count, sperm parameters, and ROS levels.**

Variable	Univariate analysis			Multivariate analysis		
	OR	95% CI	P value <sup>a</sup>	OR	95% CI	P value <sup>a</sup>
Age (y)	1.08	1.02, 1.15	.0094	1.07	0.96, 1.19	.2332
Leukocyte count ( $\times 10^6$ /mL)	14.23	0.73, 276.41	.0794	27.32	0.48, <99	.1096
Concentration ( $\times 10^6$ /mL)	0.98	0.97, 0.99	<.0001	0.99	0.98, 1.00	.0907
Motility (%)	0.92	0.90, 0.95	<.0001	0.89	0.84, 0.95	.0002
% morphology (strict criteria)	0.86	0.78, 0.94	.0009	0.83	0.64, 1.08	.1652
% morphology (WHO)	0.95	0.92, 0.98	.0042	1.09	0.97, 1.22	.1434
Log (ROS + 1)	3.84	2.27, 6.48	<.0001	4.25	1.62, 11.18	.0034

Note: WHO = World Health Organization; ROS = reactive oxygen species expressed as  $\log \text{ROS} = \log (\text{ROS} + 1)$ , where ROS is expressed as  $10^4 \text{ cpm}/20 \times 10^6 \text{ sperm}$ .

<sup>a</sup>  $P < .05$  considered significant using the Wald  $\chi^2$  test.

Agarwal. ROS and MFI. Fertil Steril 2006.

A significant correlation was observed between the predictive variables and  $\log (\text{ROS} + 1)$ . The most striking observation was the negative correlation of  $\log \text{ROS}$  with concentration and motility and morphology ( $r = -0.37$ ,  $-0.35$  [ $P < .0001$ ], and  $r = -0.35$  [ $P < .0001$ ]).

## DISCUSSION

Oxidative stress associated with increased ROS generation and reduced antioxidant capacity is negatively correlated with sperm concentration and motility in infertile men (20–25). In addition, individual reports also point to the possible relationship between seminal oxidative stress and the presence of immature spermatozoa and structural abnormalities in the spermatozoa (25–28).

Our present results also indicated a correlation between ROS and sperm concentration, motility, and morphology (Table 4). However, it is unclear in the literature if ROS

levels are dependent on sperm parameters or are directly related to the diagnosis of MFI.

We conducted a univariate analysis to further clarify the association between MFI and all the variables examined (e.g., age, leukocyte count, sperm concentration, percent motility, morphology [both WHO and Tygerberg's criteria], and ROS levels). The overall MFI group, as well as both the MFI patient subgroups had significantly poorer sperm parameters and elevated ROS levels compared with normal donors (Table 1). Within the MFI patient groups, mean ROS levels in the subgroup of those patients who had all abnormal sperm parameters were significantly higher than those with normal sperm parameters, indicating that ROS levels are partly related to sperm parameters.

When taken as a sole predictor, a significant association was observed between MFI and age, and sperm parameters with  $\log \text{ROS}$  levels (Table 2). However, this significance

**TABLE 3**

**Multivariate analysis—association between MFI and  $\log (\text{ROS} + 1)$  (using different cutoff values of ROS) and the ORs adjusting for all other variables (age, leukocyte count, concentration, motility, strict criteria morphology, WHO morphology).**

Cutoff values $\log (\text{ROS} + 1)$	ROS	OR	95% CI	P value
1.2	14.8	68, 64	6.97, 676.48	.0003
1.4	24.1	105, 36	7.895, >999.99	.0004
1.8	62.1	>999, 99	25.56, >999.99	.0089

Note: ROS = reactive oxygen species expressed as  $\log \text{ROS} = \log (\text{ROS} + 1)$ , ROS values are expressed as  $10^4 \text{ cpm}/20 \times 10^6 \text{ sperm}$ .

<sup>a</sup>  $P < .05$  considered significant using the Wald  $\chi^2$  test.

Agarwal. ROS and MFI. Fertil Steril 2006.

**TABLE 4**

**Correlation coefficients between all the study variables.**

Variable	Concentration ( $\times 10^6$ /mL)		Leukocyte count ( $\times 10^6$ /mL)		Morphology (%)		ROS log (ROS + 1)	
	Motility (%)			WHO	Tygerberg's	Age (y)		
Concentration	1							
Motility	0.38 ( $P < .0001$ )	1	-0.08 ( $P = .324$ )	0.25 ( $P = .0010$ )	0.24 ( $P = .0016$ )	0.06 ( $P = .508$ )	-0.37 ( $P < .0001$ )	
Leukocyte count	-0.08 ( $P < .32$ )	1	-0.03 ( $P = .6572$ )	0.43 ( $P < .0001$ )	0.38 ( $P < .0001$ )	-0.33 ( $P = .0002$ )	-0.35 ( $P < .0001$ )	
WHO morphology	0.25 ( $P = .001$ )		1	-0.15 ( $P = .0590$ )	-0.04 ( $P = .6145$ )	-0.07 ( $P = .4530$ )	0.33 ( $P < .0001$ )	
Tygerberg's morphology	0.24 ( $P = .001$ )			1	0.78 ( $P < .0001$ )	-0.02 ( $P = .8374$ )	-0.35 ( $P < .0001$ )	
Age	0.06 ( $P = .51$ )			0.78 ( $P < .0001$ )	1	-0.02 ( $P = .8599$ )	-0.28 ( $P = .0004$ )	
ROS	-0.37 ( $P < .0001$ )			-0.02 ( $P = .8374$ )	-0.02 ( $P = .8599$ )	1	0.18 ( $P = .0575$ )	
				-0.35 ( $P < .0001$ )	-0.27 ( $P = .0004$ )	0.18 ( $P = .0575$ )	1	

Note: WHO = World Health Organization; ROS = reactive oxygen species.

Agarwal. ROS and MFI. Fertil Steril 2006.

was not observed with leukocyte count, and this could be due to the exclusion of patients and donors with leukocytospermia in our study. Some forms of infertility are caused by age-related degenerative disorders of the testis. The idiopathic MFI may be explained as a form of premature or differential aging of the testis induced by ischemia and oxidative stress associated with a defective mitochondrial genome that controls oxidative phosphorylation (29). The presence of excessive residual cytoplasm on spermatozoa due to imperfect spermiation in aging testis may be a sign of reduced fertility. In addition, Sertoli cell abnormality in infertile men may well be central to the development of spermatogenic failure due to faulty spermiation and may be related to genetic defects, oxidative stress, or even aging of the gonads (30). Increased levels of lipofuscin are indicative of the presence of mitochondrial dysfunction, which is possibly compounded by oxidative stress in the older population. It is considered to be due to ROS-induced lipid peroxidation with age (31, 32).

Although age-related changes in the male reproductive system are universally recognized, the question of declining fecundity with age is controversial (33). A significant age-dependent decrease has repeatedly been reported for sperm motility and morphology (34). Among couples who ultimately conceive, a man >35 years old has twice the likelihood of requiring more than 12 months to impregnate his female partner than does a man <25 years old (33). In addition, a study suggests that paternal age  $\geq 40$  years significantly increases the risk of miscarriage in couples where the woman is >35 years old (35, 36). This may be attributed to an age-dependent accumulation of DNA damage in sperm, possibly as a result of a less efficient mechanism of apoptosis; age is one likely factor that may be responsible for an increase in DNA damage to sperm (37).

Several studies have demonstrated the physiologic and pathologic functions of ROS in the male reproductive system (7, 38–40). Defining the cutoff values for pathologic levels of ROS is a critical issue. However, consensus on the cutoff value of ROS levels to determine the pathologic values is lacking. There is a lack of a common, standardized methodology for the measurement of ROS among the researchers; this is largely due to the differences in the methodologies used for the measurement of ROS and units of ROS production. Using our established methodology, we were interested in providing a cutoff value for ROS that could be considered pathologic. A multiple logistic regression model was constructed after adjusting for all other variables to see whether ROS was an independent marker of MFI. Log ROS was found to be a significant predictor (OR = 4.25;  $P = .0034$ ) of MFI, even after adjusting for all other variables (age, leukocyte count, concentration, motility, morphology) (Table 2). A 1-unit increase in the log ROS value results in a fourfold increase of the odds of the patient being categorized as MFI compared with no increase in the log ROS level.

Receiver operating characteristic curve analysis was performed to find a cutoff value that could be used to differentiate MFI patients from normal males. In our recent study, we reported the cutoff values that differentiated normal from abnormal ROS levels in neat and washed sperm samples (41). However, the units of ROS expression in this study were different. The optimum  $\log(\text{ROS} + 1)$  cutoff value was found to be between 1.2 and 1.48, with a corresponding sensitivity between 0.71 and 0.78 and specificity of 0.82. These values indicate that ROS levels can predict MFI with an accuracy of  $\leq 80\%$ . It is interesting to note that no significant association was observed between sperm concentration and MFI in the univariate model that accounted for all other factors.

Although sperm concentration and morphology (WHO and Tygerberg's) are the two well-established predictors of MFI, it is likely that an association between ROS and sperm concentration may confound this relationship. If that were the case,  $\log$  ROS would have to be independently associated with the MFI as well as with sperm concentration. No clear evidence exists as to whether high ROS levels are a cause or an effect of abnormal semen parameters. From multivariate analysis, it is clear that  $\log$  ROS is independently associated with MFI. It is also evident that sperm concentration and morphology are strongly correlated with  $\log$  ROS (Table 4).

However, this observation is applicable only to the clinical population examined in this study. Future studies will be needed to examine the relationship between  $\log$  ROS and MFI with routine testing of all the sperm parameters in a more general population to provide definitive information regarding the usefulness of ROS as a better predictor of MFI. Conflicting reports make it difficult to establish the utility of ROS in fertility practice. In one report, however, Whittington et al. (23) found no significant relationship between sperm motility and the levels of ROS production. In another report, seminal oxidative stress in patients presenting with prostatitis was not associated with a reduction in sperm concentration, percentage of motility, and morphology compared with normal controls (24).

Previous prospective studies from our center and other individual reports have demonstrated the causative relationship between oxidative stress and the induction of sperm chromatin damage by means of single- and double-stranded DNA breakage (42–44). It has been demonstrated that sperm DNA damage has a significant negative effect on the developing embryo quality (45) and reportedly leads to postfertilization failure (46–49). It may be hypothesized that DNA damage in normal spermatozoa might have contributed to the etiology of infertility in our subset of infertile patients (44).

Postulated mechanisms for infertility among nonleukocytospermic patients with normal semen parameters and high or normal ROS levels are: [1] direct generation of oxygen radicals by low concentrations of leukocytes; [2] significant DNA damage in samples containing abnormal levels of

ROS-producing spermatozoa; [3] diminished levels of antioxidants in the seminal plasma; or [4] the presence of immature sperm in substantial quantities in semen.

Another major source of ROS production in leukocyte-free semen samples in infertile patients may be the presence of substantial quantities of immature spermatozoa (50). Immature spermatozoa, besides being associated with a high generation of ROS, also exhibit enhanced DNA damage indicative of derangement in the regulation of spermiogenesis (43). Thus, in the absence of leukocytospermia, damage of mature spermatozoa by ROS-producing immature spermatozoa during comigration from the seminiferous tubules to the epididymis could be a potential cause of MFI in males with normal semen parameters.

Several studies also suggested that decreased levels of antioxidants in seminal plasma might be a potential cause of infertility in these patients. It has been demonstrated that infertile patients possess low levels of antioxidants and exhibit a lower total antioxidant capacity (TAC) (15). Furthermore, ample clinical and laboratory evidence supports the efficacy of antioxidants in improving semen characteristics and achieving greater IVF success with improved pregnancy rates (51–53). However, because seminal TAC scores were not incorporated as part of our analysis, the role of decreased TAC scores in causing infertility in these patients could not be verified.

In light of our findings, a significant association was observed between MFI and ROS levels after adjusting the semen characteristics in a multiple logistic regression model following the exclusion of leukocytospermic samples. This suggests that ROS may play a significant role in the etiology of MFI. Although any one of the previously mentioned causes may be involved, perhaps the increased ROS levels in the male reproductive tract are the most important contributing factor. Our results support the idea that the presence of abnormal levels of ROS in semen could serve as an independent marker of MFI. Our results are in good agreement with other studies suggesting that ROS-induced oxidative stress is a significant risk factor associated with MFI (24).

In conclusion, MFI patients have high ROS levels in semen. The ROS levels in semen could then be used as an independent marker in the diagnosis of MFI, especially in cases of idiopathic infertility. All the sperm parameters examined, except sperm motility, proved to be poor predictors of MFI. This is a clinically significant finding, in addition to ruling out the female factor as a potential cause of infertility in the couple. It may be important to perform laboratory tests to determine the presence of oxidative stress in males presenting with idiopathic infertility. As ROS testing becomes a part of routine semen analysis, additional prospective studies will help in better defining a reference range. The treatment strategy in these men should include antioxidant supplements that will help reduce oxidative stress and thereby improve sperm quality and fertility.

## REFERENCES

- Jouannet P, Feneux D. Sperm analysis. *Ann Biol Clin* 1987;45:335–9.
- Ombelet W, Bosmans E, Janssen M, Cox A, Vlasselaer J, Gyselaers W, et al. Semen parameters in a fertile versus subfertile population: a need for change in the interpretation of semen testing. *Hum Reprod* 1997;12:987–93.
- Tomlinson MJ, Kessopoulou E, Barratt CL. The diagnostic and prognostic value of traditional semen parameters. *J Androl* 1999;20:588–93.
- Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, et al. Sperm morphology, motility, and concentration in fertile and infertile men. *NEJM* 2001;345:1388–93.
- De Jonge C. Attributes of fertile spermatozoa: an update. *J Androl* 1999;20:463–73.
- The Male Infertility Best Practice Policy Committee of the American Urological Association and The Practice Committee of the American Society for Reproductive Medicine. Report on optimal evaluation of the infertile male. *Fertil Steril* 2004;82:123–30.
- de Lamirande E, Gagnon C. Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Rad Biol Med* 1993;14:157–66.
- Saleh RA, Agarwal A. Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* 2002;23:737–52.
- Sikka SC. Relative impact of oxidative stress on male reproductive function. *Curr Med Chem* 2001;8:851–62.
- Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. *Fertil Steril* 2003;79:829–43.
- Sharma RK, Agarwal A. Role of reactive oxygen species in male infertility. *Urology* 1996;48:835–50.
- Ochsendorf FR. Infections in the male genital tract and reactive oxygen species. *Hum Reprod Update* 1999;5:399–420.
- Aitken RJ, Sawyer D. The human spermatozoon—not waving but drowning. *Adv Exp Med Biol* 2003;518:85–98.
- Lewis SE, Boyle PM, McKinney KA, Young IS, Thompson W. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertil Steril* 1995;64:868–70.
- Sharma RK, Pasqualotto FF, Nelson DR, Thomas AJ, Agarwal A Jr. The reactive oxygen species—total antioxidant capacity score is a new measure of oxidative stress to predict male infertility. *Hum Reprod* 1999;14:2801–7.
- 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;164:542–51.
- Agarwal A, Allamaneni SSR, Nallella KP, George AT, Mascha E. Correlation of reactive oxygen species (ROS) levels with fertilization rate following in vitro fertilization (IVF): a meta-analysis. *Fertil Steril* 2005;84:228–31.
- World Health Organization. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. New York: Cambridge University Press, 1999.
- Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Veeck LL, et al. New method of evaluating sperm morphology with predictive value for human in vitro fertilization. *Urology* 1987;30:248–51.
- Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, Wu FC. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J Androl* 1989;10:214–20.
- Agarwal A, Ikemoto I, Loughlin KR. Relationship of sperm parameters with levels of reactive oxygen species in semen specimens. *J Urol* 1994;152:107–10.
- Zalata A, Hafez T, Comhaire F. Evaluation of the role of reactive oxygen species in male infertility. *Hum Reprod* 1995;10:1444–51.
- Whittington K, Harrison SC, Williams KM, Day JL, McLaughlin EA, Hull MG, et al. Reactive oxygen species (ROS) production and the outcome of diagnostic tests of sperm function. *Int J Androl* 1999;22:236–42.
- Pasqualotto FF, Sharma RK, Nelson DR, Thomas AJ, Agarwal A. Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation. *Fertil Steril* 2000;73:459–64.
- Aziz N, Saleh RA, Sharma RK, Lewis-Jones I, Esfandiari N, Thomas AJ Jr, et al. Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril* 2004;81:349–54.
- Aitken J, Krausz C, Buckingham D. Relationships between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. *Mol Reprod Dev* 1994;39:268–79.
- Aitken RJ, Fisher HM, Fulton N, Gomez E, Knox W, Lewis B, et al. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev* 1997;47:468–82.
- Gil-Guzman E, Ollero M, Lopez MC, Sharma RK, Alvarez JG, Thomas AJ Jr, et al. Differential production of reactive oxygen species by subsets of human spermatozoa at different stages of maturation. *Hum Reprod* 2001;16:1922–30.
- Cummins JM, Jequier AM, Kan R. Molecular biology of human male infertility: links with aging, mitochondrial genetics, and oxidative stress? *Molecular Reproduction & Development* 1994;37:345–62.
- Sikka SC. Oxidative stress and role of antioxidants in normal and abnormal sperm function. *Front Biosci* 1996;1:78–86.
- Reichel W. Lipofuscin pigment accumulation and distribution in five rat organs as a function of age. *J Gerontol* 1968;23:145–53.
- Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. *Proc Natl Acad Sci USA* 1993;90:7915–22.
- Ford WCL, North K, Taylor H, Farrow A, Hull MGR, Golding J, et al. Increasing paternal age is associated with delayed conception in a large population of fertile couples: evidence for declining fecundity in older men. *Hum Reprod* 2000;15:1703–8.
- Kidd SA, Eskenazi B, WYROBEK AJ. Effects of male age on semen quality and fertility: a review of the literature. *Fertil Steril* 2001;75:237–48.
- Henkel R, Maass G, Schuppe SC, Jung A, Schubert J, Schill WB. Molecular aspects of declining sperm motility in older men. *Fertil Steril* 2005;84:1430–7.
- de la Rochebrochard E, Thonneau P. Paternal age  $\geq 40$  years: an important risk factor for infertility. *Am J Obstet Gynecol* 2003;189:901–15.
- Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil Steril* 2003;80:1420–30.
- de Lamirande E, Gagnon C. Redox control of changes in protein sulfhydryl levels during human sperm capacitation. *Free Rad Biol Med* 2003;35:1271–85.
- Baker MA, Aitken RJ. The importance of redox regulated pathways in sperm cell biology. *Mol Cell Endocrinol* 2004;216:47–54.
- Aitken RJ, Ryan AL, Baker MA, McLaughlin EA. Redox activity associated with the maturation and capacitation of mammalian spermatozoa. *Free Rad Biol Med* 2004;36:994–1010.
- 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;83:800–3.
- Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum Reprod* 1998;13:1429–36.
- Ollero M, Gil-Guzman E, Lopez MC, Sharma RK, Agarwal A, Larson K, et al. Characterization of subsets of human spermatozoa at different

stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum Reprod* 2001;16:1912–21.

44. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, et al. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 2003;79:1597–1605.
45. Haaf T, Ward DC. Higher order nuclear structure in mammalian sperm revealed by in situ hybridization and extended chromatin fibers. *Exp Cell Res* 1995;219:604–11.
46. Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. *Rev Reprod* 1999;4:31–7.
47. Virro MR, Larson-Cook KL, Evenson DP. Sperm chromatin structure assay (SCSA<sup>®</sup>) related to blastocyst rate, pregnancy rate and spontaneous abortion in IVF and ICSI cycles. *Fertil Steril* 2004;8:1289–95.
48. Bungum M, Humaidan P, Spano M, Jepson K, Bungum L, Giwercman A. The predictive value of sperm chromatin structure assay (SCSA<sup>®</sup>) parameters for the outcome of intrauterine insemination, IVF and ICSI. *Hum Reprod* 2004;19:1401–8.
49. Evenson DP, Wixon R. Comparison of the Halosperm<sup>®</sup> test kit with the Sperm Chromatin Structure Assay (SCSA<sup>®</sup>) infertility test in relation to patient diagnosis and prognosis. *Fertil Steril* 2004;8:846–9.
50. Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. *J Reprod Fertil* 1992;94:451–62.
51. Lenzi A, Culasso F, Gandini L, Lombardo F, Dondero F. Placebo-controlled, double-blind, cross-over trial of glutathione therapy in male infertility. *Hum Reprod* 1993;8:1657–62.
52. Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* 1996;17:530–7.
53. Agarwal A, Nallella KP, Allamaneni SS, Said TM. Role of antioxidants in treatment of male infertility: an overview of the literature. *Reprod Biomed Online* 2004;8:616–27.