

# Relationship between oxidative stress, semen characteristics, and clinical diagnosis in men undergoing infertility investigation

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**Objective:** To determine whether particular semen characteristics in various clinical diagnoses of infertility are associated with high oxidative stress and whether any group of infertile men is more likely to have high seminal oxidative stress. Reactive oxygen species (ROS) play an important role in sperm physiological functions, but elevated levels of ROS or oxidative stress are related to male infertility.

**Design:** Measurement of sperm concentration, motility, morphology, seminal ROS, and total antioxidant capacity (TAC) in patients seeking infertility treatment and controls.

**Setting:** Male infertility clinic of a tertiary care center.

**Patient(s):** One hundred sixty-seven infertile patients and 19 controls.

**Intervention(s):** None.

**Main Outcome Measure(s):** Semen characteristics, seminal ROS, and TAC in samples from patients with various clinical diagnoses and controls.

**Result(s):** Fifteen patients (9.0%) were Endtz positive and 152 (91.0%) Endtz negative. Sperm concentration, motility, and morphology were significantly reduced in all groups compared with the controls ( $P = .02$ ), except in varicocele associated with infection group. Mean ( $\pm$ SD) ROS levels in patient groups ranged from  $2.2 \pm 0.13$  to  $3.2 \pm 0.35$ , significantly higher than controls ( $1.3 \pm 0.3$ ;  $P < .005$ ). Patient groups had a significantly lower mean ( $\pm$ SD) TAC from  $1014.75 \pm 79.22$  to  $1173.05 \pm 58.07$  than controls ( $1653 \pm 115.28$ ,  $P < .001$ ), except in the vasectomy reversal group ( $1532.02 \pm 74.24$ ). Sperm concentration was negatively correlated with ROS both overall and within all groups ( $P \leq .007$ ), with the exception of idiopathic infertility.

**Conclusion(s):** Irrespective of the clinical diagnosis and semen characteristics, the presence of seminal oxidative stress in infertile men suggests its role in the pathophysiology of infertility. Medical or surgical treatments for infertility in these men should include strategies to reduce oxidative stress. (Fertil Steril® 2000; 73:459–64. ©2000 by American Society for Reproductive Medicine.)

**Key Words:** Spermatozoa, male infertility, reactive oxygen species, oxidative stress, antioxidants

Impaired sperm function is an obvious and general cause of male infertility (1). The controlled generation of reactive oxygen species (ROS) in spermatozoa is associated with normal physiological functions. Uncontrolled and excessive production of ROS, however, seems to have a significant role as one of the major factors leading to an infertile status (2–6). Studies have shown that 40%–88% of non-selected infertile patients have high levels of seminal ROS (4).

stress, resulting in decreased sperm motility, viability, and increased midpiece sperm defects that impair sperm capacitation and acrosome reaction (7–9). Human spermatozoa are rich in polyunsaturated fatty acids, and therefore are susceptible to ROS attack. To counteract the harmful effects of ROS, sperm and seminal plasma possess a number of antioxidant systems that scavenge ROS and prevent internal cellular damage (10, 11).

We determined the oxidative stress in semen specimens of men undergoing fertility

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Excessive ROS production causes oxidative

evaluation by measuring the levels of ROS and total antioxidant capacity (TAC) in the seminal plasma. The results from those patients with different clinical diagnoses were compared with those results obtained from men with normal semen parameters (controls). The purpose of this study was to determine whether any semen characteristics associated with a variety of clinical diagnoses were related to levels of high oxidative stress. Also, we examined the levels of oxidative stress in different clinical diagnoses.

## MATERIALS AND METHODS

### Subjects

The study was approved by the institutional review board of the Cleveland Clinic Foundation. Semen specimens were obtained from 167 patients attending the male infertility clinic in the Urology department for infertility evaluation between 1997 and 1998. Nineteen healthy men with normal semen characteristics according to the World Health Organization (WHO) guidelines were recruited to serve as controls (12). All patients were evaluated with a complete medical history, physical examination, and semen analyses. Patients with azoospermia or those with a history of <1 year of infertility were excluded from this study.

Patients were divided into four groups according to their clinical diagnosis: group I, varicocele ( $n = 77$ ); group II, vasectomy reversal ( $n = 43$ ); group III, idiopathic infertility ( $n = 36$ ); and group IV, varicocele associated with infection ( $n = 11$ ). Patients with leukocytes present in the semen analysis were considered as having infection of the male reproductive tract. Semen samples were obtained by masturbation after at least 48 hours of abstinence. Samples were collected into sterile containers and allowed to liquefy at 37°C for 30 minutes and analyzed for sperm concentration, percent motility, and morphology according to WHO criteria (12).

### Semen Analysis

Computer-assisted semen analysis (CASA) was performed on all specimens, with a Motion Analysis VP 50 semen analyzer (Motion Analysis Corporation, Santa Rosa, CA). For each measurement, a 5- $\mu\text{L}$  aliquot was loaded on a counting chamber (MicroCell; Conception Technologies, La Jolla, CA). Four to eight representative fields containing 200 or more spermatozoa were examined. Samples were analyzed for concentration, percent motility, and complex motion characteristics. To ensure the accuracy of the CASA results, a manual assessment was also performed.

### Myeloperoxidase (Endtz Test) for White Blood Cell Measurement

The presence of white blood cells (WBC), especially the granulocytes in semen specimens was assessed by the Endtz test (myeloperoxidase) (13). A 20- $\mu\text{L}$  volume of liquefied specimen was placed in a Corning 2.0-mL cryogenic vial (Corning Costar Corp., Cambridge, MA): 20  $\mu\text{L}$  of phos-

phate-buffered saline (PBS, pH 7.0) and 40  $\mu\text{L}$  of benzidine solution were added. The mixture was vortexed and allowed to sit for 5 minutes. Five microliters of the specimen was placed on a Makler chamber (Sefi Medical, Haifa, Israel) and counted in all 100 squares. Peroxidase positive WBCs staining dark brown were counted. The results, after correction for dilution, were recorded as counts into  $10^6/\text{mL}$ . Leukocytospermia was defined as the presence of at least  $1 \times 10^6$  WBC/mL. In our study, we included both patients with and without leukocytospermia.

### Reactive Oxygen Species

Aliquots of liquefied semen were centrifuged at  $300 \times g$  for 7 minutes. Seminal plasma was aliquoted and frozen at  $-20^\circ\text{C}$  for later measurement of total antioxidant levels. The sperm pellet was washed twice with (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 by the chemiluminescence assay method using luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as the probe (13). 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 with a luminometer (LKB 953, Wallac Inc., Gaithersburg, MD) in the integrated mode for 15 minutes, and results were expressed as  $10^4$  counted photons per minute (cpm) per  $20 \times 10^6$  sperm.

### Total Antioxidant Capacity

Total antioxidant capacity was measured in seminal plasma with use of the enhanced chemiluminescence assay (14). Aliquots of the seminal plasma stored at  $-20^\circ\text{C}$  were thawed at room temperature and immediately assessed for their antioxidant capacity as follows. Seminal plasma was diluted 1:10 with deionized water ( $\text{dH}_2\text{O}$ ) and filtered through a 0.20- $\mu\text{m}$  Millipore filter (Allegiance Healthcare Corporation, McGaw Park, IL). Signal reagent was prepared with a chemiluminescence kit (Amersham Life Science, Buckinghamshire, United Kingdom). Twenty microliters of horseradish peroxidase-linked immunoglobulin (HRP-linked Ig; Amersham Life Science) was added to 4.98 mL of  $\text{dH}_2\text{O}$ . This was further diluted 1:1 to give a working solution with the desired luminescence output ( $3 \times 10^7$  cpm). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble tocopherol analogue, was added as the standard at concentrations between 50 and 150  $\mu\text{M}$ .

With the luminometer set in the kinetic mode, 100  $\mu\text{L}$  of signal reagent and 100  $\mu\text{L}$  of HRP were added to 700  $\mu\text{L}$  of  $\text{dH}_2\text{O}$  and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between 2 and  $3 \times 10^7$  cpm) for 100 seconds. One hundred microliters of the prepared seminal plasma was added to the signal reagent and HRP, and the chemiluminescence was measured. Suppression of chemiluminescence and the time from the addition of seminal plasma to 10% recovery of the initial chemi-

**TABLE 2**

Comparison of semen characteristics, ROS, and TAC in control and in infertile men with various clinical diagnoses.

Clinical diagnosis	Semen characteristics			Oxidative stress		
	Sperm concentration (10 <sup>6</sup> mL)	Sperm motility (%)	Sperm morphology (WHO)	Log (ROS + 1) (cpm)	TAC (Trolox equivalents)	
Control	69.42 ± 10.03	55.45 ± 4.83	39.78 ± 3.16	1.3 ± 0.3	1653.98 ± 115.29	—
Varicocele	25.89 ± 4.77	38.40 ± 2.33	29.05 ± 1.41	2.2 ± 0.13	1173.05 ± 58.07	.0005
Vasectomy reversal	34.18 ± 6.30	28.97 ± 3.03	30.12 ± 1.84	2.4 ± 0.17	1532.02 ± 74.24	.40
Idiopathic infertility	37.53 ± 6.89	37.93 ± 3.36	30.39 ± 2.23	2.3 ± 0.21	1014.75 ± 79.22	.0001
Varicocele associated with infection	48.24 ± 12.47	48.17 ± 6.00	36.81 ± 3.56	3.2 ± 0.25	1026.33 ± 150.32	.002

Values are means ± SD.

\* Comparison between patient groups and control,  $P < .05$  was considered significant.

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**TABLE 1**

Leukocytospermia in control and in infertile patients with clinical diagnoses.

Clinical diagnosis	Endtz positive (%)	Endtz negative (%)
Control	0 (0/19)	100 (19/19)
Varicocele	7.8 (6/77)	92.2 (71/77)
Vasectomy reversal	7 (3/43)	93 (40/43)
Idiopathic infertility	5.6 (2/36)	94.4 (34/36)
Varicocele associated with infection	36.4 (4/11)	63.6 (7/11)
All groups	9.0 (15/167)	91.0 (152/167)

Note: Numbers in parentheses indicate the number of Endtz-positive patients to the total number of patients.

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luminescence were recorded. Antioxidant capacity was expressed as molar Trolox equivalents.

### Statistical Analysis

Endtz-positive status was compared among the different clinical groups with Fisher's exact test. Semen characteristics, ROS, and TAC levels were compared among the five diagnostic groups and the control group with the analysis of variance (ANOVA). If significant differences were found, each clinical diagnosis was compared with the control group to determine pairwise significance with Student's *t*-test. The correlation between semen characteristics, ROS, and TAC were performed with use of Spearman rank method. Statistical significance was assessed with  $P < .05$  and two-tailed tests. All summary statistics are presented as means ± SE. The SAS statistical software package (SAS Institute Inc., version 6.12, Cary, NC) was used for all analyses.

## RESULTS

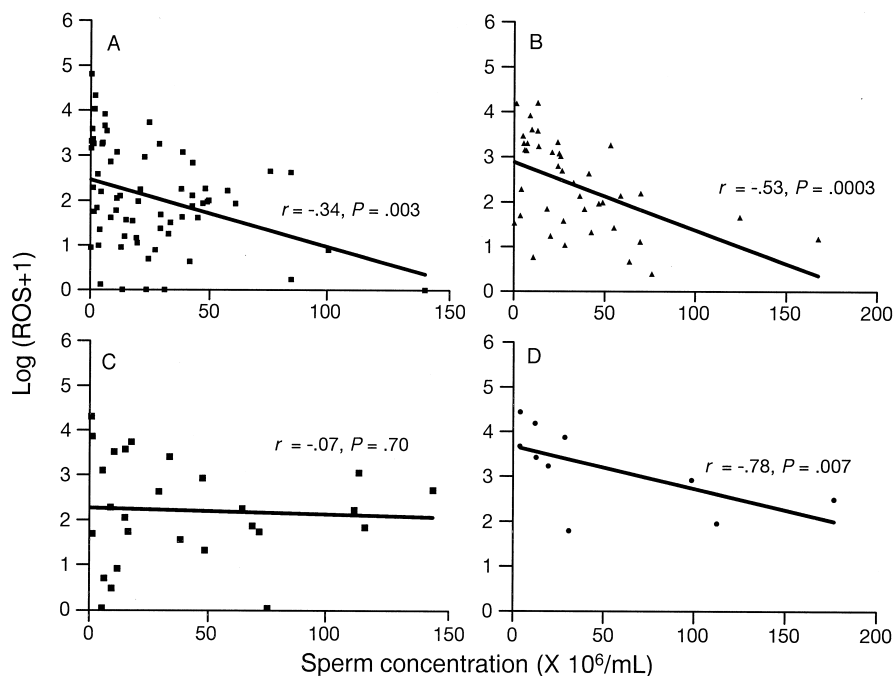
The patient sample consisted of both Endtz-positive and Endtz-negative patients. Of the 167 patients, 91.0% (152 of 167) were Endtz-negative and 9.0% (15 of 167) Endtz-positive. Among the four groups, a significantly higher percent of Endtz-positive patients was present in group IV (36.4%, 4/11;  $P = .04$ ), whereas there was no difference in the number of Endtz-positive patients in groups I, II, and III ( $P = 1.00$ ) (Table 1).

Results of semen analyses are shown in Table 2. Compared with controls, sperm concentration, motility, and morphology were significantly reduced in all groups ( $P \leq .02$ ), except group IV. This may, in part, be attributable to the small sample size of the group ( $n = 11$ ). In addition, the patients in this group had the highest sperm concentration, motility, and normal morphology of the four groups.

Higher levels of ROS were seen in all patients' groups compared with controls (Table 2). The highest levels of ROS were seen in the varicocele associated with infection group

**FIGURE 1**

Relationship between ROS and sperm concentration ( $r$ ) within four groups of patients: (A) varicocele, (B) vasectomy reversal, (C) idiopathic infertility, and (D) varicocele associated with infection.



Pasqualotto. Oxidative stress. Fertil Steril 2000.

( $3.2 \pm 0.35$ ). Sperm concentration was the parameter most correlated with ROS in groups I, II, and III ( $P \leq .007$ ) (Fig. 1). A strong correlation was also seen between sperm morphology and ROS in group II ( $P \leq .005$ ) (Table 3).

Decreased TAC levels were observed in all four patient groups compared with controls and the lowest TAC levels were found in group III ( $1014.75 \pm 79.22$ ) (Table 2). The TAC was negatively related to morphology when the patient had varicocele or vasectomy reversal (Table 3). Overall, a significant correlation was observed between TAC and percent motility ( $r = -0.17$ ;  $P = .04$ ).

## DISCUSSION

Controlled generation of ROS has a physiological role in spermatozoal functions such as hyperactivation, capacitation, and acrosome reaction (8, 15). Increased levels of ROS have been found in the semen of infertile men (16, 17). A prospective study demonstrated that men with high levels of ROS generation had sevenfold less chance of initiating a pregnancy compared with those with low ROS (17). The ROS can cause an increase in DNA fragmentation (18), and pretreatment with antioxidants that dispose, scavenge, and

**TABLE 3**

Correlation between ROS and TAC levels with semen characteristics in infertile patients with various clinical diagnoses.

Clinical diagnosis	ROS			TAC		
	Concentration	Motility	Morphology	Concentration	Motility	Morphology
Varicocele	-0.34 ( $P = .003$ )	-0.07 ( $P = .052$ )	-0.23 ( $P = .06$ )	-0.003 ( $P = .97$ )	-0.07 ( $P = .17$ )	-0.29 ( $P = .02$ )
Vasectomy reversal	-0.53 ( $P = .0003$ )	-0.13 ( $P = .41$ )	-0.41 ( $P = .009$ )	0.27 ( $P = .08$ )	-0.05 ( $P = .75$ )	0.32 ( $P = .04$ )
Idiopathic infertility	-0.07 ( $P = .70$ )	-0.253 ( $P = .20$ )	-0.03 ( $P = .86$ )	0.005 ( $P = .97$ )	0.05 ( $P = .77$ )	0.05 ( $P = .79$ )
Varicocele associated with infection	-0.78 ( $P = .007$ )	0.40 ( $P = .20$ )	-0.12 ( $P = .72$ )	0.018 ( $P = .96$ )	-0.26 ( $P = .46$ )	-0.38 ( $P = .27$ )
Overall	-0.33 ( $P < .0001$ )	-0.11 ( $P = .19$ )	-0.21 ( $P = .01$ )	0.08 ( $P = .31$ )	-0.17 ( $P = .04$ )	-0.06 ( $P = .45$ )

$P < .05$  was considered significant.

Pasqualotto. Oxidative stress. Fertil Steril 2000.

suppress the formation of ROS can reduce DNA damage (19).

Among the well-known biological antioxidants, superoxide dismutase, catalase, and glutathione peroxidase/reductase system have a significant role in protecting the sperm against peroxidative damage (3, 8). Depressed seminal antioxidant capacity has been implicated in male infertility. Both TAC and individual antioxidant levels have been shown to be lower in the semen of infertile men (4, 10). Furthermore, studies of infertile men empirically treated with antioxidants have demonstrated improved semen characteristics, fertilization in vitro, and higher pregnancy rates in the treatment group (20–22).

Excessive ROS levels are related to an increase in lipid peroxidation (23). Spermatozoa from oligospermic patients are susceptible to lipid peroxidation, and at least half of these men have elevated levels of ROS in seminal plasma (5, 6). We found abnormally high levels of ROS in infertile men attending our infertility clinic, irrespective of their clinical diagnosis. Most of the patients in our study had poor semen characteristics (concentration, motility, and morphology); however, the presence of these abnormal semen characteristics was insufficient to predict which groups of patients were more likely to exhibit high levels of oxidative stress. Although patients in the varicocele associated with infection group had the highest level of ROS, they showed no differences in semen characteristics from controls.

Higher generation of ROS in patients with varicocele associated with infection in our study was similar to our previous finding of a fourfold increase in ROS generation in the incidental varicocele patients compared with normal healthy men (24). The ROS was also negatively related to sperm concentration. In patients with varicocele associated with infection in the semen, leukocytospermia due to inflammation may have a higher percentage of activated neutrophils, which are considered the main producers of ROS.

Reactive oxygen species generation by the WBCs, especially polymorphonuclear leukocytes (PML) or granulocytes, can exert a deleterious effect on human spermatozoa as indicated by a marked loss of sperm motility and a reduced capacity for oocyte penetration. That contaminating PML is a major source of the reactive oxygen species activity recorded in the human sperm is incontrovertible.

The impact of contaminating leukocytes on sperm function clearly depends on the number of cells involved, their state of activation, the levels of free radical generation, and the point at which they are added to the sperm suspensions. Therefore, the development of efficient techniques for selectively removing PML from sperm suspensions or neutralizing their adverse effects could be important for improving fertilization and pregnancy rates. Although leukocyte infection may not have a major impact on male infertility in vivo, these cells do influence fertilization rates in vitro (25).

In the absence of varicoceles, high levels of leukocyte contamination may be associated with damage to accessory glands and the disruption of the sperm function via mechanisms that are not related directly to peroxidative damage to sperm plasma membrane.

Recent data suggest that cytokines and ROS may interact in mediating the toxic effects of inflammation. In subfertile patients with or without leukocytospermia, increase in the number of WBC was associated with lower  $\alpha$ -glucosidase levels and  $\gamma$ -glutamyltransferase activity. These were correlated with the overproduction of ROS, interleukin-1 (IL-1), and IL-receptor antagonist, suggesting that in cases with male accessory gland infection, the deleterious effects on sperm quality may be exerted through the production of ROS and/or of particular cytokines produced locally and by WBC. The measurement of these cytokines in semen may provide clinically useful information for the diagnosis of male accessory gland infection and in the absence of WBC where it can provide information about certain mechanisms of male reproductive function and dysfunction. Again, in patients with or without male accessory gland infection, ROS and IL-6 concentration had a comparable sensitivity in discriminating between cases with or without male accessory gland infection (26).

Previous reports have described that 25%–40% of patients with idiopathic infertility have elevated levels of ROS. This would suggest that lipid peroxidation of sperm membrane may be one of the key mechanisms involved in the pathophysiology of male infertility (2). We have confirmed the generation of high ROS levels in this patient population and demonstrated that this group had the second highest level of oxidative stress among the different clinical diagnosis groups. The presence of high ROS, lack of correlation with sperm concentration, and low percentage of patients with leukocytospermia in patients with idiopathic infertility is unclear.

Vasectomy reversal patients are also capable of producing abnormally high levels of ROS (14). In these men, ROS was negatively related to sperm concentration and morphology, and TAC was positively related to morphology. Postulated mechanisms for the infertility among these patients are direct generation of oxygen radicals by leukocytes, sperm damage as a result of partial epididymal blockage, alteration within the immune mechanisms, and presence of immature sperm as a result of testicular damage due to prior obstruction (27).

Total antioxidant levels were lower in all groups of patients in our study compared with controls. Total antioxidant capacity in the idiopathic infertility, varicocele and infection, and varicocele groups were significantly less than the levels in the control group. The TAC levels may contribute strongly to the pathophysiology of male infertility, irrespective of the clinical diagnosis.

The high levels of ROS and low TAC in infertile men

with male factor diagnoses suggest that oxidative stress is clearly associated with a variety of male infertility diagnoses. Treatment approaches for these male factors should include a strategy to decrease ROS and increase TAC levels.

In conclusion, patients attending an infertility clinic, irrespective of their clinical diagnosis, have high ROS and depressed TAC levels. Semen characteristics are unable to predict groups of patients who will have higher ROS levels. The highest levels of ROS are found in men with varicocele associated with infection group. Treatment strategy in these men might include antioxidant supplements that may reduce oxidative stress and improve sperm quality.

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