

Impact of clinical varicocele and testis size on seminal reactive oxygen species levels in a fertile population: a prospective controlled study

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Objective: To investigate: 1) the impact of clinical varicocele on reactive oxygen species (ROS) levels in neat and washed semen in a proven fertile population; and 2) the correlation between ROS levels, testicular volume, and varicocele grade in the same population of fertile men.

Design: Prospective controlled clinical study.

Setting: Andrology laboratory at tertiary-care hospital.

Patient(s): One hundred fourteen healthy fertile men (81 normal fertile and 33 fertile with clinical varicocele) and 30 infertile patients (control subjects).

Intervention(s): Standard semen analysis and measurement of sperm ROS production.

Main Outcome Measure(s): Seminal parameters, seminal ROS levels, seminal leukocyte levels, clinical varicocele, and testis size.

Result(s): Thirty-three of the 114 (29%) fertile men had clinical varicocele (grade 1, n = 14; grade 2, n = 11; and grade 3, n = 8), and the remaining 81 (71%) had a normal physical examination. Levels of ROS and semen quality did not differ significantly between the fertile men with or without varicocele. No significant differences in ROS levels in neat and washed semen were observed compared with fertile men with grades 2 and 3 varicocele and with fertile men with varicocele grade 1. The ROS levels in neat and washed semen were not significantly correlated with varicocele grade in fertile men. No significant correlations between ROS levels and testis volume were observed between the fertile groups.

Conclusion(s): The presence of clinical varicocele in fertile men is not associated with higher seminal ROS levels or abnormal semen parameters. Levels of ROS are not correlated with varicocele grade or testis volume in the same population of fertile men. (Fertil Steril® 2007; ■: ■–■. ©2007 by American Society for Reproductive Medicine.)

Key Words: Normal fertility, varicocele, testis volume, reactive oxygen species, male infertility

Varicocele is associated with both primary infertility (19% to 41% of men) and secondary male factor infertility (45% to 81%). However, more than 85% of men with varicoceles are not infertile (1, 2). No single physiologic mechanism satisfactorily accounts for the development of varicocele. In all likelihood, the etiology is multifactorial. Although men with varicocele frequently have decreased testicular volume and impaired sperm quality, the majority of them are still able to initiate a pregnancy (3, 4).

The exact mechanism by which varicocele affects male fertility and spermatogenesis is unknown (5). Clearly, the factors contributing to abnormal sperm function caused by varicocele that lead to infertility are ambiguous. Research conducted during the last decade has provided growing support to the concept that oxidative stress (OS) is one of the main causes of male infertility. Oxidative stress occurs either as a result of increased levels of reactive oxygen species or insufficient antioxidant capacity (6, 7).

Studies have reported increased levels of ROS in semen from infertile patients with varicocele and in men with incidental varicocele (8, 9). However, ROS levels in fertile men with incidental varicocele who have initiated at least one pregnancy have not been assessed. Even men presenting with normal semen parameters can not be considered fertile

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because seminal analysis is not a proof of fertility. We believe that because the majority of fertile men with varicocele have “normal” semen analyses, they should be able to conceive without difficulty. The objective of the present study was to evaluate ROS levels in fertile men with and without clinical varicocele. We also assessed the correlation between ROS levels in semen, testicular volume, and varicocele grade in the same population of fertile men.

MATERIAL AND METHODS

Study Population

The Institutional Review Board approved this study, and written informed consent was obtained from all participants by the time of their first appointment. This observational prospective controlled study included 114 men who underwent voluntary sterilization by vasectomy between May 2004 and January 2006. The fertile group included healthy men who had initiated at least one pregnancy and were therefore considered to have proven fertility. Thirty-three of the 114 men had a diagnosis of clinical varicocele, and 81 had normal genital examinations. Thirty men from couples who had failed to conceive after at least 1 year of regular unprotected intercourse were used as a positive control. The control group comprised patients with clinical varicocele as the only identifiable cause of infertility. The female partners of those men underwent gynecologic evaluations, and all results were normal.

Two male infertility specialists evaluated all participants. Varicocele was clinically classified as grade 1 (palpable with Valsalva), grade 2 (palpable without Valsalva), or grade 3 (visible through the scrotal skin). Testicular volume was measured with a Prader orchidometer (10). Patients were excluded from the study if 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, epididymal, or vas deferens alterations, and scrotal or inguinal surgery. To reduce the heterogeneity of the groups, the same exclusion criteria were applied to all of the male participants.

Semen Analysis

Semen was collected by masturbation after 48 hours to 72 hours of sexual abstinence. All semen analyses were performed manually after liquefaction. The macroscopic and microscopic parameters were assessed according to World Health Organization (WHO) guidelines (11). Complete semen analysis, leukocytospermia (Endtz test), and determination of ROS levels in neat and washed semen were performed in all samples. Subjects with semen samples containing $\geq 1 \times 10^6$ leukocytes/mL semen were excluded to avoid a potential source of ROS generation. Semen parameters, including sperm concentration, total motility, grade A sperm, grade B sperm, grade C sperm, total motile sperm, and total sperm

and morphology according to WHO and Kruger's strict criteria (3, 12) were assessed.

Leukocytospermia

Leukocyte concentrations in semen were measured by a myeloperoxidase-staining test (13). A 20- μ L volume of liquefied specimen was placed in a 1.8-mL microtube, and 20 μ L phosphate-buffered saline (pH 7.0) with 40 μ L benzidine solution was added. The mixture was examined for cells that had stained brown, indicating that they contained peroxidase and were therefore granulocytes (13).

Reactive Oxygen Species

Measurement of seminal ROS in neat semen and washed spermatozoa Aliquots of liquefied semen were centrifuged at 300g for 7 minutes. The sperm pellet was washed twice with human tubal fluid (HTF) and Hepes buffer (cat #90126; Irvine Scientific, Santa Ana, CA). It was then resuspended in the same medium at a concentration of 20×10^6 sperm/mL (14). The ROS production was measured by a 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 mmol/L luminol prepared in dimethyl sulfoxide (Sigma Chemical Co.) were added to 400 μ L neat semen or washed sperm suspension. The ROS levels were determined by measuring chemiluminescence with a luminometer, MicroBeta Trilux (software version 4.7; Perkin Elmer Life Sciences, Turku, Finland) for 15 minutes. Results were expressed as 10^4 counted photons per minute (cpm)/ 20×10^6 sperm.

Statistical Analysis

The Kolmogorov-Smirnov test was used to view the data graphically and analyze how the data was distributed. We compared all groups on the categorical variables using the chi-squared test and Fisher exact test. Student *t* test was used for the normally distributed continuous variables. Pair-wise comparisons between the groups were performed with Wilcoxon rank sum tests for continuous nonnormally distributed data. Correlations between variables were calculated using Spearman nonparametric method. All analyses were performed with Minitab (version 14.2; Six Sigma; Austin, TX) and SPSS (version 14.0; SPSS Institute, Cary, NC). A *P* value of $< .05$ was considered to be statistically significant.

RESULTS

The median (interquartile range [IQR]) value of age among the study groups was: 1) fertile men with varicocele, 34 years (31, 38 years); 2) fertile men with normal genital examination, 34 years (30, 39 years); and 3) infertile patients with varicocele, 33 years (26, 37 years). The differences were not statistically significant ($P=.17$). The distribution of clinical varicocele grade among fertile men was as follows: 42.4%

grade 1; 33.3% grade 2; and 24.2% grade 3. The distribution of clinical varicocele grade among the infertile men was: 20% grade 1, 43.3% grade 2, and 36.6% grade 3. The median ROS levels were significantly lower in the neat semen of the infertile men, $0.4 (0.2, 0.9) \times 10^4$ cpm, than in the washed semen, $8.1 (3, 21) \times 10^4$ cpm; $P < .0009$.

The results of standard semen parameters (sperm count, total motility, and percentage normal morphology by WHO and Kruger's criteria) and leukocyte levels in the fertile men with or without varicocele and infertile patients with varicocele are shown in Table 1. In the infertile patients with varicocele, all seminal parameters were significantly lower, and all leukocyte levels higher than that of the fertile men with or without varicocele. However, there were no significant differences between routine seminal parameters and leukocyte levels between the fertile men with varicocele and fertile men without varicocele. Also, the infertile men had lower testis volume than the fertile men, but there were no significant differences between the men with and without varicocele (Table 1). The ROS levels in neat and washed semen were significantly higher in the infertile patients than in both fertile populations. Levels of ROS in neat and washed semen did not significantly differ between fertile men with varicocele and fertile without varicocele (Table 1).

Correlations between ROS (neat and washed semen), varicocele grade and testis volume in the fertile population were

performed by considering ROS as the sole predictor in each model. No significant correlation between ROS levels and varicocele grade or testis volume was found in fertile men with or without varicocele (Table 2).

No significant differences in seminal parameters, WHO morphology, Kruger's strict criteria, leukocyte levels, and testis volumes between the fertile men with varicocele grades 2 and 3 and the men with varicocele grade 1 were observed (Table 3). Also, there were no significant differences in ROS levels in neat and washed semen when comparing fertile men with varicocele grades 2 and 3 with fertile men with varicocele grade 1 (Table 3).

DISCUSSION

The incidence of clinical varicocele in the general population is approximately 15%, although large controlled studies have reported rates up to 30% (15, 16). However, in this population, it is difficult to demonstrate a firm association between varicocele and abnormal semen parameters or decreased fertility (16, 17). Studies on unselected fertile men with and without varicocele have shown no significant differences between standard seminal parameters (mainly sperm count, motility, and morphology) (18–20).

Patients with varicocele usually undergo treatment based on the evidence that it leads to a gradual loss of normal

TABLE 1

Comparison of seminal parameters, leukocyte levels, and ROS levels in neat and washed semen among fertile men without varicocele, fertile men with varicocele, and infertile patients.

Parameter	Fertile men without varicocele (n = 81)	Fertile men with varicocele (n = 33)	Infertile patients (n = 30)			
				A	B	C
Sperm concentration ($\times 10^6$ /mL)	83 (51, 144)	92 (53, 143)	26 (16, 78)	.85	<.0001*	.0001*
Sperm motility (%)	67 (56, 74)	70 (63, 75)	48 (32, 58)	.08	<.0001*	<.0001*
WHO morphology (%)	20 (11, 27)	21 (15, 30)	11 (5, 23)	.13	.011*	.0017*
Kruger's morphology (%)	4 (2, 8)	6 (3, 9)	2.0 (0, 4)	.09	.0043*	.0005*
Leukocytes ($\times 10^6$ /mL)	0 (0, 0.2)	0 (0, 0.2)	0.4 (0, 1.6)	.23	<.0001*	.0007*
ROS in neat semen ($\times 10^4$ cpm)	0.4 (0.2, 0.9)	0.3 (0.1, 1)	1.8 (0.5, 7.9)	.85	<.0001*	.0004*
ROS in washed semen ($\times 10^4$ cpm)	8 (3, 20)	7 (2, 30)	69 (20, 714)	.95	<.0001*	<.0001*
Right testis volume (mL)	20 (20, 25)	20 (20, 20)	20 (15, 20)	.31	.028*	.03*
Left testis volume (mL)	20 (20, 20)	20 (20, 20)	20 (12, 20)	.35	.019*	.02*

Note: ROS = reactive oxygen species; WHO = World Health Organization; A = *P* value between fertile men without varicocele and fertile men with varicocele; B = *P* value between fertile men without varicocele and infertile patients; C = *P* value between fertile men with varicocele and infertile patients. Values are median and interquartile range (25%, 75%). Wilcoxon rank sum test was used for the analysis.

**P* < .05 was considered to be statistically significant.

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TABLE 2

Correlation of ROS levels (neat and washed semen) with varicocele grade and testis volume in fertile men with varicocele and fertile men without varicocele.

Variable	Fertile men without varicocele (n = 81)		Fertile men with varicocele (n = 33)		Fertile men without varicocele (n = 81)		Fertile men with varicocele (n = 33)	
	ROS in neat semen				ROS in washed semen			
	r	p value	r	p value	r	p value	r	p value
Varicocele grade	N/A	N/A	.01	.91	N/A	N/A	0.04	.78
Right testis volume (mL)	−0.10	.36	−0.12	.49	−0.08	.45	−0.13	.46
Left testis volume (mL)	−0.04	.71	−0.26	.13	−0.002	.98	−0.31	.08

Note: N/A = not applicable; ROS = reactive oxygen species. $P < .05$ was considered to be statistically significant (Spearman rho test).

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spermatogenesis over time and ultimately results in irreversible infertility (21). However, this is not accurate, and surgery is not indicated for all men presenting with varicocele, even though varicocelectomy has a low morbidity (22). In fact, varicocele repair is not currently recommended for men who are not attempting to achieve conception but have a palpable varicocele with normal semen analyses and a desire for future fertility (23). Although routine seminal analysis is the initial step in any infertility evaluation, it does not adequately reflect semen quality and the functions required for optimal fertility potential. The identification of markers that identify fertile men with varicocele who will progressively demon-

strate a decrease in sperm function will reduce unnecessary surgeries. The use of testicular volume and routine seminal parameters as a predictor of fertility potential in patients with varicocele is still controversial. Pinto et al. (4) demonstrated that testicular size was not a predictor of fertility potential in patients with varicocele.

The mechanism(s) by which varicocele grade corresponds with variable spermatogenic functions is still unknown. Nevertheless, varicocele grade in infertile patients has been inversely associated with seminal parameters and ROS levels (3, 24) but not with testis size (22).

TABLE 3

Comparison of seminal parameters, leukocyte levels, and ROS levels in neat and washed semen between fertile men with varicocele grades 2 and 3 and fertile men with varicocele grade 1.

Parameters	Fertile men, varicocele grade 1 (n = 19)	Fertile men, varicocele grade 2 and 3 (n = 14)	p value
Sperm concentration ($\times 10^6$ /mL)	91 (44, 147)	95 (55, 137)	.82
Sperm motility (%)	70 (60, 75)	69 (63, 75)	.91
WHO morphology (%)	24 (19, 30)	13 (19, 28)	.20
Kruger's morphology (%)	7 (4, 10)	5 (3, 9)	.43
Leukocytes ($\times 10^6$ /mL)	0 (0, 0.2)	0 (0, 0.2)	1.0
Right testis volume (mL)	20 (20, 20)	20 (15, 20)	.25
Left testis volume (mL)	20 (20, 20)	20 (15, 20)	.47
ROS in neat semen ($\times 10^4$ /cpm)	0.3 (0.1, 1)	0.3 (0.1, 1)	.91
ROS in washed semen ($\times 10^4$ /cpm)	8.1 (1, 17)	5 (2, 64)	.86

Note: ROS = reactive oxygen species; WHO = World Health Organization. Values are median and interquartile range (25%, 75%). Wilcoxon rank sum test was used for the analysis, and $P < .05$ was considered to be statistically significant.

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One previous study reported elevated ROS production in 80% of an infertile varicocele population (9). Those authors also reported that 77% of the incidental varicocele group had increased ROS production compared with only 20% of the control group. However, among the clinical “fertile” varicocele group, only 2 of 15 patients had ever initiated a pregnancy (9). Because the ultimate evaluation of fertility is pregnancy, we therefore believe that the data from that study is not comparable with our findings, because our population had a proven fertility status. Current investigative modalities such as routine semen analysis and physical examination are not enough to indicate which patients with varicocele should be treated when fertility potential has not been tested. We found no difference in seminal parameters, ROS levels, and testis volume between fertile men with varicocele grade 1 and men with grades 2 and 3.

Presently there is no consensus about including ROS measurements in routine exams for the evaluation of fertility potential in men with varicocele. This may be due to a lack of standardization of the normal values of ROS in the fertile and infertile populations. Levels of ROS have been assessed previously in neat and washed semen (14, 25). The determination of ROS levels in neat semen may reflect more accurately the condition of OS, supported by the fact that during the washing procedure, seminal plasma is removed and results in a decrease of the antioxidant capacity as well as an increase in ROS levels (26). On the other hand, it is also important to measure ROS in washed semen, because removal of the seminal plasma is the first step in processing samples for assisted reproductive techniques.

Clinical varicocele in infertile patients has been associated with decreased seminal parameters, high levels of ROS, and progressive testicular atrophy (3, 9). However, in the present study, the presence of varicocele did not exert a detrimental effect on these parameters in the fertile population, suggesting that the pathophysiologic mechanisms of varicocele may not have the same impact on testicular function in fertile men. The fertile population may have more efficient defense mechanisms to protect themselves against varicocele consequences such as testicular overheat, hypoxia, stasis, increased testicular pressure, elevated testicular temperature, increase in spermatic vein catecholamines, and excessive ROS levels (5). Additionally, the equilibrium between oxidants and antioxidants of the fertile population may be more efficient in the neutralization of increased ROS levels stemming from varicocele.

Sperm from infertile men with varicocele has recently been associated with significantly high levels of DNA damage (8). The finding of high seminal ROS in patients with varicoceles may indicate that OS plays an important role in the pathogenesis of sperm DNA damage in patients with this condition. It also has been reported that high levels of OS and DNA damage are associated with a decrease in the fertilizing capacity of spermatozoa (27). Although no treatment for abnormal DNA integrity has been shown to have clinical value (28), Zini et al. (29) reported that varicocelectomy can im-

prove human sperm DNA integrity in infertile men with clinical varicoceles. Moreover, Mostafa et al. (30) showed that varicocelectomy reduces seminal ROS levels, therefore infertile patients with palpable varicocele and normal seminal parameters may have experience the same benefit from varicocelectomy if high levels of ROS are found in semen. On the other hand, measurement of ROS may help in the decision to treat adolescents or men with unknown fertility potential presenting with clinical varicocele and normal seminal parameters.

According to the Best Policies Practice Groups of both the American Urological Association and the American Society for Reproductive Medicine, for men who have a palpable varicocele and abnormal semen analyses but are not attempting to conceive, varicocele repair may be offered (31). However, adolescents as well as adult men who have a palpable varicocele and normal semen analyses should be followed with semen analyses every 1 to 2 years (32).

In the present study, we found that the presence of varicocele in fertile men was not associated with higher levels of ROS. In addition, ROS levels were not correlated with varicocele grade and testis volume in fertile men. In men with unknown fertility status presenting with palpable varicocele and normal seminal parameters, the presence of increased ROS levels may be indicative of an early identification of those subjects who will experience a progressive decrease in fertility potential if left untreated.

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