

RELATIONSHIP OF INTERLEUKIN-6 WITH SEMEN CHARACTERISTICS AND OXIDATIVE STRESS IN PATIENTS WITH VARICOCELE

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ABSTRACT

Objectives. To examine levels of interleukin-6 (IL-6) in fertile semen donors and patients with varicocele and examine its association with semen characteristics and levels of reactive oxygen species (ROS).

Methods. We conducted a prospective study consisting of 15 fertile donors (controls) and 35 infertile patients with varicocele. Semen analysis was performed according to the World Health Organization guidelines. IL-6 levels were measured using the enzyme-linked immunosorbent assay. ROS ($\times 10^4$ counted photons per minute per 20×10^6 sperm) and total antioxidant capacity (molar trolox equivalents) were measured using a chemiluminescence assay.

Results. The sperm concentration and motility were significantly greater in the donors compared with the infertile patients with varicocele ($P < 0.0001$ and $P = 0.01$, respectively). The IL-6 (\log_{10} [IL-6 + 1]) and ROS (\log_{10} [ROS + 1]) levels were significantly greater in infertile patients with varicocele than in the donors (IL-6: 2.1 [1.7, 2.4] versus 0.7 [0, 1.9], $P = 0.003$; ROS: 1.8 [1.2, 2.6] versus 1.0 [0.7, 1.6], $P = 0.04$). The total antioxidant capacity levels were significantly lower in the varicocele patients (1166.7 ± 366.2) than in the donors (1556.4 ± 468.1 ; $P = 0.003$). The IL-6 levels correlated significantly with the ROS levels in the infertile patients with varicocele ($r = -0.39$; $P = 0.01$).

Conclusions. Infertile patients with varicocele exhibited elevated levels of IL-6 and ROS and decreased levels of total antioxidant capacity. Pro-inflammatory cytokine IL-6 and oxidative stress may play a role in the pathophysiology of infertility in these patients. UROLOGY 64: 1010–1013, 2004. © 2004 Elsevier Inc.

Varicocele is a common condition in men attending infertility clinics, affecting approximately 35% to 40% of those with primary infertility and up to 80% of men with secondary infertility.¹ Although the association of varicocele and infertility has long been recognized, the underlying pathophysiology has yet to be clearly elucidated. Several theories have been proposed.² According to one theory, infertility is caused by an imbalance between reactive oxygen species (ROS) and semi-

nal antioxidants in the semen, resulting in oxidative stress and spermatozoal damage.^{3,4} Several studies have supported this premise as a cause of infertility in patients with varicocele also.^{5–10}

Some research has suggested that varicocele-related infertility is also associated with cytokines. Cytokines are released by various immunocompetent cells in the male urogenital tract. They play an important role in cell signaling and perform broad pleomorphic activities.¹¹ Studies have reported that cytokines may be mediators of oxidative stress and have the potential to alter redox equilibrium.^{12–14} A study in patients with genital tract inflammation reported that cytokines may modulate pro-oxidant and antioxidant activities in the male genital tract.¹⁵ They are also capable of influencing sperm function and fertility.^{16,17} Many studies have indicated that cytokines in infertile patients with male accessory gland infection are sensitive markers of silent inflammation.^{18–20} In-

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TABLE I. Comparison of semen characteristics, reactive oxygen species, total antioxidant capacity, and interleukin-6 levels between fertile donors and men with varicocele

Variable	Normal Donors (n = 15)	Infertile Patients with Varicocele (n = 35)	P Value
Concentration ($\times 10^6$ /mL)*	65.8 (44.1, 75.3)	19.0 (7.6, 37.4)	<0.0001
Motility (%) [†]	53.0 \pm 19.2	37.9 \pm 17.3	0.01
WHO morphology (%) [†]	38.0 \pm 10	35.2 \pm 13.8	0.54
Kruger's morphology (%) [†]	11.5 \pm 2.9	10.5 \pm 5.0	0.52
Log ₁₀ (IL-6 + 1)*	0.7 (0, 1.9)	2.1 (1.7, 2.4)	0.003
Log ₁₀ (ROS + 1)*	1.0 (0.7, 1.6)	1.8 (1.2, 2.6)	0.04
TAC (Trolox equivalents) [†]	1556.4 \pm 468.1	1166.7 \pm 366.2	0.003

KEY: WHO = World Health Organization; IL-6 = interleukin-6; ROS = reactive oxygen species; TAC = total antioxidant capacity.
* Values presented as median with 25%, 75% interquartile range in parentheses; P values calculated by Mann-Whitney U test.
[†] Values presented as mean \pm standard deviation; P values calculated by unpaired t test (P <0.05 considered statistically significant).

erleukin-6 (IL-6) is a multifunctional cytokine found in seminal fluid that is produced by a number of different cells.²¹

Although a few studies have examined the role of cytokines^{16,20,22} and oxidative stress^{5,6,8} independently in patients with varicocele, to our knowledge, none have examined the association of both cytokines and oxidative stress simultaneously in such patients.

Therefore, the aim of our study was to examine IL-6 levels in fertile donors and infertile patients with varicocele and to examine the association among IL-6, semen characteristics, and oxidative stress.

MATERIAL AND METHODS

SUBJECTS

The institutional review board approved this study, and all subjects provided written informed consent. A total of 15 donors and 35 infertile patients with varicocele were enrolled through the male infertility clinic and andrology laboratory at a tertiary care hospital. A male infertility specialist (A.J.T.) confirmed the presence of clinical varicocele on physical examination. The control group consisted of 15 healthy volunteers who had initiated a pregnancy within the past 2 years and had normal semen analysis results according to the World Health Organization (WHO) criteria (1999).²³ Azoospermic men and men with leukocytospermia (more than 10^6 white blood cells/mL) were excluded from the study.

Semen samples were obtained by masturbation after at least 48 hours of sexual abstinence. Samples were collected into sterile containers and allowed to liquefy at 37°C for 30 minutes. The semen was analyzed according to the WHO guidelines for sperm concentration ($\times 10^6$) and percent motility. Sperm morphology was analyzed using both WHO and Tygerberg strict criteria.^{23,24} The presence of leukocytes in semen specimens was assessed using a myeloperoxidase (Endtz) test.

MEASUREMENT OF IL-6

IL-6 levels were measured with a double antibody "sandwich assay" (enzyme-linked immunosorbent assay) using monoclonal antibody specific for IL-6 (Carmen Chemical, Ann Arbor, Mich). The immobilized end product was read at 410 nm. The intensity of color was proportional to the absorbance of acetylcholine esterase, which in turn was propor-

tional to the IL-6 levels. Samples for each patient group were measured in parallel and in duplicate to avoid interassay variance. The sensitivity of the IL-6 was 0.7 pg/mL, and the standard curve range was 3.12 to 300 pg/mL.

MEASUREMENT OF ROS

Aliquots of liquefied semen were centrifuged at 300g for 7 minutes. The sperm pellet was washed twice with phosphate-buffered saline (pH 7.4) and resuspended in the same medium at a concentration of 20×10^6 sperm/mL. ROS production was measured with the chemiluminescence assay, using luminol (5-amino-2, 3-dihydro-1, 4-phthalazinedione; Sigma Chemical, St. Louis, Mo) as the probe. A total of 10 μ L of 5 mM luminol prepared in dimethyl sulfoxide (Sigma Chemical) was added to 400 μ L of the washed sperm suspension. The levels of ROS were determined by measuring chemiluminescence with an Autolumat LB 953 luminometer (Berthold Technologies, Bad-Wildbad, Germany) in the integrated mode for 15 minutes. The results are expressed as $\times 10^4$ counted photons per minute per 20×10^6 sperm.

MEASUREMENT OF TOTAL ANTIOXIDANT CAPACITY

Total antioxidant capacity was measured in the seminal plasma using the enhanced chemiluminescence assay.²⁵ Aliquots of the seminal plasma stored at -20°C were thawed at room temperature and immediately assessed for their antioxidant capacity. Signal reagent was prepared using a chemiluminescence kit (Amersham Life Science, Buckinghamshire, England). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble alpha-tocopherol analogue, was used as the standard.

With the luminometer set in the kinetic mode, 100 μ L of signal reagent and 100 μ L of horseradish peroxidase were added to 700 μ L of dH₂O and mixed. The solution was then equilibrated to the desired level of chemiluminescence output (between 2 and 3×10^7 counted photons per minute) for 100 seconds. A total of 100 μ L of the prepared seminal plasma was added to the signal reagent and horseradish peroxidase, and the chemiluminescence was measured. Antioxidant capacity is expressed as molar trolox equivalents.

STATISTICAL ANALYSIS

Log-transformed values of ROS and IL-6 were used for all comparisons. Pairwise comparisons were performed using the unpaired *t* test and Mann-Whitney *U* test. Correlations between variables were assessed using Spearman's rank correlation. A *P* value of less than 0.05 was considered statistically significant using a two-tailed test. Data were analyzed using

TABLE II. Correlation between interleukin-6 and sperm concentration and motility and reactive oxygen species

Variable	Normal Donors (n = 15)		Infertile Varicocele (n = 35)	
	r	P Value	r	P Value
Concentration ($\times 10^6$ /mL)	-0.35	0.23	-0.14	0.58
Motility (%)	-0.47	0.07	-0.11	0.53
ROS Log ₁₀ (ROS + 1)	0.38	0.15	0.39	0.01*

KEY: ROS = reactive oxygen species; r = Spearman rank-correlation coefficient.
P < 0.05 considered statistically significant.
* Statistically significant.

GraphPad Software, version 3.20 (1998, GraphPad Software, San Diego, Calif).

RESULTS

The mean age was similar between the 35 men with varicocele (32.4 ± 1.1 years) and the 15 healthy donors (31.1 ± 2.1 years). Table I details the semen characteristics and levels of ROS, total antioxidant capacity, and IL-6 in the fertile donors and infertile men with varicocele. The median sperm concentration and sperm motility in the fertile donors were significantly greater than in the infertile patients with varicocele ($P < 0.0001$ and $P = 0.01$, respectively). No statistically significant difference was observed in sperm morphology using WHO and Kruger's criteria between the donors and the infertile patients with varicocele (WHO: $P = 0.54$; Kruger's: $P = 0.52$).

The IL-6 levels in the infertile patients with varicocele [\log_{10} (IL-6 + 1)] were significantly greater than the levels in the donors (2.1 [1.7, 2.4] versus 0.7 [0, 1.9]; $P = 0.003$). The levels of ROS in infertile patients with varicocele [\log_{10} (ROS + 1)] were significantly greater than the levels in the donors (1.8 [1.2, 2.6] versus 1.0 [0.7, 1.6]; $P = 0.04$). The levels of total antioxidant capacity in the infertile patients with varicocele were significantly lower than the levels in the donors ($P = 0.003$; Table I).

Table II shows the correlation among IL-6, semen characteristics (sperm concentration and motility), and ROS. IL-6 was directly correlated with ROS in infertile varicocele patients ($r = 0.39$; $P = 0.01$). In addition, no statistically significant correlation was observed between IL-6 and the semen characteristics.

COMMENT

The role of cytokines in male reproductive function has been the subject of recent re-

ports.^{17,26} Most immune responses are probably local, and cytokines released by immunocompetent cells during the defense of bacterial infections act either in a paracrine or autocrine manner.^{17,26} Although the immune system may be the major source of these cytokines, other cells in the reproductive tract such as spermatozoa may also secrete cytokines.²⁷

Our study analyzed the role of pro-inflammatory cytokine IL-6 and oxidative stress in varicocele-related infertility. Our results agree with earlier reports that found semen parameters to be significantly abnormal in infertile patients with varicocele. Our results also showed that the levels of IL-6 and ROS in the patients with varicocele were significantly greater than those in the fertile donors.

The cause of infertility and the elevated levels of IL-6 and ROS in the infertile varicocele group may be a result of an unknown molecular process associated with varicocele pathologic features. It is possible that a subclinical inflammation may occur in patients with varicocele that results in these cellular changes. Our data indicate that IL-6 may be directly correlated with infertility in patients with varicocele. Thus, it may be relevant to measure the IL-6 levels both before and after varicocelectomy surgery and that continuing levels after surgery may predict infertility.

Our results also showed that levels of total antioxidant capacity in the patients with varicocele were lower than those levels in the fertile donors. Antioxidants^{12,28} and glutathione precursors^{14,29} have been shown to downregulate cytokine transcription and biosynthesis. Glutathione depletion, however, is associated with the augmentation of a pro-inflammatory signal by upregulating ROS.^{14,21,29-31} It was reasoned that a differential manipulation of glutathione homeostasis and shuttling might antagonistically affect pro-inflammatory cytokines, thus bearing potential consequences for the treatment of diseases in which cytokines and oxidative stress are recognized as major participants in their pathophysiology.³⁰ Our finding of a statistically significant association between IL-6 and ROS levels in infertile varicocele patients suggests that an interaction occurs between cytokines and ROS.

Even though the conclusions of this study may be limited by the sample size, IL-6 and ROS levels may still be considered as potential markers of infertility in patients with varicocele. However, additional studies recruiting additional infertile patients presenting with different etiologies of infertility are needed to address the role of IL-6 in male infertility.

CONCLUSIONS

Infertile patients with varicocele have greater levels of cytokines and oxidative stress than healthy patients as indicated by elevated levels of IL-6 and ROS and decreased levels of total antioxidant capacity. Pro-inflammatory cytokine IL-6 and oxidative stress may contribute to the pathophysiology of the infertility in men with varicocele. Measuring these biochemical markers may be helpful in the clinical diagnosis of male infertility in these patients.

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