

## Infertility

# Reactive Oxygen Species in the Internal Spermatic and Brachial Veins of Patients with Varicocele-Induced Infertility

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**Purpose:** The aim of this study was to evaluate the relationship between the levels of reactive oxygen species (ROS) and the clinical characteristics of varicoceles among patients with varicocele-induced infertility.

**Materials and Methods:** Ninety-eight patients with infertile varicocele and 22 control subjects without varicocele were enrolled. Blood samples were drawn from the brachial vein (BV) and the dilated internal spermatic vein (ISV) on the side of the varicocele during surgery. ROS levels were determined by spectrophotometry, and comparisons between the varicocele and control groups were performed. In addition, the ROS levels were analyzed according to the characteristics of the varicocele, and ROS levels in the ISV and the BV were compared.

**Results:** The ROS levels measured in the ISV of men with varicocele were higher than in the control group regardless of the varicocele grade except for subclinical grade; however, in the BV, a difference was noted only for grade III. When the difference in testis volume between sides was greater than 3 ml, and the varicocele had been present for more than 3 years, ROS levels were higher in the ISV than in the BV. Sperm viability was significantly associated with ROS levels. Serum hormone levels were not correlated with ROS levels.

**Conclusions:** Serum ROS levels were higher in infertile men with a varicocele than in controls. They were correlated with varicocele grade, varicocele duration, the degree of testicular hypotrophy, and sperm viability. In addition, ROS levels and their associations with clinical characteristics were higher in the ISV than in the BV.

**Key Words:** Infertility; Reactive oxygen species; Varicocele

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## INTRODUCTION

Varicocele is an abnormal enlargement of the pampiniform plexus of the spermatic cord. It is the most common cause of male infertility and occurs in approximately 30% to 81% of infertile men [1,2]. The association of a varicocele with infertility has been recognized for centuries. However, the exact mechanism that causes this abnormality remains to be determined. Several theories have been suggested to explain the mechanism by which a varicocele impairs male fertility. These theories include an increase in apoptosis, increased sperm DNA damage [3], oxidative stress, tissue hypoxia [4,5], degenerative changes in the seminiferous tubule, and immunological infertility caused by a decreased production of Fas protein [6]. These changes are considered to occur gradually as the duration of the varicocele in-

creases [7]. However, the presence of a varicocele does not necessarily cause infertility or worsen the semen parameters. Therefore, studies on the association of a varicocele with male infertility are still needed to improve our understanding of the correlation between a varicocele and male infertility. Reactive oxygen species (ROS) are oxygen metabolites that include superoxide anions, hydrogen peroxide, hydroxyl radical, hydroperoxyl radical, and nitric oxide (NO). When they are in excess, they can cause pathological damage by inducing oxidative changes in cellular lipids, proteins, and DNA [8]. Significant increases of superoxide anions and free radical activity have been demonstrated in some conditions, such as leukocytospermia in men and with varicoceles where great oxidative stress produces different unstable, potentially toxic products [9]. Varicoceles have been found to be associated with elevated

sperm ROS production and diminished seminal plasma antioxidant activity [10]. In addition, surgical varicocelectomy improves seminal parameters and is associated with decreased ROS production and increased levels of seminal plasma antioxidants [11]. Therefore, the ROS level is a useful parameter for assessing the treatment of varicocele-induced infertility. The aim of this study was to evaluate the relationship between the ROS level and the clinical characteristics of varicoceles in infertile men. In addition, the ROS levels from the internal spermatic vein (ISV) were compared with those in the brachial vein (BV).

## MATERIALS AND METHODS

### 1. Subjects

The study group consisted of 98 infertile men selected from patients who presented for varicocelectomy between January 2006 and December 2008. As a control group, 22 patients were enrolled who underwent herniorrhaphy, orchietomy, or primary closure for testicular rupture. Collection and analyses of all samples were approved by the institutional review board, and informed consent was obtained from each subject. The varicoceles were graded according to their physical characteristics: grade III (large, visible through the scrotal skin), grade II (moderately sized, easily palpable without a Valsalva maneuver), and grade I (small, palpable only with a Valsalva maneuver) [12]. To minimize interobserver bias, one experienced senior urologist performed all examinations. Patients with no abnormal findings on the physical examination but with a diameter of the internal spermatic vein > 3.5 mm and reflux seen by color Doppler ultrasonography (Viking 2400, B-K Medical Inc., Denmark) were considered to have subclinical varicoceles.

### 2. Varicocelectomy technique

The ISV was ligated with the aid of a surgical microscope (Universal S3, Zeiss, Germany) by the inguinal or subinguinal approach.

### 3. Blood sampling

Whole blood samples (1 ml) were drawn with a 26-gauge needle during surgery from the BV and from the ISV on the affected side in men with varicocele. The dilated spermatic vein was punctured immediately after exposing the spermatic cord and before any further manipulations. A second blood sample was drawn simultaneously from the BV. In the control group, whole blood samples were drawn by the same method from the ISV and the BV during surgery.

### 4. ROS measurement

The level of ROS radicals was estimated to assess the extent of oxidative stress associated with the varicocele. Aliquots of sampled serum (20 µl) were washed in a microtube with chromogen-buffered saline (CrNH<sub>2</sub>, an amine derivative; reagent R1 of the FORT kit) and resuspended fully and then centrifuged at 3500 rpm for 1 min in FORT kit tubes containing H<sub>2</sub>O<sub>2</sub> (0.26 mg/l). The levels of ROS were deter-

mined by spectrophotometry (Form CR 2000, Callegari, Parma, Italy). The data were expressed in arbitrary units (A.U.).

### 5. Serum testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) level measurement

Measurement of the levels of testosterone, LH, and FSH was carried out by radioimmunoassay (WALLAC 1470 Wizard Automatic Gamma Counter, WALLAC Inc., Gaithersburg, MD, USA).

### 6. Semen parameters and testicular volume

Sperm concentration and motility were measured by using computer-assisted semen analysis (Sperm Analysis Imaging System, Medical Supply Inc., Korea). Sperm viability was measured by using eosin-nigrosin staining, and unstained sperm were regarded as viable. The testicular volume was determined by using a Prader orchidometer. Ipsilateral testicular hypotrophy was defined as a testicular size discrepancy of greater than 3 ml [13].

### 7. Statistical analysis

All data were analyzed by using SPSS for Windows statistical software (version 12.0, SPSS Inc., Chicago, IL, USA), and the statistics are presented as the Mean±SD. Intergroup analysis was performed by using the Mann-Whitney nonparametric U test. Statistical significance was accepted at a  $p < 0.05$ .

## RESULTS

### 1. Demographics

The mean age of the 98 study patients was 28.4±5.6 years.

TABLE 1. Baseline patient characteristics

	Varicocele group (n=98)	Control group (n=22)
Age (years)	28.4±5.6	25.2±6.8
BMI (kg/m <sup>2</sup> )	23.6±7.4	24.2±6.4
History of smoking, n (%)		
Current	43 (43.9)	11 (50.0)
Former	13 (13.3)	5 (22.7)
Never	42 (42.8)	6 (27.3)
History of drinking alcohol, n (%)		
Yes	86 (87.8)	19 (86.4)
No	12 (12.2)	3 (13.6)
Concomittant disease, n (%)		
Hepatic disease	3 (3.1)	2 (9.1)
Hypertension	2 (2.0)	1 (4.5)
Diabetes	0 (0.0)	0 (0.0)
Dyslipidemia	8 (8.2)	3 (13.6)
Grade of varicocele, n (%)		
Subclinical	8 (8.2)	NA
Grade I	8 (8.2)	NA
Grade II	22 (22.4)	NA
Grade III	60 (61.2)	NA

BMI: body mass index, NA: not applicable

The varicoceles were classed as subclinical grade for 8 patients (8.2%), grade I for 8 patients (8.2%), grade II for 22 patients (22.4%), and grade III for 60 patients (61.2%). The control group included 22 patients, and their mean age was 25.2±6.8 years. Eighteen had undergone surgery for testicular injury and four for a hernia. There were no significant differences between the groups in the baseline pa-

tient characteristics (Table 1).

## 2. ROS levels according to clinical characteristics

**1) Grade of varicocele:** As shown in Table 2, the ROS level measured in the ISV for men with all grades of varicocele except subclinical varicocele was significantly higher than in the control group (grade I, p=0.012; grade II, p=0.002;

**TABLE 2.** Comparison of ROS levels between the varicocele and control groups

	Control group (x10 <sup>3</sup> A.U.)	Varicocele group			
		Subclinical (x10 <sup>3</sup> A.U.)	Grade I (x10 <sup>3</sup> A.U.)	Grade II (x10 <sup>3</sup> A.U.)	Grade III (x10 <sup>3</sup> A.U.)
Internal spermatic vein	162.4±46.2	174.2±46.8	197.6±73.5	197.6±74.8	216.4±62.4
p-value		0.058	0.012	0.002	0.001
Brachial vein	164.8±30.3	178.4±41.4	177.6±58.5	185.5±64.2	205.4±49.2
p-value		0.065	0.055	0.052	0.021

ROS: reactive oxygen species, A.U.: arbitrary unit

**TABLE 3.** ROS levels according to clinical characteristics among the men with a varicocele

	n	Internal spermatic vein (x10 <sup>3</sup> A.U.)	Brachial vein (x10 <sup>3</sup> A.U.)	p-value
<b>Varicocele grade</b>				
Subclinical	8	174.2±46.8	178.4±41.4	0.175
Grade I	8	197.6±73.5	177.6±58.5	0.019
Grade II	22	197.6±74.8	185.5±64.2	0.024
Grade III	60	216.4±62.4	205.4±49.2	0.022
<b>Testicular volume (ml)<sup>a</sup></b>				
< 12	9	208.6±40.2	190.5±38.4	0.232
13-15	40	211.6±74.4	184.2±79.2	0.577
16-20	40	201.8±64.5	182.3±66.6	0.052
> 21	9	203.5±56.4	179.2±52.2	0.332
<b>Testicular volume difference (ml)</b>				
≤ 3	51	180.6±59.3	170.7±66.2	0.059
> 3	47	232.6±41.3	215.3±67.2	0.045
<b>Semen parameters</b>				
<b>Sperm concentration (x10<sup>6</sup>/ml)</b>				
< 10	18	227.4±62.4	218.6±42.9	0.117
10-19	54	216.8±47.4	207.5±48.9	0.185
20-40	14	190.5±56.1	177.4±37.2	0.066
> 40	12	177.9±59.4	169.3±44.6	0.086
<b>Motility (%)</b>				
< 30	66	222.6±50.4	207.6±60.9	0.068
30-50	20	200.4±57.6	177.6±61.4	0.051
> 50	12	182.8±58.2	171.8±47.4	0.086
<b>Viability (%)</b>				
< 40	38	241.2±50.1	215.8±59.4	0.048
40-59	18	214.7±67.2	207.6±47.8	0.088
60-80	42	182.3±65.4	174.6±69.9	0.102
> 80	10	179.6±72.8	172.3±85.4	0.332
<b>Duration of varicocele (months)</b>				
< 6	28	201.6±46.2	187.6±56.7	0.057
6-11	32	203.7±44.7	184.1±53.1	0.063
12-36	19	215.8±59.4	192.8±74.4	0.052
> 36	19	236.6±59.1	194.7±79.2	0.002

ROS: reactive oxygen species, A.U.: arbitrary unit, <sup>a</sup>: ipsilateral testis

grade III,  $p=0.001$ ). However, levels in the BV differed only for the patients with grade III varicoceles ( $p=0.021$ ).

**2) Testicular volume:** When the difference in testicular volume between the affected and the unaffected sides in men with varicocele was less than 3 ml, the ROS levels measured in the ISV did not differ from those measured in the BV (Table 3). However, for men with a difference of greater than 3 ml, the ROS level measured in the ISV was significantly higher than that measured in the BV ( $p=0.045$ ).

**3) Duration of varicocele:** In men with a varicocele present for longer than 3 years, the ROS level measured in the ISV was significantly higher than the level in the BV ( $p=0.002$ ) (Table 3).

**4) Semen parameters:** The mean ROS levels in the ISV of men with a varicocele were not significantly different according to sperm concentration. However, in men with deterioration of sperm motility and viability, the ROS levels were high in both the ISV and the BV. In particular, for men with sperm viability of lower than 40%, the ROS levels were significantly higher in the ISV than in the BV ( $p=0.048$ ) (Table 3).

**5) Serum hormone levels:** The ROS levels were not significantly different between the ISV and the BV in relation to serum levels of testosterone, LH, or FSH (Table 4).

## DISCUSSION

The possible causes of varicocele-induced male infertility include hormone dysfunction, scrotal hyperthermia, the retrograde flow of adrenal or renal metabolites, and impairment of testicular artery perfusion [14]. Recently, nu-

merous studies have shown that the oxidative stress associated with the increase in ROS synthesis or the reduction of antioxidant capacity is associated with male infertility in patients with a varicocele. A marked increase in the level of chemoattractive ROS has been reported in men with a varicocele [15]. Testicular histological findings have shown that as the grade of the varicocele increases, the concentration of malondialdehyde (an end product of lipid peroxidation) also increases [16]. In addition, it has been shown that the ROS level can be reduced by varicocelectomy [11]. Furthermore, an association of ROS levels with the pregnancy rate was reported by Aitken et al [17].

Several mechanisms associated with ROS levels and infertility have been suggested. ROS are produced physiologically in the sperm midpiece mitochondria, and spermatozoa are especially vulnerable to oxidative stress because of the high content of polyunsaturated fatty acids in their cell membrane [18]. Also, oxidative stress induces chromosomal telomere shortening, which is directly associated with genomic instability [19]. Leydig cell damage induced by ROS leads to a reduction in testosterone secretion [20]. Oxidative stress exerts its effects not only on the testis but also on the epididymis and the accessory glands, which mediate normal sperm maturation and seminal volume [21]. In the testicular venous system, the main venous drainage associated with a varicocele is the pampiniform plexus that passes through the external inguinal ring and includes the ISV and testicular artery. In the testes of men with a varicocele, several studies have used the ISV to evaluate the level of oxidative stress caused by an increase of ROS. Shiraishi and Naito evaluated the level of testicular oxidative stress by measuring the ROS levels of the ISV immediately adjacent to the testis [22]. Thus, we hypothesized that there would be a more significant relationship of ROS and clinical characteristics in the ISV of infertile varicocele patients than in the BV.

Whether the grade of the varicocele is associated with infertility has not been determined. However, an association of the quality of semen with the grade of varicocele was reported recently [23]. In the present study, the ROS levels in the ISV and the BV of the patients with a varicocele were higher than in the control group, and they were significantly higher in the patients with a grade III varicocele. On the other hand, in a study by Villanueva-Diaz et al [24], there was no correlation between the semen parameters and the grade of varicocele detected.

The ipsilateral testis of patients with varicocele is usually hypotrophied compared with the contralateral side [25]. In this study, in men with less than a 3-ml difference in testicular volume, the ROS levels in the ISV did not differ significantly from those in the BV. However, in men with a difference in volume of greater than 3 ml, the ROS levels in the ISV were significantly higher. These findings suggest that there is an association of testicular hypotrophy with the increase in ROS associated with a varicocele.

The ROS levels in the ISV was significantly higher in those patients who had experienced a varicocele for longer

**TABLE 4.** ROS levels in men with a varicocele according to semen hormone levels

	n	Internal spermatic vein (x10 <sup>3</sup> A.U.)	Brachial vein (x10 <sup>3</sup> A.U.)
<b>Testosterone (ng/ml)</b>			
< 2.0	2	214.4±23.5	220.4±32.4
2.0-2.4	2	208.9±43.4	207.6±13.8
2.5-2.9	14	206.4±70.5	192.5±58.8
3.0-3.5	20	198.3±53.4	186.4±47.4
> 3.5	60	203.8±56.1	194.7±49.2
<b>LH (mIU/ml)</b>			
< 0.5	2	211.6±74.4	199.4±65.4
0.5-2.4	22	202.4±61.5	188.6±67.2
2.5-5.0	42	205.4±58.2	196.1±50.1
> 5.0	32	192.6±50.1	192.8±56.4
<b>FSH (mIU/ml)</b>			
< 1.0	0	NA	NA
1.0-4.9	18	197.7±67.8	189.4±50.4
5.0-9.9	62	207.6±53.4	195.5±59.1
10.0-15.0	15	NA	NA
> 15.0	3	201.2±43.2	198.4±28.9

ROS: reactive oxygen species, A.U.: arbitrary unit, LH: luteinizing hormone, FSH: follicle stimulating hormone, NA: not applicable

than 3 years. This is consistent with the study by Gürdal et al showing that testicular damage caused by apoptosis increases with the duration of the varicocele [7].

In infertile patients, poor semen quality is associated with high ROS levels and a low ROS-total antioxidant capacity score [26]. In this study, the ROS level and sperm concentration, motility, and viability were measured to evaluate the association between semen quality and ROS levels. Overall, the deterioration in seminal parameters was inversely correlated with ROS level. In particular, when the sperm viability was less than 40%, the ROS levels in the ISV were significantly higher than the levels in the BV.

This study showed no significant differences in ROS levels according to serum hormone levels. However, it has been reported that Leydig cell damage caused by ROS is associated with a decreased testosterone level [27]. Such differences might have been due to the relatively short duration of the varicocele in this study, resulting in insufficient time for the varicocele to affect Leydig cell function.

The significant findings of this study were that the ROS level in the BV differed from that in the ISV. This is because the BV is a peripheral vein and thus the ROS levels can be diluted by the effects of the systemic circulation. On the other hand, in the ISV, the ROS produced by Leydig cells and spermatozoa accumulate locally. Therefore, ROS as a marker for infertility are likely to be more useful on the basis of the levels in the ISV. The limitations of this study include the small sample size and the use of patients with testicular injury as the control group. For blood sampling from the ISV, we enrolled patients with a testis injury for the control group. However, changes in the ROS levels associated with testicular injury in the controls might have affected the data.

## CONCLUSIONS

The serum ROS levels of infertile men with a varicocele were high. In addition, the levels were correlated with the varicocele grade, its duration, and degree of testicular hypotrophy. The relationship between ROS levels and the clinical characteristics of infertile men with a varicocele was significantly stronger for samples taken from the ISV than for those taken from the peripheral venous blood. Further studies are required to ascertain the possibility of using the ROS level as a predictive marker of fertility in patients with a varicocele.

## Conflicts of Interest

The authors have nothing to disclose.

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