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Reactive oxygen species (ROS) production in seminal fluid correlate with the severity of varicocele in infertile men

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Abstract

Background: Reactive oxygen species (ROS) are a group of free radicals that in excessive amounts have negative influence on sperm quality and function.

Objective: To study the effect of varicocele and its severity on the level of ROS in infertile men with clinical varicocele.

Materials and Methods: In this controlled prospective study, 42 men with clinically diagnosed left varicocele and 30 fertile men were studied. Patients were asked about history of urogenital infection, using any antioxidant medication and smoking. Grade of varicocele was determined by physical examination. Levels of ROS in seminal plasma were measured in each group by a chemiluminiscence assay. The sperm parameters were also determined and compared in different groups.

Results: The ROS levels were significantly higher in patients with varicocele than normal men (mean: 1575.42 RLU (Radio Luminescence Unit) vs. 53.79 RLU, p=0.005). In total 20 patients had grade I, 20 patients grade II and 2 patients had grade III varicocele. The mean ROS levels were 669.12 RLU, 2406.87 RLU and 2324 RLU in patients with grade I, II and III varicocele respectively (p=0.144). In case group, 15 patients were smoker and 27 were non-smokers. The mean ROS levels in patients with the history of smoking was 1367.71 RLU while in non-smokers it was 897.672 RLU (p=0.729).

Conclusion: Our study showed that increased levels of ROS production in the seminal fluid can be an important factor in the etiology of male infertility in patients with varicocele, and this effect is more prominent with higher grade of varicocele.

***Key words:** Male infertility, Varicocele, Reactive Oxygen Species (ROS), Smoking.*

Introduction

Seminal abnormalities in male contribute to nearly half of the cases of infertility (1). Varicocele is the most common and correctable cause of male factor infertility and varicocelectomy results in seminal improvement in 60-80% of cases (2).

In total, 35-40% of men presenting to an infertility clinic with primary infertility have varicocele. Varicocele is also seen in 69-81% of men with secondary infertility. Varicocele is also a physical abnormality that is present in 2-22 % of the adult male population (3).

Several mechanisms have been proposed that can explain the testicular dysfunction which accompanies varicocele. One of the most recent proposed mechanisms is the over- production of reactive oxygen species (ROS) in semen of patients with varicocele (4). Steckel and co-workers found that men with large varicoceles have poorer semen qualities than men with small varicoceles (5).Oxygen is required to support life, but its metabolites such as ROS, can modify cell function or endanger cell survival (6). Hence, ROS must be continuously inactivated to only the small

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amounts necessary to maintain normal cell functions (7). A strong body of evidence suggests that small amounts of ROS are necessary for spermatozoa to enhance and regulate some sperm functions like the ability to bind Zona pellucida, stimulate sperm capacitation, hyper activation, acrosome reaction and oocyte fusion (8-10). But at high levels they have potential toxic effects on sperm quality and function. Koksal *et al* study showed that severe pathologic changes in the testicular tissue are associated with a high level of lipid peroxidation and suggested that overproduction of ROS may play a role in the mechanism of testicular degeneration associated with infertility (11). ROS are highly reactive oxidizing agents belonging to the class of free radicals. Spermatozoa are particularly susceptible to oxidative stress induced damage, because their plasma membrane contains large quantities of poly unsaturated fatty acids (PUFA) and their cytoplasm contains low concentration of scavenging enzymes (8). The generation of ROS has become a real concern because of their potential toxic effects at high levels on sperm quality and functions (12). Several studies showed increased seminal ROS in infertile patients with varicocele (4, 13). It also has been shown that total antioxidant activity is lower in semen of patients with varicocele (4, 14). But as we know, there is only one paper that addressed the severity of varicocele and the amount of ROS production in semen of infertile patients with varicocele.

In that study, seminal ROS level showed significant correlation with varicocele grade and significantly elevated seminal ROS levels were seen in men with left varicocele grade II and III compared to grade I (15).

Therefore, due to lack of sufficient study in this subject (only one published study), in this study, we assessed the level of ROS production in patients with left varicocele compared with control group, and also the effect of varicocele grade on ROS levels in these patients. We also studied the role of cigarette smoking on ROS levels in patients with varicocele.

Materials and methods

Clinical characteristics

The institutional review board approved this study and it was supported by a grant from the Research Deputy of Yazd University of Medical Sciences. In this cross sectional descriptive-analytic study, we selected 42 infertile men who were referred to Andrology Clinic of Yazd Research and Clinical Center for Infertility and diagnosed to have left varicocele.

All men were examined and evaluated by the same andrologist (Dr. M.R. Moein). Patients were examined both in supine and standing position and instructed to do Valsalva maneuver during examination. Varicocele grade was determined classically as grade I (n=20) if spermatic veins were palpable only with Valsalva, grade II (n=20) if the veins were palpable in standing position without Valsalva, and grade III (n=2) if the veins were visible through scrotal skin and palpable in standing position (16). Patients were asked about their smoking habits and also the medications that they probably had been received for their problem. Patients who had history of taking antioxidant medications (Vitamin C, Vitamin E, Carnitine, Pentoxyphylline, Vitamin A) within the past three months were excluded from study.

Patients also were asked about any history of genital or urinary tract infections and if they had such a history, they also were excluded from the study. We also selected 30 married men as control group who were referred for infertility and work up showed that they have pure female factor as a causative factor for their infertility. They were also examined by the same physician and checked to have not varicocele.

Methods

Routine semen analysis was done for all of cases and control group according to WHO laboratory manuals (17). Semen samples were also checked for peroxidase positive leukocytes (Endz test), and all positive samples with more than 1×10^6 white blood cells/ml were excluded from study.

Levels of ROS were measured by a chemiluminescence assay. Fresh, neat semen specimens were centrifuged at 300×g for 7 minutes and seminal plasma was removed. The pellet was washed with phosphate buffer saline (PBS, Sigma Chemical Co., USA) and re-suspended in the same media. 10µl of luminol (5-amino-2, 3, -dihydro-1, 4-phtalazinedione; Sigma Chemical Co., USA) used as a probe and was added to the aliquot. A negative control was prepared by adding 10µl of PBS. The ROS levels were assessed by measuring chemiluminiscence activity with an Autolamat LB

935 Luminometer (Berthold technologies, Bad-wildbad, Germany) in the integrated mode for 15 minutes (18). The results were expressed as RLU (Radio Luminescence Unit) per 20 million spermatozoa. Routine semen analysis, Endz test and ROS levels in semen were checked for control group too. All of them had normal semen analysis. Data from this group compared with case group and results were expressed.

Statistical analysis

Data were expressed in mean±SD. Differences between control and patients groups were assessed using Mann-whitney U test. A p-value of <0.05 was considered statistically significant. SPSS software version 15 was used for data analysis.

Results

The mean age of control group was 30.90±3.72 years ranging from 22 to 38 years. The mean age of patients in study group, who had clinical varicocele, was 29.79±4.07 years ranging from 23 to 42 years, with no significant difference (p=0.240). Patients were divided according to the severity of varicocele. In total 20 patients had grade I, 20 patients had grade II varicocele and 2 patients had grade III in physical examination. Sperm parameters including sperm count, motility and morphology in both groups are shown in Table I. Also sperm parameters in patients with different grade of varicocele is shown and compared in Table I. All sperm parameters were significantly lower in infertile patients with varicocele as compared to control group (p=0.000). As it shows the sperm count, motility and morphology are significantly different in these three groups (p=0.002, p=0.010, p=0.027 respectively). The mean ROS level in control group was 53.79 RLU, and in infertile patients with varicocele was 1575.42 RLU, which was significantly higher in case group (p=0.005) (Table II).

The mean ROS level in patients with grade I varicocele was 669.12 RLU, in patients with grade II varicocele was 2406.87 RLU and in grade III was 2324 RLU (p=0.144) (Table II). Fifteen patients were smoker and 27 were non-smokers. The mean ROS levels in patients with the history of smoking were 1367.71 RLU, and in patients without history of smoking were 897.67 RLU, which was not significantly different (p=0.729).

Table I. Semen parameters in control group and patients with varicocele (grade I, II and III).

Variable	Control group	Patients group (varicocele)				
	(n =30)	Grade I (n =20)	Grade II (n =20)	Grade III (n =2)	p^a	p^b
Sperm count ($\times 10^6$/ml)	80.50±36.83	60.00±36.93	24.85±19.18	56.50±36.06	0.000	0.002
Sperm motility (%)	53.53±10.24	34.44±20.93	16.40±16.99	42.50±10.60	0.000	0.010
Sperm morphology (%)	41.50±10.34	25.94±11.63	16.30±9.68	22.50±4.95	0.000	0.027

a: p-value between control group and patient group.
b: p-value between grade I , II and III varicocele.

Table II. Maximum, minimum and mean of reactive oxygen species in control group and patients with varicocele (grade I, II and III).

ROS	Control group	Patients group (varicocele)			p-value
	(n =30)	Grade I (n =20)	Grade II (n =20)	Grade III (n =2)	
Minimum	0.050	1.600	1.600	64.000	p^a=0.005
Maximum	498.000	5340.000	9700.000	4584.000	p^b=0.055
Median	12.750	67.500	378.500	2324.000	p^c=0.426
Interquartile range	47.530	751.350	3615.750	-----	p^d=0.968

a: p-value between control group and patient group.
b: p-value between grade I and grade II varicocele.
c: p-value between grade I and grade III varicocele.
d: p-value between grade II and grade III varicocele

Discussion

Recently the over-production of ROS in the male reproductive tract has become a real concern.Recent reports have indicated that high levels of ROS are detected in semen samples of 25-40% of infertile men (8, 19). There are some well known potential sources of ROS production in semen like immature sperm and peroxidase positive leukocyte. Also, several clinical entities have been implicated as a cause of oxidative stress in semen. One of these entities is varicocele.

Varicocele is a well known cause of male infertility in up to 40% of male patients. More recent series have demonstrated that larger varicoceles have a greater improvement in seminal parameters following correction, suggesting that size is a factor (5, 20). Treatment is mainly based on surgical correction of varicocele, although immobilization techniques have been tried for some type of small varicocele. It is also one of the clinical entities that are associated with increased level of ROS in semen. A recent meta-analysis reported that oxidative stress parameters (such as ROS and lipid peroxidation) are significantly increased and antioxidant concentrations significantly decreased in varicocele patients compared with normal sperm donors (21). Although some studies have shown the relation of varicocele and increased level of ROS, but there is a few study that addressed the impact of varicocele grade on the level of ROS production. Therefore, in this prospective, controlled study we assessed the level of ROS production in patients with varicocele comparing to control group. We divided the patients in case group according to the grade of varicocele and measured the ROS level in each group. We also studied the additional effect of cigarette smoking on the level of ROS production in patients with varicocele.

Our study showed that patients with varicocele have totally decreased semen quality relative to fertile men and all parameter of sperm including sperm count, quick and slow progressive motility and sperm morphology are lower in patients with varicocele (Table I). Also levels of ROS production in semen of infertile patients with varicocele was significantly higher than in control groups (p=0.005). This is in accordance with previous studies that showed increasing production of reactive oxygen species and decrease in antioxidant capacity in infertile men with varicocele (4, 14). We divided the patients according to the grade of varicocele into three groups and our study showed that levels of ROS in seminal fluid was higher in patients with grade II and III varicocele compared to the patients with grade I varicocele (p=0.144). The difference was statistically significant between grade I and II varicocele, (p=0.05) but not between grade II and III (p=0.968). This may be due to the relatively small sample size and wide variation of ROS levels, that must be confirmed with larger samples in the future studies. Of course one of the limitations of our study was the low number of patients with grade III varicocele, which can be due to the fact that many of these patients are treated before marriage. In another study, Allamaneni and co-workers also found that ROS levels were significantly greater in patients with high grade varicocele (15). The sperm parameters, as we shown, were higher in patients with grade I varicocele compared to more severe grades, and the differences were statistically significant. Our results are in accordance with the study of Steckel and co-workers who found that men with larger varicoceles have poorer sperm quality than men with small varicoceles (5). More recent series have demonstrated that larger varicoceles have a greater improvement in seminal parameters following correction (5, 20).

While, another study found no correlation between semen parameters and the varicocele grade (22). The lack of correlation between sperm parameters and severity of varicocele in infertile patients in some studies may be due to direct effects of increased ROS impairing the sperm-fertilizing capacity without affecting the normal semen parameters (15). It has been shown that cigarette smoking is one of the causes of increased ROS production in seminal fluid of infertile men (23). In this study we also divided patients with varicocele according to their smoking habit and it showed that ROS levels are higher in smoker patients with varicocele than nonsmokers but it was not significant. Therefore, in contrast to the others, our study didn’t show the additional deteriorating effect of smoking on sperm dysfunction in these patients (23).

Conclusion

It is clear that varicocele is one of the major causes of male infertility. Although different mechanisms have been proposed for its effect on sperm dysfunction, but one of the most recent one, that is over-production of ROS, is not fully studied. Our study showed that varicocele can cause increased level of ROS in seminal fluid of infertile men that is more prominent with higher grade of varicocele but smoking does not aggravate this effect.

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