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Seminal reactive oxygen species-antioxidant relationship in fertile males with and without varicocele

T. Mostafa[1], T. Anis[1], H. Imam[2], A. R. El-Nashar[1] & I. A. Osman[1]

1 Andrology & Sexology Department, Faculty of Medicine, Cairo University, Cairo, Egypt;
2 Medical Biochemistry Department, Ophthalmology Institute, Giza, Egypt

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Correspondence
Dr Taymour Mostafa, MD, Andrology & Sexology Department, Faculty of Medicine, Cairo University, Cairo 11562, Egypt.
Tel.: +20 10 515 0297;
E-mail: taymour1155@link.net

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Summary

The aim of this study was to assess seminal reactive oxygen species (ROS)-antioxidants relationship in fertile and infertile men with and without varicocele. One hundred and seventy six males were studied; fertile healthy volunteers (n = 45), fertile men with varicocele (n = 45), infertile oligoasthenozoospermia (OA, n = 44) without varicocele and infertile OA with varicocele (n = 42). In their seminal plasma, two ROS parameters (malondialdehyde, hydrogen peroxide) and five antioxidants (superoxide dismutase, catalase, glutathione peroxidase, vitaminE, vitaminC) were estimated. Compared with fertile healthy men, in all other studied groups, estimated seminal ROS were significantly higher and estimated antioxidants were significantly lower. Infertile men with varicocele showed the same relationship as infertile men without varicocele. Sperm concentration, total sperm motility as well as sperm normal forms were negatively correlated with seminal malondialdehyde and were positively correlated with vitaminC. It is concluded that varicocele has an oxidative stress (OS) in fertile normozoospermic bearing conditions. This may allow understanding that, within men with varicocele, there is a threshold value of OS over which male fertility may be impaired.

Introduction

Oxidative stress (OS) as a result of the imbalance between reactive oxygen species (ROS) and antioxidants in the semen can lead to sperm damage, deformity and eventually male infertility. This involves peroxidative damage to sperm membrane and DNA fragmentation at both nuclear and mitochondrial levels. OS has been implicated as the major aetiological factor leading to sperm DNA damage (Agarwal *et al.*, 2008).

Varicocele, a varicosity of the pampiniform plexus, is an important physical finding in infertile men and is thought to be the most treatable factor of male infertility. Its overall prevalence is approximately 15% in post-pubertal populations, with prevalence in infertility clinics approaching 40% (Levinger *et al.*, 2007). Varicocele may be associated with a variety of spermatogenetic conditions, ranging from normal semen parameters to moderate oligoasthenoteratozoospermia or azoospermia. The effect of varicocele on spermatogenesis in subfertile men is often reflected by low sperm count, decreased sperm motility or increased sperm abnormal morphology (Pasqualotto *et al.*, 2005; Zucchi *et al.*, 2006). Köksal *et al.* (2007) added that varicocele-induced testicular dysfunction may be associated with disruption of the blood-testis barrier.

Different studies demonstrated high levels of seminal oxidative stress in men with varicocele, suggesting that sperm dysfunction may be related to oxidative stress (Hendin *et al.*, 1999; Pasqualotto *et al.*, 2000; Zini *et al.*, 2000; Meucci *et al.*, 2003). In addition, oxidative stress has been shown to affect the integrity of the sperm genome by causing high frequencies of single- and double-strand DNA breaks (Mostafa *et al.*, 2001; Saleh & Agarwal, 2002; Wang *et al.*, 2003; Smith *et al.*, 2006). Varicocele-associated OS was evidenced also at spermatic vein blood (Mitropoulos *et al.*, 1996; Ozbek *et al.*, 2000; Mostafa *et al.*, 2006a) as well as testicular tissues (Köksal

et al., 2000, 2007). Allamaneni *et al.* (2004) correlated increased ROS with varicocele grade, but not with the testis size. Further, Shiraishi & Naito (2006) speculated that surgical varicocele repair reduces OS in the testis.

The aim of this study was to assess seminal ROS-antioxidants relationship in fertile and infertile males with and without varicocele.

Materials and methods

We enrolled a total of 176 males with matched age who were recruited prospectively from the Andrology Department of the University Hospital after institutional review board approval. Their mean age was 31.4 ± 6.2 years (range 23–47 years) and all of them were married for more than 2 years. They were divided into groups such as (Gp1, $n = 45$) fertile healthy volunteers without varicocele as controls, fertile men with varicocele (Gp2, $n = 45$), infertile oligoasthenozoospermia without varicocele (Gp3, $n = 44$) and infertile oligoasthenozoospermia with unilateral varicocele (Gp4, $n = 42$). Inclusion criteria of fertile men were having a child within the last year in addition to two successive normozoospermic semen analyses. Mean duration of infertility was 3.2 ± 0.62 years. Exclusion criteria included secondary infertility, leucocytospermia and smoking that could elevate ROS levels (Mostafa *et al.*, 2006b; Athayde *et al.*, 2007). A detailed medical history was gathered and physical examination was carried out. Varicocele was diagnosed by physical examination confirmed by using scrotal colour Doppler ultrasound (Acuson 128XB, Golden, Colorado, USA) in both supine and upright position. Grades I, II, III were included.

Ejaculates were obtained (08:00–10:00) after 4 days of sexual abstinence into sterile containers for immediate analysis. More than one sample provided by each subject was examined according to the guidelines of the World Health Organization (1999) by computer-assisted (Autosperm) method (Kamtchouing *et al.*, 1993). Sperm tracks in freshly liquefied semen were followed manually with the help of a cursor on the digitising tablet superimposed at the centre of the microscope field (Olympus microscope CH30/CH40, Tokyo, Japan) magnified X500. This tablet generated data that can be analysed by a microcomputer programme to calculate sperm concentration and percentage of total sperm motility. Sperm morphology was evaluated by phase contrast microscopy of the native sample. Also, air-dried smears were prepared and fixed in equal parts of ethanol-ether, then stained using simplified Papanicolaou stain counting minimal 200 spermatozoa using oil-immersion lenses. Samples with white blood cell >1 million ml^{-1} or positive bacterial culture were excluded.

Semen samples were centrifuged at 1800 ***g*** for 15 min and the supernatant seminal plasma was subjected to estimation of two ROS parameters (malondialdehyde [MDA], hydrogen peroxide [H_2O_2]) and five antioxidants (superoxide dismutase [SOD], catalase [Cat], glutathione peroxidase [GPx], vitaminE and vitaminC). MDA was assayed by thiobarbituric acid reaction (Placer *et al.*, 1966). H_2O_2 was estimated by the modified spectrophotometric method (Feigl, 1958). SOD was measured according to Winterbourn *et al.* (1975). Cat was measured calorimetrically (Sinha, 1972). Total PGx was measured using the method of Paglia & Valentine (1967). Vitamin E was measured spectrofluorometrically by the method of Taylor *et al.* (1976). Saponification and extraction of vitaminE from seminal plasma were performed according to Desai (1984). VitaminC was measured calorimetrically as described by Jagota & Dani (1982).

Statistical analysis

Numerical data were expressed as mean ± SD. Comparisons were performed by one-way ANOVA to test the null hypothesis that there were no differences and then Bonferroni *post-hoc* test was conducted. Correlation between variables was calculated using Spearman's nonparametric methods. Statistical significance was set at $P < 0.05$.

Results

Data of the investigated groups are presented in Table 1. Seminal ROS parameters; MDA, H_2O_2 were significantly higher and estimated antioxidants were significantly lower in fertile men with varicocele, OA men with and without varicocele compared with healthy controls. Also, there was a significant decrease in seminal ROS parameters and a significant increase in seminal antioxidants in infertile men without varicocele compared with infertile men with varicocele.

Sperm concentration showed a significant negative correlation with seminal MDA ($r = -0.857$, $P = 0.001$), H_2O_2 ($r = -0.680$, $P = 0.001$) and significant positive correlation with seminal vitaminC ($r = 0.629$, $P = 0.005$), vitaminE ($r = 0.619$, $P = 0.006$). Total sperm motility percentage showed a significant negative correlation with seminal MDA ($r = -0.859$, $P = 0.001$), H_2O_2 ($r = -0.718$, $P = 0.001$) and a significant positive correlation with seminal vitaminC ($r = 0.705$, $P = 0.001$), SOD ($r = 0.662$, $P = 0.014$), catalase ($r = 0.538$, $P = 0.0225$). Sperm abnormal forms percentage showed a significant positive correlation with seminal MDA ($r = 0.733$, $P = 0.001$), H_2O_2 ($r = -0.766$, $P = 0.001$) and a significant negative correlation with seminal vitaminC ($r = -0.569$, $P = 0.009$), SOD ($r = -0.509$, $P = 0466$), GPx ($r = -0.518$,

Table 1 Data of the studied groups (mean ± SD)

	Gp1 (n = 45) Controls	Gp2 (n = 45) Fertile with varicocele	Gp3 (n = 44) OA without varicocele	Gp4 (n = 42) OA with varicocele
Age (years)	30.3 ± 4.5	27.7 ± 6.7	34.8 ± 5.6	32.5 ± 6.7
Sperm concentration (10^6 per ml)	89.5 ± 41.3	59.8 ± 24.5[a]	12.2 ± 11.1[a,b]	8.0 ± 6.0[a,b,c]
Total sperm motility (%)	69.5 ± 2.1	66.0 ± 5 .0	33.0 ± 11.7[a,b]	23.3 ± 10.4[a,b,c]
Sperm abnormal forms (%)	25.0 ± 8.8	35.7 ± 6.6[a]	52.2 ± 11.1[a,b]	68.6 ± 14.6[a,b]
MDA (nmol ml^{-1})	5.2 ± 0.6	12.5 ± 1.2[a]	15.1 ± 2.1[a]	23.5 ± 2.2[a,b,c]
H_2O_2 (μmol ml^{-1})	18.0 ± 3.7	37.0 ± 4.1[a]	34.2 ± 4.2[a]	40.7 ± 4.6[a,c]
SOD (U ml^{-1})	22.1 ± 3.2	14.9 ± 3.5[a]	14.3 ± 2.9[a]	6.1 ± 1.4[a,b,c]
Cat (μmol ml^{-1})	15.2 ± 2.1	8.6 ± 2.0[a]	6.9 ± 2.1[a]	5.7 ± 0.7[a,b]
GPx (U ml^{-1})	5.4 ± 1.8	4.1 ± 1.3[a]	3.6 ± 1.4[a,b]	1.1 ± 0.2[a,b,c]
Vit. E (μg dl^{-1})	45.5 ± 5.0	32.3 ± 4.2[a]	33.5 ± 33.1[a]	31.5 ± 4.7[a]
Vit. C (mg dl^{-1})	0.50 ± 0.09	0.24 ± 0.09[a]	0.25 ± 0.04[a]	0.3 ± 0.07[a,b]

[a]Significant comparison Gr1 versus all groups ($P < 0.05$).
[b]Significant comparison Gr2 versus all groups ($P < 0.05$).
[c]Significant comparison Gr3 versus all groups ($P < 0.05$).

$P = 0.0047$). Other relations demonstrated nonsignificant correlations.

Discussion

Oxidative stress results from the production of oxygen radicals in excess of the antioxidant capacity of the stressed tissue. Varicocele has been demonstrated to increase testicular oxidative stress, which leads to an increase in germ cell apoptosis and subsequent hypospermatogenesis. Such stress conditions can cause changes in the dynamics of testicular microvascular blood blow, endocrine signaling and germ cell apoptosis (Turner & Lysiak, 2008).

Most of the articles that discussed the influence of varicocele in infertile males demonstrated increased seminal ROS in varicocele-associated conditions being negatively correlated with sperm concentration, sperm motility and sperm normal forms percentage (Hendin *et al.*, 1999; Mostafa *et al.*, 2001; Mancini *et al.*, 2004). In this study, this issue was investigated as well in fertile normozoospermic males with and without varicocele. Estimated seminal ROS parameters were significantly higher and seminal antioxidants were significantly lower in fertile normozoospermic men with varicocele compared with those without varicocele.

Several studies suggested that an individual with varicocele even with a normal semen analysis or documentation of previous fertility is at risk of subsequent loss of testicular function and fertility status (Cozzolino & Lipshultz, 2001; Marmar, 2001). Pasqualotto *et al.* (2001) showed increased ROS-total antioxidant capacity (TAC) scores in normozoospermic infertile patients compared with their levels in the controls concluding that presence of OS in infertile normozoospermia may explain the previously unexplained cases of infertility attributed to female factors. Nakamura *et al.* (2002) added that elevated OS in subfertile groups was shown even in the sperm fraction considered being mature and normal. In normal rats, Cam *et al.* (2004) found that increased rate of apoptosis with experimental varicocele suggested a molecular alteration that involved ROS overproduction as a triggering mechanism. Smith *et al.* (2006) showed that varicocele is associated with high levels of oxidative damage and DNA-damaged spermatozoa even in the presence of normal semen profile. Compared with healthy subjects, Pasqualotto *et al.* (2008) observed that infertile men with varicocele had significantly lower ROS-TAC scores, but these scores were nonsignificantly different from those in fertile men with varicocele. They warned that the fertility potential in fertile varicocele patients can decline due to OS.

There is consensus that there are two main sources of increased ROS in semen; seminal leucocytes and spermatozoa with abnormal morphology, particularly mid-piece defects. As these two factors were excluded in our fertile men with varicocele cases, the origin of excess ROS in this group is suggested to be due to qualitative defect in the voided spermatozoa. Another explanation could be related to the association between the disturbances in the concentration of metabolites by metabolomic profiling in semen reported in varicocele associated conditions (Saleh *et al.*, 2003; Deepinder *et al.*, 2007).

With the background of evidence-based medicine, several controlled studies were performed on the effectiveness of varicocele repair with controversial results (Madgar *et al.*, 1995; Hargreave, 1997; Nieschlag *et al.*, 1998; Heaton, 2006). Proving that OS in varicocele-associated cases is present even in normozoospermic men may shed a light on the impact of such a condition. Marmar (2001)

supported a 'co-factor' hypothesis that for varicocele to be associated with infertility, they exist as 'co-factors' along with other molecular/genetic problems. Gallardo (2007) showed that the measurement of the antioxidative and oxidative agents could serve to evaluate human infertility in those men in whom the result of the spematobioscopy appears normal.

It is concluded that varicocele has an OS impact even in fertile normozoospermic bearing conditions. It could be understood that within men with varicocele, there is a threshold value of OS over which male fertility may be influenced.

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