

Polymorphisms of glutathione S-transferase M1 and male infertility in Taiwanese patients with varicocele

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BACKGROUND: To examine glutathione S-transferase M1 (*GST M1*) gene polymorphism and male infertility in Taiwanese patients with varicocele, 80 young male patients with varicocele (group 1), 62 young male patients with subclinical varicocele (group 2) and 60 normal young males (group 3) were recruited in this study. **METHODS:** *GST M1* null homozygous genotype [*GST M1* (–)] and the occurrence of a 4977 bp deletion of sperm mitochondrial DNA (mtDNA) were determined by polymerase chain reaction. The 8-hydroxy-2'-deoxyguanosine (8-OHdG) content of sperm DNA was measured by high-performance liquid chromatography. **RESULTS:** The frequencies of *GST M1* (–) genotype were 43.8, 41.9 and 45% for patients in groups 1, 2 and 3 respectively. In group 1 patients with *GST M1* (–) genotype, the frequency of the presence of the 4977 bp deletion in sperm mtDNA (54.3%) was significantly higher than that of the patients without the 4977 bp deletion in sperm mtDNA (45.7%, OR: 2.63, *P* = 0.04). Patients of groups 1 and 2 with *GST M1* (–) genotype had significantly higher 8-OHdG content in sperm DNA and lower protein thiols and ascorbic acid in seminal plasma than those with *GST M1* (+) genotype. **CONCLUSION:** *GST M1* (–) genotype predisposes to increased oxidative damage to sperm of patients with varicocele.

Key words: 8-hydroxy-2'-deoxyguanosine/*GST M1*/polymorphism/sperm/varicocele

Introduction

Varicocele is the abnormal tortuosity and dilatation of the veins of the pampiniform plexus within the spermatic cord and is one of the causes of male infertility. The aetiology of varicocele is as yet unknown. Previous studies suggest that varicocele is associated with elevated levels of reactive oxygen species (ROS) in sperm and reduced seminal plasma antioxidant capacity. Moreover, these studies revealed that oxidative stress plays an important role in sperm dysfunction among varicocele-afflicted patients (Weese *et al.*, 1993; Lewis *et al.*, 1995; Smith *et al.*, 1996; Alkan *et al.*, 1997; Sanocka *et al.*, 1997; Barbieri *et al.*, 1999; Hedin *et al.*, 1999; Chen *et al.*, 2001).

It has been postulated that human semen contains a significant amount of glutathione S-transferase (GST) and that the enzyme could attenuate the toxicity of ROS to sperm (Mukhtar *et al.*, 1978). The *GST* gene family produces isoenzyme that is important in protection against oxidative stress (Mann *et al.*, 2000), and an increase of ROS associated with reduced activity of GST may lead to sperm membrane damage (Gopalakrishnan and Shana, 1998). *GST M1* is a member of the *GST* family, which are proteins involved in the detoxification of several chemical carcinogens by conjugating glutathione or binding

them directly. About half of all people from different racial groups lack the enzyme activity of GST, which has a polymorphic expression, and its deficiency is due to homozygous deletion of the gene (Board *et al.*, 1990). *GST M1* (–) genotype has been reported to be associated with an increased risk of cancer of the lung, colorectum, urinary bladder and stomach (Harada *et al.*, 1987; Zhong *et al.*, 1991, 1993; Bell *et al.*, 1993; Hirvonen *et al.*, 1993; Gao and Zhang, 1999; Hou *et al.*, 2000). However, its role in varicocele is still unknown.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) is one of the most abundant oxidative products of DNA induced by ROS (Ames, 1998), which can reflect extremely low levels of oxidative DNA damage because it can be detected by high-performance liquid chromatography and an electrochemical detector (HPLC-ECD) system. It has been suggested that sperm DNA damage is closely related to male infertility and that 8-OHdG is a sensitive marker of oxidative DNA damage caused by ROS in human sperm (Shen *et al.*, 1999, 2000).

Mitochondrial DNA (mtDNA) is a naked and compact DNA molecule, which is rapidly replicated without proofreading. Moreover, mitochondria do not have efficient DNA repair systems. Thus, the mutation rate of mtDNA is 10–20-fold higher than that of nuclear DNA (Clayton *et al.*, 1974; Wallace

Table I. Incidence of *GST M1* genotypes for patients of groups 1 and 2 with respect to infertility and 4977 bp deletion of sperm mtDNA

Patient group	Infertile	Fertile	<i>P</i>	OR	95% CI
Group 1 (80)					
<i>GST M1</i> (-) (35)	80% (28)	20% (7)	0.19 ^a	2.00	0.71–5.63
<i>GST M1</i> (+) (45)	66.7% (30)	33.3% (15)			
Group 2 (62)					
<i>GST M1</i> (-) (26)	69.2% (18)	30.8% (8)	0.28 ^a	1.80	0.62–5.20
<i>GST M1</i> (+) (36)	55.6% (20)	44.4% (16)			
	mtDNA (+)	mtDNA (-)			
Group 1 (80)					
<i>GST M1</i> (-) (35)	54.3% (19)	45.7% (16)	0.04 ^b	2.63	1.05–6.58
<i>GST M1</i> (+) (45)	31.1% (14)	68.9% (31)			
Group 2 (62)					
<i>GST M1</i> (-) (26)	26.9% (7)	73.1% (19)	0.12 ^b	2.95	0.76–11.40
<i>GST M1</i> (+) (36)	11.1% (4)	88.9% (32)			

The number of patients in each group is indicated in parentheses.

^aInfertile patients versus fertile ones.

^bPatients with 4977 bp deletion of sperm mtDNA versus those without; *GST M1* (-) = *GST M1* null homozygous genotype; *GST M1* (+) = without *GST M1* null homozygous genotype; mtDNA (+) = sperm mtDNA 4977 bp deletion; mtDNA (-) = without sperm mtDNA 4977 bp deletion; OR = odds ratio; CI = confidence interval.

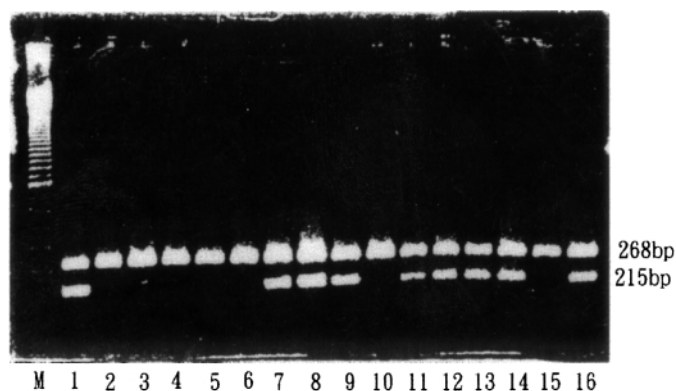


Figure 1. Gel electrophoretogram of PCR products of *GST M1* gene in human sperm. The sizes of the DNA fragments of *GST M1* gene amplified by PCR were 268 and 215 bp respectively, but only 268 bp was amplified from total DNA of the patients with *GST M1* (-) genotype (lanes 2, 3, 4, 5, 6, 10 and 15). M = DNA size marker.

et al., 1987). We have demonstrated in a previous study that human sperm with lower motility have a higher frequency of occurrence of the 4977 bp deletion of mtDNA (Kao *et al.*, 1995).

Smoking increases oxidative damage to sperm DNA (Fraga *et al.*, 1996). It has been suggested that varicocele and smoking might affect sperm function (El Mulla *et al.*, 1995). Our previous studies showed no difference in antioxidant capacity between patients with unilateral and those with bilateral varicocele (Chen *et al.*, 2001).

To evaluate the role of *GST M1* (-) genotype in patients with varicocele, we conducted this study to evaluate the genetic polymorphism of *GST M1* and its association with male infertility and 4977 bp deletion of sperm mtDNA. In addition, sperm function [by computer-assisted semen analysis (CASA)], the levels of 8-OHdG in sperm, and seminal plasma antioxidant

capacity were also analysed with respect to *GST M1* (-) genotype, infertility, 4977 bp deletion of sperm mtDNA, smoking, and unilateral or bilateral varicocele.

Materials and methods

Patients

From January 1998 to February 2001, 80 young male patients with varicocele (group 1), 62 young men with subclinical varicocele (group 2) and 60 normal young men without palpable and subclinical varicocele (group 3) were recruited for this study. Patients were examined in a warm room while standing up and their scrotums were inspected and palpated. All received Doppler ultrasonography of the scrotum. Diagnosis of the subclinical varicocele was made when one or more veins had a maximum diameter >3 mm and a retrograde flow either at rest or under the Valsalva manoeuvre (McClure and Hricak, 1986). Male infertility was diagnosed on the basis of the results from at least two successive semen analyses according to published criteria (World Health Organization, 1992). Infertile male patients in this study had a history of infertility for at least 1 year with their spouses having a confirmed normal gynaecological evaluation. The cause of infertility of males examined in this study was varicocele or subclinical varicocele. All fertile patients in groups 1 and 2 and normal controls had normal semen characteristics. Smokers were defined as patients who smoked more than one pack of cigarettes per day for more than 1 year. All of the patients signed informed consent and agreements before the collection of semen.

GST M1 gene polymorphism

Five millilitres of blood was harvested from each patient. DNA was isolated from peripheral leukocytes by standard procedures using proteinase K digestion and phenol/chloroform extraction. *GST M1* gene deletion was detected by published methods (Bell *et al.*, 1993) using a pair of primers for *GST M1* gene (G5: 5'-GAACTCCCTGAAAGCTAAAGC-3'; G6: 5'-GTTGGGCTCAAATATACGGTGG-3') and two primers for β -globin gene (PC04: 5'-CAACTTCATCCACGTTACACC-3'; GH20: 5'-GAAGAGCCAAGGACAGGTAC-3').

Table II. Motility characteristics of sperm in patients with varicocele in terms of *GST M1* genotype

Motility parameters	<i>GST M1</i> genotype		<i>P</i>
	<i>GST M1</i> (-)	<i>GST M1</i> (+)	
Motility	50.0 (40.0–65.0)	55.0 (50.0–73.0)	0.005
Morphology	50.0 (41.0–60.0)	56.0 (50.0–72.0)	0.008
Concentration	20.0 (14.0–39.0)	20.0 (12.5–70)	0.573
PMOT	38.0 (27.0–51.0)	41.0 (39.0–54.0)	0.007
LIN	61.2 (60.4–63.1)	63.2 (61.4–64.9)	0.002
STR	83.7 (82.1–85.4)	85.7 (84.3–87.9)	<0.001
ALH	3.6 (3.4–3.7)	3.8 (3.6–4.0)	<0.001
BCF	12.0 (12.0–14.0)	13.0 (13.0–15.0)	0.003
VCL	56.0 (53.0–66.0)	60.0 (57.5–69.0)	0.008
VSL	36.0 (34.0–41.0)	38.0 (36.0–43.0)	0.019
VAP	42.0 (39.0–48.0)	45.0 (42.0–49.0)	0.005

Data are expressed as median with interquartile range; Mann–Whitney test was used for analysis.

GST M1 (-) = *GST M1* null homozygous genotype; *GST M1* (+) = without *GST M1* null homozygous genotype; motility (%) = percentage of motile sperm; morphology (%) = percentage of sperm with normal morphology; concentration = $\times 10^6$ /ml; PMOT (%) = progressive sperm motility with a linearity >75%; LIN (%) = VSL/VCL; STR (%) = VSL/VAP; ALH (μ m) = amplitude of lateral head displacement; BCF (Hz) = beat cross frequency (number of times the sperm head goes across the average path); VCL (μ m/s) = curvilinear velocity (total distance travelled by the sperm head in 1 s); VSL (μ m/s) = straight line velocity (straight line distance travelled by sperm head in 1 s); VAP (μ m/s) = average path velocity (calculated using a 5-point smoothing algorithm).

PCR was carried out for 35 cycles in a DNA thermal cycler (Perkin–Elmer/Cetus) using a thermal profile of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and primer extension at 72°C for 40 s. The PCR products were then separated on a 3% agarose/synergel gel (5:1, w/w) (Diversified Biotech, Newton Center, MA, USA) at 150 V for 1.5 h and stained with 1 μ g/ml ethidium bromide at 25°C for 10 min. DNA fragments of the *GST M1* gene amplified by PCR were 268 and 215 bp in size, but only the 268 bp band was observed in patients with *GST M1* (-) genotype (Figure 1).

Preparation of sperm

Semen was collected from the patients and controls after sexual abstinence for 3–4 days. Leukospermia and viscous semen were excluded. After the semen sample had been liquefied at room temperature, sperm were separated from the seminal plasma by centrifugation at 300 g for 10 min at 25°C, and then fractionated by self-migration into a discontinuous Percoll gradient. Semen analysis was done for all the patients by a CASA system (HTM-2000) (Kao *et al.*, 1995).

Sperm DNA extraction

Total DNA of human sperm was extracted according to the method developed in our laboratory (Kao *et al.*, 1995).

Measurement of 8-OHdG content in sperm DNA

8-OHdG level in sperm DNA was measured by HPLC–ECD (Bio-analytical Systems, West Lafayette, IN, USA) as previously described by (Shigenaga *et al.*, 1994). Deoxyguanosine (dG) and 8-OHdG were used as standards. 8-OHdG levels in sperm DNA are expressed as the number of 8-OHdG molecules per 10^5 dG.

Detection of mtDNA deletion in sperm

Sperm mtDNA deletions were detected by the method developed in this laboratory (Kao *et al.*, 1998). PCR was carried out for 35 cycles

Table III. Motility characteristics of sperm in patients with subclinical varicocele in terms of *GST M1* genotype

Motility parameters	<i>GST M1</i> genotype		<i>P</i>
	<i>GST M1</i> (-)	<i>GST M1</i> (+)	
Motility	55.0 (48.0–70.3)	56.5 (42.0–75.0)	0.667
Morphology	54.5 (49.5–68.8)	60.0 (48.0–65.0)	0.616
Concentration	20.0 (12.8–66.3)	25.0 (11.0–70.0)	0.943
PMOT	41.0 (37.0–53.3)	41.0 (31.0–54.0)	0.731
LIN	62.4 (61.1–64.1)	61.8 (60.9–64.7)	0.764
STR	84.4 (83.5–86.2)	84.7 (83.3–87.1)	0.721
ALH	3.7 (3.7–4.0)	3.7 (3.6–4.0)	0.462
BCF	14.0 (13.0–15.0)	13.0 (12.0–15.0)	0.122
VCL	58.0 (57.5–67.3)	61.5 (53.0–70.0)	0.886
VSL	38.0 (36.0–42.3)	39.0 (35.0–42.0)	0.595
VAP	44.5 (42.0–49.0)	44.5 (41.0–48.0)	0.567

Data are expressed as median with interquartile range; Mann–Whitney test was used for analysis.

For definitions, see Table II.

Table IV. Motility characteristics of sperm in normal control in terms of *GST M1* genotype

Motility parameters	<i>GST M1</i> genotype		<i>P</i>
	<i>GST M1</i> (-)	<i>GST M1</i> (+)	
Motility	70.0 (65.0–75.0)	72.0 (65.0–85.0)	0.138
Morphology	67.0 (61.0–75.0)	72.0 (62.0–84.0)	0.090
Concentration	71.0 (63.0–85.0)	71.0 (63.0–98.0)	0.348
PMOT	55.0 (52.0–55.0)	55.0 (52.0–72.0)	0.024
LIN	63.6 (61.2–64.7)	63.2 (63.1–68.4)	0.459
STR	86.9 (85.9–87.8)	87.8 (85.4–89.7)	0.636
ALH	4.0 (3.7–4.0)	4.0 (3.9–4.1)	0.175
BCF	15.0 (14.0–15.0)	15.0 (15.0–17.0)	0.011
VCL	68.0 (67.0–70.0)	68.0 (65.0–76.0)	0.310
VSL	42.0 (41.0–44.0)	44.0 (41.0–52.0)	0.272
VAP	49.0 (48.0–49.0)	49.0 (48.0–58.0)	0.112

Data are expressed as median with interquartile range; Mann–Whitney test was used for analysis.

For definitions, see Table II.

in a DNA thermal cycler (Perkin–Elmer/Cetus) using a thermal profile of denaturation at 94°C for 40 s, annealing at 55°C for 40 s and primer extension at 72°C for 40 s. PCR products were then separated on a 1.5% agarose/synergel gel (5:1, w/w) (Diversified Biotech) at 150 V for 1.5 h and visualized by staining with 1 μ g/ml ethidium bromide at 25°C for 20 min (usually >20 min).

Primer-shift PCR

In order to avoid artefacts in the detection of mtDNA deletions, primer-shift PCR was employed to ascertain that the amplified DNA fragment was not a result of misannealing of primers to the DNA template (Lee *et al.*, 1994). We consistently obtained PCR products of 719, 587, 524 and 392 bp from the 4977 bp-deleted mtDNA by using primer pairs L8150–H13845, L8282–H13845, L8150–H13650 and L8282–H13650 respectively.

Measurement of protein thiols of the seminal plasma

Protein thiols of the seminal plasma were measured by a previously described spectrophotometric method by (Boyne and Ellman, 1972). An equal volume of 10% perchloric acid was added to 50 μ l of seminal plasma. The pellet was dissolved in 1.4 ml of 50 mmol/l

Table V. Protein thiols of seminal plasma in patients of three different groups with respect to *GST M1* genotype, sperm mtDNA 4977 bp deletion, smoking, fertility and unilateral or bilateral varicocele

	Protein thiols of seminal plasma (nmol/ml)		
	Group 1	Group 2	Group 3
<i>GST M1</i> (-)	0.06 (0.02–1.37)	1.10 (0.64–2.29)	3.37 (2.97–3.91)
<i>GST M1</i> (+)	1.80 (0.64–2.77)	2.43 (1.18–2.94)	5.60 (3.34–8.57)
<i>P</i>	< 0.001	0.001	0.002
mtDNA 4977 bp deletion (+)	0.05 (0.02–0.67)	0.64 (0.23–0.65)	
mtDNA 4977 bp deletion (-)	1.37 (0.64–2.75)	2.28 (1.12–2.63)	
<i>P</i>	< 0.001	< 0.001	
Smokers	0.64 (0.02–1.37)	1.00 (0.64–1.89)	3.34 (2.90–5.60)
Non-smokers	1.30 (0.17–2.75)	2.43 (1.12–2.59)	4.66 (3.37–7.42)
<i>P</i>	0.004	0.014	0.008
Infertile	0.34 (0.03–1.02)	1.10 (0.64–1.67)	
Fertile	2.77 (1.95–4.41)	2.46 (2.28–2.59)	
<i>P</i>	< 0.001	0.001	
Unilateral	1.02 (0.02–2.09)	2.21 (0.72–2.63)	
Bilateral	1.00 (0.02–2.06)	1.67 (0.84–2.52)	
<i>P</i>	0.153	0.580	

Data are expressed as median with interquartile range; Mann–Whitney test.
 Group 1 = varicocele; group 2 = subclinical varicocele; group 3 = normal control; *GST M1* (-) = *GST M1* null homozygous genotype; *GST M1* (+) = without *GST M1* null homozygous genotype.

Table VI. Ascorbic acid of seminal plasma in patients of three different groups with respect to *GST M1* genotype, sperm mtDNA 4977 bp deletion, smoking, fertility, and unilateral or bilateral varicocele

	Ascorbic acid of seminal plasma (mg/dl)		
	Group 1	Group 2	Group 3
<i>GST M1</i> (-)	1.50 (1.01–1.97)	2.16 (2.06–2.26)	2.38 (2.36–2.45)
<i>GST M1</i> (+)	2.13 (2.01–2.90)	2.25 (2.14–2.26)	3.90 (2.41–5.80)
<i>P</i>	< 0.001	0.027	0.002
mtDNA 4977 bp deletion (+)	1.87 (1.38–2.03)	1.99 (1.92–2.10)	
mtDNA 4977 bp deletion (-)	2.12 (1.92–2.90)	2.25 (2.16–2.26)	
<i>P</i>	0.001	< 0.001	
Smokers	1.93 (1.49–2.12)	2.14 (2.10–2.24)	2.38 (2.31–4.00)
Non-smokers	2.05 (1.87–2.90)	2.26 (2.16–2.27)	2.45 (2.38–5.50)
<i>P</i>	0.037	0.001	0.023
Infertile	1.94 (1.49–2.10)	2.15 (2.10–2.21)	
Fertile	2.90 (2.16–3.50)	2.26 (2.25–2.27)	
<i>P</i>	< 0.001	0.001	
Unilateral	2.05 (1.76–2.22)	2.20 (2.11–2.27)	
Bilateral	1.75 (1.01–2.13)	2.25 (2.14–2.26)	
<i>P</i>	0.292	0.579	

Data are expressed as median with interquartile range; Mann–Whitney test.
 For definitions, see Table V.

phosphate buffer containing 1% sodium dodecyl sulphate and 50 mmol/l EDTA (pH 7.4). An aliquot of 23 µl of 100 mmol/l 5,5'-dithiolbis-2-nitrobenzoic acid was added and thoroughly mixed. The absorbance of the reaction product at 412 nm was recorded and the protein thiol content was calculated from a standard curve. The value of protein thiol is expressed as nmol/ml seminal plasma.

Measurement of ascorbic acid in the seminal plasma

Ascorbic acid level of the seminal plasma was measured by a spectrophotometric method (Kyaw, 1978). The colour reagent was prepared by mixing 0.3 mmol/l sodium tungstate, 1.2 mmol/l sodium hydrogen phosphate and 18 mol/l sulphuric acid. Absorbance at 700 nm was read against a reagent blank and plasma ascorbic acid was calculated from a standard curve, and this value is expressed as mg/dl seminal plasma.

Statistical analysis

Data are reported as median with interquartile range because they are not normally distributed. We used the Mann–Whitney test, Kruskal–Wallis test, Pearson correlation and odds ratio (OR) with a 95% confidence interval for statistical analysis. The software used was SPSS 8.0 for Windows. *P* < 0.05 was considered to be statistically significant.

Results

Mean ages were 29.7 ± 4.3 years for patients with varicocele, 30.6 ± 5.4 years for patients with subclinical varicocele, and 30.2 ± 4.9 years for the healthy controls (range: 25–35). Of the 80 patients with varicocele, 58 were infertile and 33 had

Table VII. The levels of sperm 8-hydroxy-2'-deoxyguanosine (8-OHdG) in patients of three different groups with respect to *GST M1* genotype, sperm mtDNA 4977 bp deletion, smoking, fertility, and unilateral or bilateral varicocele

	8-OHdG of sperm (10^{-5} dG)		
	Group 1	Group 2	Group 3
<i>GST M1</i> (-)	8.30 (6.40–10.50)	5.70 (3.98–7.55)	1.90 (1.80–3.10)
<i>GST M1</i> (+)	5.20 (3.80–6.20)	4.20 (3.00–4.90)	1.40 (1.10–1.95)
<i>P</i>	< 0.001	0.003	0.075
mtDNA 4977 bp deletion (+)	9.20 (6.00–10.95)	7.70 (7.30–7.90)	
mtDNA 4977 bp deletion (-)	5.20 (3.90–6.40)	4.20 (3.20–5.10)	
<i>P</i>	< 0.001	< 0.001	
Smokers	6.90 (5.40–10.15)	5.10 (4.40–7.00)	2.10 (1.90–3.10)
Non-smokers	5.50 (3.90–7.80)	3.90 (3.00–4.70)	1.20 (1.10–1.80)
<i>P</i>	0.006	< 0.001	< 0.001
Infertile	7.50 (5.68–9.83)	5.40 (4.50–7.15)	
Fertile	3.75 (3.15–4.93)	3.10 (2.73–3.90)	
<i>P</i>	< 0.001	< 0.001	
Unilateral	5.90 (4.90–8.55)	4.50 (3.30–6.75)	
Bilateral	7.30 (4.80–11.40)	4.50 (3.95–5.35)	
<i>P</i>	0.434	0.835	

Data are expressed as median with interquartile range; Mann–Whitney test. For definitions, see Table V.

a history of smoking. Sixty-five patients with varicocele had left varicocele, and the rest (15) had bilateral varicocele. Among the 62 subjects with subclinical varicocele; 38 were infertile and 27 were smokers; 37 had left subclinical varicocele, while 25 had bilateral subclinical varicocele. Among 96 infertile patients in groups 1 and 2, 83 were diagnosed as oligozoospermia, 87 exhibited asthenozoospermia, and 30 were diagnosed as teratozoospermia.

The frequencies of *GST M1* (-) genotype were 43.8, 41.9 and 45% for subjects in groups 1, 2 and 3 respectively, and there are no differences between the three groups. However, in group 1 patients with *GST M1* (-) genotype, the frequency of the presence of 4977 bp deletion in sperm mtDNA (+) (54.3%) was significantly higher than that of the absence of 4977 bp deletion in sperm mtDNA (-) (45.7%, OR: 2.63, $P = 0.04$), but no significant difference was found for patients in group 2 (OR: 2.95, $P = 0.12$, Table I). In groups 1 and 2 patients with *GST M1* (-) genotype, the incidence of infertility was higher than that of fertility, but no significant difference was noticed (OR: 2.0, $P = 0.19$ for group 1 patients and OR: 1.8, $P = 0.28$ for group 2 patients). The incidence of 4977 bp deletion in sperm mtDNA were 41.3, 17.7 and 1.67% for subjects in groups 1, 2 and 3 respectively. Significantly higher incidence was found in patients of group 1 as compared with those of groups 2 and 3.

The CASA parameters for semen quality in 80 patients with varicocele, 62 patients with subclinical varicocele, and the 60 controls in terms of *GST M1* (-) are summarized in Tables II–IV. CASA parameters of the sperm, except sperm concentration, decreased significantly for the patients of group 1 associated with *GST M1* (-), but no such differences were noticed in subjects of groups 2 and 3. For all patients with *GST M1* (-) genotype, group 3 patients had better sperm function than did those in groups 1 and 2.

Protein thiols and ascorbic acid levels of the seminal plasma

as well as the levels of sperm 8-OHdG in patients of three different groups with respect to *GST M1* genotype, 4977 bp deletion of mtDNA in sperm, smoking, fertility and unilateral or bilateral varicocele are summarized in Tables V–VII. Patients in group 1 had significantly lower protein thiols and ascorbic acid levels in seminal plasma and higher levels of sperm 8-OHdG than those of groups 2 and 3. Patients of groups 1 and 2, who were characterized by *GST M1* (-) genotype, sperm mtDNA 4977 bp deletion, infertility and smoking, had significantly lower seminal plasma protein thiols and ascorbic acids levels and higher levels of sperm 8-OHdG compared with those without *GST M1* (-) genotype, sperm mtDNA 4977 bp deletion, infertility and smoking. No such difference was noticed between patients with unilateral and those with bilateral varicocele. For the normal controls, significantly higher levels of sperm 8-OHdG and lower antioxidant capacity of the seminal plasma were found in patients with a history of smoking as compared to those without (Tables V–VII). Besides, the control patients with *GST M1* (-) genotype had significantly lower antioxidant capacity of the seminal plasma than those without, but no significant difference was noticed in the levels of 8-OHdG in sperm DNA (Tables V–VII). Furthermore, a negative correlation was found between the content of 8-OHdG in spermatozoa and sperm function including motility, morphology and sperm concentration (Figures 2–4).

Discussion

Increasing evidence suggests that varicocele has a detrimental effect on spermatogenesis. However, many unsolved clinical problems associated with varicocele still exist. One of these is subclinical varicocele, which is not palpable clinically but detectable by Doppler ultrasonography. To date, there has been no gold standard for diagnosis of subclinical varicocele. It has been suggested that to make the final diagnosis of subclinical

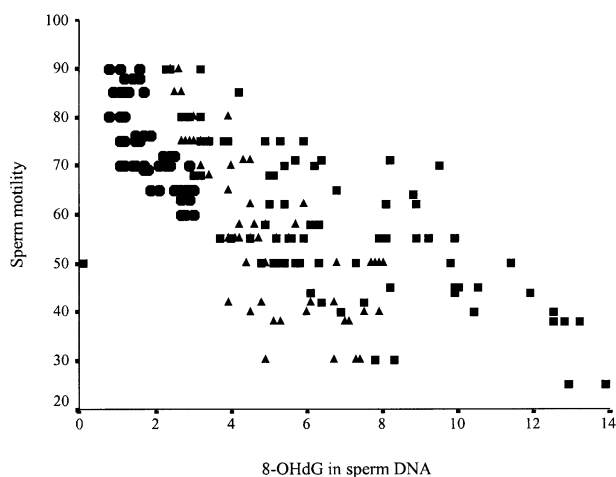


Figure 2. The correlation between 8-hydroxy-2'-deoxyguanosine (8-OHdG) content in sperm DNA and sperm motility for patients in different groups. The symbols ■, ▲ and ● represent the data points for patients in groups 1, 2 and 3 respectively ($\gamma = -0.67$, $P < 0.001$ for group 1; $\gamma = -0.77$, $P < 0.001$ for group 2; $\gamma = -0.78$, $P < 0.001$ for group 3).

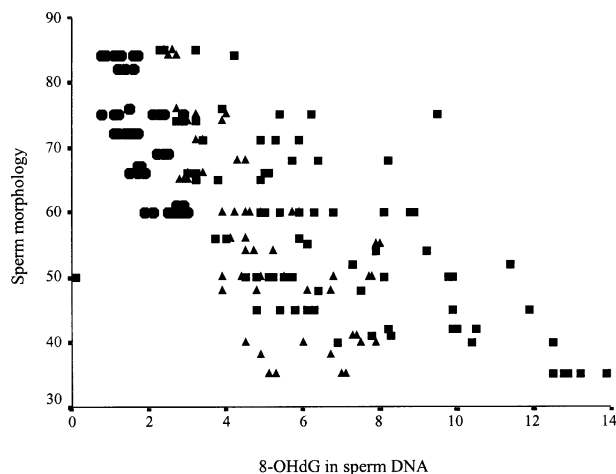


Figure 3. The correlation between 8-hydroxy-2'-deoxyguanosine (8-OHdG) content in sperm DNA and sperm morphology for patients in different groups. The symbols ■, ▲ and ● represent the data points for patients in groups 1, 2 and 3 respectively ($\gamma = -0.64$, $P < 0.001$ for group 1; $\gamma = -0.73$, $P < 0.001$ for group 2; $\gamma = -0.75$, $P < 0.001$ for group 3).

varicocele, the internal spermatic vein diameter must be >3.0 mm with clinically palpable varicocele (McClure and Hriack, 1986). In this study, internal spermatic vein diameter >3 mm plus an associated reversal flow detectable via Doppler ultrasonography were used as diagnostic criteria for diagnosis of subclinical varicocele.

Ascorbate, urates and thiols in the seminal plasma are major antioxidants for which significantly lower levels have been recorded in infertile compared with fertile men (Lewis *et al.*, 1997). Therefore, we used thiols and ascorbic acid levels to evaluate antioxidant capacity of the seminal plasma. CASA provides an accurate quantitative evaluation of semen quality in patients with varicocele. Our previous study demonstrated that protein thiols and ascorbate in the seminal plasma are good markers for evaluating oxidative stress in patients with

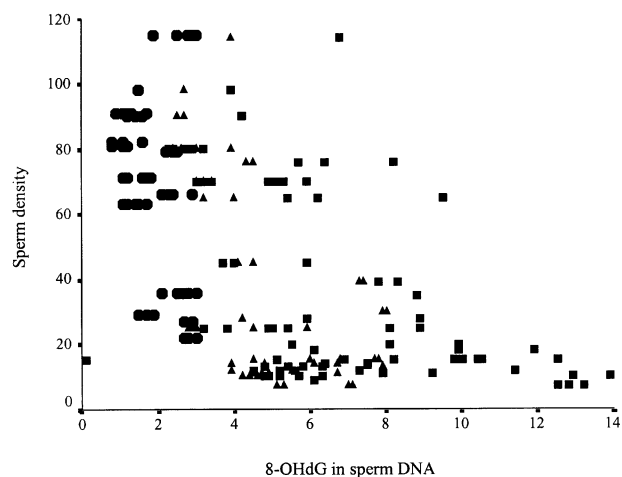


Figure 4. The correlation between 8-hydroxy-2'-deoxyguanosine (8-OHdG) content in sperm DNA and sperm concentration for patients in different groups. The symbols ■, ▲ and ● represent the data points for patients in groups 1, 2 and 3 respectively ($\gamma = -0.48$, $P < 0.001$ for group 1; $\gamma = -0.56$, $P < 0.001$ for group 2; $\gamma = -0.32$, $P = 0.013$ for group 3).

varicocele (Chen *et al.*, 2001). In this study, we found that varicocele and subclinical varicocele patients with *GST M1* (-) genotype had lower seminal plasma antioxidant capacity, but only patients with palpable varicoceles and this genotype had lower CASA parameters (except sperm concentration). However, no such difference was found for patients with subclinical varicocele. These results suggest that *GST M1* (-) genotype plays a role in lowering seminal plasma antioxidant capacity, which may result in sperm dysfunction for patients with varicocele.

Deoxyribonucleic bases are susceptible to oxidative stress and a high level of oxidative DNA damage of the sperm may be involved in male infertility (Hideya *et al.*, 1997). It has been reported that the 8-OHdG contents of sperm DNA in smokers were higher than those in non-smokers (Shen *et al.*, 1998). In this study, we found that the level of sperm 8-OHdG was significantly higher in patients with varicocele and subclinical varicocele, and *GST M1* (-) genotype, 4977 bp deletion of mtDNA, smoking and infertility. However, no difference was found between control patients with *GST M1* (-) genotype and those without. Besides, there is a negative correlation between sperm function and the level of 8-OHdG of sperm DNA for all the patients. Moreover, the level of sperm 8-OHdG was the highest in patients with varicocele followed by those with subclinical varicocele and normal controls. This confirms that sperm 8-OHdG is a good marker for evaluating oxidative stress in patients with varicocele.

It has been demonstrated that mtDNA mutations play an important role in some pathophysiological conditions of human spermatozoa, and that multiple mtDNA deletions occur more frequently in the sperm with low motility and infertile patients (Kao *et al.* 1995, 1998). In this study, patients with varicocele had a higher frequency of 4977 bp deletion of sperm mtDNA and infertility than patients with subclinical varicocele and normal controls. Also, patients with varicocele and subclinical varicocele associated with 4977 bp deletion in sperm mtDNA

had higher levels of sperm 8-OHdG and lower antioxidant capacity of the seminal plasma than those without the 4977 bp deletion of mtDNA.

Genetic factors that could mediate the pathogenesis of male infertility are mostly unclear and increased frequencies of cytochrome *P4501A1* gene polymorphisms have been found in infertile men (Fritsche *et al.*, 1998; Schuppe *et al.*, 2000). Asthenozoospermia is associated with mtDNA halogroups in Caucasians and it was suggested that genetic alterations of mtDNA can induce sperm dysfunction (Ruiz-Pesini *et al.*, 2000). One study found that long androgen receptor CAG alleles are associated with male infertility and defective spermatogenesis (Mifsud *et al.*, 2001). *GST M1* is located at human chromosome 1p13, which displays three alleles: *GST M1a*, *GST M1b* and *GST M1 null* (–) (Board, 1981a,b; Suzuki *et al.*, 1987). GST plays an important role in biotransformation and detoxification of many xenobiotics. GST activity is widely distributed in hepatic and extrahepatic tissues including ovary, testes, and serum. It has been demonstrated that GST might have a relevant protective role during spermatogenesis (Castellon, 1999). It has been suggested that polymorphism of the *GST M1* gene might be an important factor in determining the susceptibility of a patient to develop alcohol-induced disorders of human spermatogenesis (Pajarinen *et al.*, 1996). Mu and theta class GST play an important role in the detoxification of products of oxidative damage, such as lipid hydroperoxides, alkenals and DNA hydroperoxides, as well as some carcinogens including methyl halides and benzo(a)pyrene epoxides (Smith *et al.*, 1995). However, Gopalakrishnan and Shaha (1998) argued that the role of GST in the antioxidant defence mechanism of sperm is still not clear although increased ROS production by the cell was noticed when GST activity was suppressed (Gopalakrishnan and Shaha, 1998). Therefore, in this study we used antioxidant capacity of seminal plasma and the level of sperm 8-OHdG to evaluate the effect of *GST M1* (–) genotype instead of GST enzyme because patients with *GST M1* (–) genotype had deficiency of this enzyme.

We demonstrated for the first time that there is an association between *GST M1* gene polymorphism and 4977 bp deletion of sperm mtDNA for patients with varicocele. In group 1 patients with *GST M1* (–) genotype, the frequency of the presence of the 4977 bp deletion in sperm mtDNA (54.3%) was significantly higher than that of the absence of the 4977 bp deletion in sperm mtDNA (45.7%, OR: 2.63, *P* = 0.04). Besides, significantly higher 8-OHdG content in sperm DNA and lower protein thiols and ascorbic acid in seminal plasma were found in patients of groups 1 and 2 with *GST M1* (–) genotype than those with *GST M1* (+) genotype. These results suggest that the sperm of varicocele patients with *GST M1* (–) genotype are more vulnerable to oxidative damage. Therefore, we should pay more attention to oxidative stress-related pathological manifestations in varicocele-bearing patients with *GST M1* (–) genotype.

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