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Original Article

Genetic polymorphisms in glutathione *S*-transferase *T1* affect the surgical outcome of varicocelectomies in infertile patients

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Abstract

Glutathione *S*-transferases (*GSTs*), superoxide dismutase 2 (*SOD2*) and NAD(P)H:quinone oxidoreductase 1 (*NQO1*) are anti-oxidant enzyme genes. Polymorphisms of *GSTs*, *SOD2* and *NQO1* have been reported to influence individual susceptibility to various diseases. In an earlier study, we obtained preliminary findings that a subset of glutathione *S*-transferase *T1 (GSTT1)*-wt patients with varicocele may exhibit good response to varicocelectomy. In this study, we extended the earlier study to determine the distribution of genotype of each gene in the infertile population and to evaluate whether polymorphism of these genes affects the results of surgical treatment of varicocele. We analyzed 72 infertile varicocele patients, 202 infertile patients without varicocele and 101 male controls. Genotypes of *GSTs* were determined by polymerase chain reaction (PCR). Genotyping of *SOD2* and *NQO1* was performed using the PCR-restriction fragment length polymorphism (PCR-RFLP) method. A significantly better response to varicocelectomy was found in patients with the *GSTT1*-wt genotype (63.2%) and *NQO1*-Ser/Ser genotype (80.0%) than in those with *GSTT1*-null genotype (35.3%) and *NQO1*-Pro/Pro or *NQO1*-Pro/Ser genotype (45.2%), respectively. The frequencies of glutathione *S*-transferase *M1/T1*, *SOD2* and *NQO1* genotypes did not differ significantly among the varicocele patients, idiopathic infertile patients and male controls. *GSTT1* genotype is associated with improvement of semen parameters after varicocelectomy. As the number of patients with *NQO1*-Ser/Ser genotype was not sufficient to reach definite conclusions, the association of *NQO1* genotype with varicocelectomy requires further investigation.

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1 Introduction

The most common and correctable known risk factor for male infertility is varicocele. Varicoceles are found in approximately 40% of infertile men, whereas the incidence in the general male population is approximately 15% [1–3]. Surgical correction of varicocele in infertile men has been shown to improve semen parameters [3–5]. However, not all men with varicocele are infertile, and not all infertile patients with varicocele show an improvement in seminal findings following surgical repair. Thus, a clinically important issue is the preoperative determination of which subset of patients, among all infertile men with varicocele, will respond to varicocelectomy.

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Reactive oxygen species (ROS) are groups of free radicals that have deleterious effects on many organs. In varicocele patients, ROS production is enhanced [6, 7], and oxidative stress is present in spermatic vein blood and seminal plasma [8–10]. Varicocele treatment can significantly improve sperm parameters, by decreasing ROS concentration and increasing antioxidant capacity, and these effects lead to improved sperm parameters finally [11]. Oxidative stress results from an imbalance between the production and reduction of ROS, and several types of anti-oxidant enzyme genes are associated with the removal of ROS.

The glutathione *S*-transferases (*GSTs*) are a family of cytosolic or microsomal enzymes [12] that play important roles in the detoxification of products resulting from oxidative damage and exposure to some carcinogens [13]. Four classes of cytosolic *GSTs* have been identified in mammals, including the alpha, pi, theta and mu classes. Two human *GST* isoenzymes—the mu class enzyme, glutathione *S*-transferase *M1* (*GSTM1*), and the theta class enzyme, glutathione *S*-transferase *T1* (*GSTT1*)—have been shown to be polymorphic. In both cases, a gene deletion is responsible for the existence of a null allele. Individuals who are homozygous with respect to a given null allele lack that specific enzyme function. About half of all the individuals from various racial groups lack *GST* activity [14].

Superoxide dismutase 2 (*SOD2*) is the only known superoxide scavenger in the mitochondria. Several nucleotide polymorphisms in the *SOD2* gene have been reported, one of which is the alanine (Ala)-to-valine (Val) polymorphism at codon 16 in exon 2 (rs4880 at double-strand single-nucleotide polymorphism [dbSNP]). The Ala-to-Val substitution may affect the mitochondrial targeting rate of *SOD2*, which is responsible for mitochondrial damage and a decrease in the activity of this enzyme [15].

NAD(P)H:quinone oxidoreductase 1 (*NQO1*) is a cytosolic enzyme that induces two-electron reduction of quinoid compounds to hydroquinones, a less toxic form, thus avoiding free radical formation. A sequence variant at position 609 (C-to-T, proline [Pro]-to serine [Ser], rs1800566 at dbSNP) in the *NQO1* gene encodes for an enzyme with reduced quinone reductase activity, which has been hypothesized to affect cancer susceptibility [16, 17].

These polymorphisms of *GSTs*, *SOD2* and *NQO1* have been reported to influence individual susceptibility to various diseases, including malignancy [18–25], although the relationship between polymorphisms and male infertility remains to be elucidated [26, 27]. We therefore speculated that individual susceptibility to varicocele-induced ROS may depend on one or more of these genetic polymorphisms and that the outcome of varicocelectomy may be influenced by an individual's genetic susceptibility to ROS. In our earlier study, we obtained preliminary findings that a subset of *GSTT1*-wt patients with varicocele showed good responses to varicocelectomy [28]. However, the number of patients in that study was not sufficient to yield definitive conclusions. We therefore extended the earlier study and analysed a larger number of clinical samples to determine the distribution of genotypes for the *GSTM1, GSTT1, SOD2* and *NQO1* genes in the infertile population and to evaluate whether polymorphisms of these genes affect the results of varicocele surgical treatment.

2 Materials and methods

2.1 Subjects

Seventy-two male patients with clinical varicocele, 202 infertile patients without varicocele and 101 male controls at the Kyoto University Hospital and Akita University Hospital were enrolled in this study. All patients had the same ethnic and geographical origin. Male infertility was diagnosed on the basis of the results from at least two semen analyses, according to published criteria [29]. Patients with azoospermia were excluded from this study. Chromosomal analysis of peripheral blood was performed using a G-banding method in patients with severe oligozoospermia ($\leq$ 5 million sperm per mL), and patients with a chromosomal abnormality were excluded from this study. A varicocele testis was identified on physical examination. The control group consisted of healthy volunteers and patients from the same clinics who had a benign urological disease. This study was approved by the Institutional Review Board of Kyoto University Hospital (Kyoto, Japan). Written informed consent was obtained from all patients.

2.2 Varicocelectomy and postoperative evaluation

Seventy-two patients with clinical varicocele underwent varicocelectomy. All varicocelectomies were performed using an inguinal approach under microscopic magnification, according to a method reported earlier [30]. At least two semen analyses were

performed at 3, 6, 9 or 12 months postoperatively. A positive response to varicocele repair was defined as a 100% increase in the concentration of total motile sperm (sperm concentration × motile fraction) in the postoperative study.

2.3 DNA extraction, amplification and GSTM1/GSTT1/SOD2/NQO1 polymorphism genotyping

Genomic DNA was extracted from blood samples by one of two methods: samples were processed using a QIAamp Blood Kit (QIAGEN, Hilden, Germany) or by the standard method, involving a proteinase K digestion followed by phenol-chloroform extraction.

Multiplex polymerase chain reaction (PCR) was performed to detect the presence or absence of *GSTM1* and *GSTT1* genes, as described [31]. Individuals with homozygous null alleles lack the respective enzyme function, whereas individuals with two wild-type alleles and heterozygotes with one active and one inactive allele were classified as one group having enzyme function. The primer sequences were 5'-GAACTCCCTGAAAAGCTAAAGC-3' and 5'-GTTGGGCTCAAATATACGGTGG-3' for *GSTM1*, and 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3' for *GSTT1*. As an internal positive control for successful PCR, the β-globin gene was amplified with the primers 5'-CAACTTCATCCACGTTCACC-3' and 5'-GAAGAGCCAAGGACAGTTAC-3'. The PCR conditions were 10 min at 95ºC for one cycle, and 60 s at 95ºC, 60 s at 58ºC and 90 s at 72ºC for 35 cycles. *GSTM1* and *GSTT1* wild or null genotypes were indicated by the presence or absence of a 215 bp band and a 480 bp band, respectively. The genotypes of *GSTM1* and *GSTT1* were not scored if the PCR product from the internal reference β-globin gene (268 bp) was not evident.

We also analysed single-nucleotide polymorphisms (SNPs) of *SOD2* and *NQO1*. The SNP of *SOD2* is a C-to-T conversion in exon 2 that substitutes a Val for an Ala in the signal peptide at codon 9 (*Ala16Val*). The SNP of *NQO1* is a C-to-T conversion that substitutes a Ser for a Pro at codon 187 (*Pro187Ser*). To determine *SOD2* and *NQO1* genotypes, DNA was amplified by PCR and genotyped by restriction analysis, as described [24, 32]. The PCR primers used were 5'-ACCAGCAGGCAGCTGGCGCCGG-3' and 5'-GCGTTGATGTGAGGTTCCAG-3' for the *Ala16Val SOD2* polymorphism, and 5'-TCCTCAGAGTGGCATTCTGC-3' and 5'-TCTCCTCATCCTGTACCTCT-3' for the *Pro187Ser NQO1* polymorphism. The PCR conditions for *SOD2* were 10 min at 95ºC for one cycle, 30 s at 95ºC, 30 s at 55ºC and 60 s at 72ºC for 35 cycles, and 5 min at 72ºC for one cycle. For *NQO1*, the PCR proceeded for 10 min at 95ºC for one cycle, 30 s at 95ºC, 30 s at 58ºC and 45 s at 72ºC for 35 cycles, and 5 min at 72ºC for one cycle. Each PCR product was digested with *NaeI* for the *Ala16Val SOD2* polymorphism and with HinfI for the *Pro187Ser NQO1* polymorphism. DNA samples, together with those that were examined earlier, served as quality controls and were concomitantly digested. For the *Ala16Val SOD2* polymorphism, restriction fragments were 106 and 21 bp for the Ala allele, and 127 bp for the Val allele. For the *Pro187Ser NQO1* polymorphism, restriction fragments were 195 and 35 bp for the Pro allele, and 151, 44 and 35 bp for the Ser allele.

2.4 Statistical methods

The distribution of each genotype was analysed with the χ^2 test. Differences in clinical parameters among subgroups were examined with the Mann-Whitney *U* test and the χ^2 test. Comparison of the distribution of responders and non-responders was performed with the χ^2 test. Odds ratios in the combination groups were calculated using logistic regression analysis. $P < 0.05$ was considered significant.

3 Results

3.1 Patient background and outcome of varicocelectomy

We evaluated the clinical characteristics of 72 patients who underwent a varicocelectomy. In the preoperative evaluation, 40 (55.6%) and 57 (79.2%) of the patients had oligozoospermia and asthenozoospermia, respectively. Patient background factors are shown stratified by genotype in Table 1. There were no significant differences among genotype subgroups with regard to age, preoperative or postoperative seminal parameters, or the distribution of varicocele grades. Positive responses to varicocele repair, based on motile sperm concentrations, were observed in 36 (50%) out of 72 patients. Preoperative and postoperative seminal parameters in the responder and non-responder subgroups are shown in Table 2.

The rates of response to varicocelectomy were also examined in each genotype using motile sperm concentration (Table 3). A significantly higher response

Table 1. Background characteristics of patients stratified by *GSTM1/T1*, *SOD2* and *NQO1* genotypes (mean ± SD).

	Age (years)	Grade of varicocele			Preoperative semen parameters		Postoperative semen parameters	
		G1	G2	G3	Concentration ($\times 10^6$)	Motility (%)	Concentration ($\times 10^6$)	Motility (%)
Overall (n = 72)	33.3 ± 4.7	17	32	23	22.8 ± 21.6	34.1 ± 21.6	31.7 ± 32.6	43.7 ± 21.4
GSTM1								
Null (n = 45)	33.1 ± 4.9	9	23	13	21.1 ± 20.4	35.9 ± 23.6	35.2 ± 36.5	43.6 ± 23.0
Wt (n = 27)	33.6 ± 4.4	8	9	10	25.6 ± 23.7	31.0 ± 18.0	25.9 ± 24.2	44.0 ± 19.0
GSTT1								
Null (n = 34)	33.6 ± 5.2	10	16	8	26.1 ± 26.2	31.4 ± 18.6	31.8 ± 37.7	44.1 ± 21.9
Wt (n = 38)	32.9 ± 4.2	7	16	15	19.8 ± 16.3	36.4 ± 24.0	31.6 ± 27.7	43.4 ± 21.3
MnSOD								
Ala/Ala+Ala/Val (n = 13)	33.8 ± 4.4	2	5	6	17.2 ± 16.8	39.9 ± 27.1	37.2 ± 35.5	48.5 ± 22.4
Val/Val (n = 59)	33.1 ± 4.8	15	27	17	24.0 ± 22.5	32.8 ± 20.3	30.5 ± 32.1	42.7 ± 21.3
NQO1								
Pro/Pro+Pro/Ser (n = 59)	33.5 ± 4.7	15	28	19	23.1 ± 22.0	35.5 ± 22.5	30.5 ± 33.4	44.8 ± 21.8
Ser/Ser (n = 10)	31.9 ± 4.8	2	4	4	20.4 ± 20.2	25.0 ± 12.6	39.3 ± 27.5	37.5 ± 19.0

Abbreviations: Ala, alanine; *GSTM1*, glutathione *S*-transferase *M1; GSTT1*, glutathione *S*-transferase *T1; NQO1*, NAD(P)H:quinone oxidoreductase; Pro, proline; Ser, serine; Val, valine.

Table 2. Background characteristics and pre- and postoperative seminal parameters for patients in the responder and non-responder subgroups (mean ± SD).

	Overall (n = 72)	Responder (n = 36)	Non-responder (n = 36)	P-value
Age (year)	33.3 ± 4.7	33.3 ± 4.3	33.2 ± 5.1	NS
Grade of varicocele (N)				
G1	17	6	11	
G2	32	16	16	NS
G3	23	14	9	
Preoperative semen parameters				
Concentration ($\times 10^6$)	22.8 ± 21.6	17.8 ± 17.5	27.7 ± 24.3	NS
Motility (%)	34.1 ± 21.6	31.4 ± 19.8	36.7 ± 23.3	NS
Postoperative semen parameters				
Concentration ($\times 10^6$)	31.7 ± 32.6	46.3 ± 37.5	17.1 ± 17.7	< 0.001
Motility (%)	43.7 ± 21.4	50.8 ± 20.5	36.7 ± 20.2	0.0061

Abbreviation: NS, not significant.

rate was observed in patients with the *GSTT1*-wt genotype (63.2%) and the *NQO1*-Ser/Ser genotype (80.0%), compared with the *GSTT1*-null genotype (35.3%) and the *NQO1*-Pro/Pro or *NQO1*-Pro/Ser genotype (45.2%).

3.2 Predictive values in combined subgroups

The response rates of the groups with combinations of *GSTT1* and *GSTM1* genotypes, and of *GSTT1* and *NQO1* genotypes were analysed (Table 3). A higher response rate was observed in the *GSTT1*-wt/*M1*-wt group (75%) than in the groups with combinations of other *GSTT1/M1* genotypes, although the differences were not statistically significant. A favourable response rate was also noted in the *GSTT1*-wt/*NQO1*-Ser/Ser group (88.9%), compared with the groups having combinations of other *GSTT1/NQO1* genotypes, although the differences were not statistically significant.

3.3 Comparison of the distribution of each genotype in the controls, the overall group of infertile patients and

Table 3. The response rates for varicocelectomy in each genotype and in combination subgroups of *GSTM1/T1* and *GSTT1/NQO1* genotypes.

	Response rate	*P*-value	Odds ratio (95% CI)
Genotype			
Over all	50.0% (36/72)		
GSTM1			
null	51.1% (23/45)	NS	NS
wt	48.1% (13/27)		
GSTT1			
null	35.3% (12/34)	0.018	3.14 (1.20–8.24)
wt	63.2% (24/38)		
MnSOD			
Ala/Ala+Ala/Val	61.5% (8/13)	NS	NS
Val/Va	47.5% (28/59)		
NQO1			
Pro/Pro+Pro/Ser	45.2% (28/62)	0.041	NS
Ser/Ser	80.0% (8/10)		
Combination groups			
GSTT1-wt/GSTM1-wt	66.7% (8/12)		
GSTT1-null/GSTM1-wt and GSTT1-wt/GSTM1-null	51.2% (21/41)	NS	NS
GSTT1-null/GSTM1-null	36.8% (7/19)		
GSTT1-wt/NQO1-Ser/Ser	88.9% (8/9)		
GSTT1-null/NQO1-Ser/Ser and GSTT1-wt/NQO1-Pro/Pro or -Pro/Ser	53.3% (16/30)	NS	NS
GSTT1-null/NQO1-Pro/Pro or -Pro/Ser	36.4% (12/33)		

Abbreviations: Ala, alanine; CI, confidence interval; *GSTM1*, glutathione *S*-transferase *M1; GSTT1*, glutathione *S*-transferase *T1; NQO1*, NAD(P)H:quinone oxidoreductase; NS, not significant; Pro, proline; Ser, serine; Val, valine.

Table 4. The frequencies of each genotype in the controls and the subgroups of infertile patients.

Genotype	Controls (n = 101)	Infertile patients			
		Overall (n = 274)	*vs.* controls	Varicocele patients (n = 72)	*vs.* controls
GSTM1					
Null	52.5% (53)	58.0% (159)	NS	62.5% (45)	NS
wt	47.5% (48)	42.0% (115)		37.5% (27)	
GSTT1					
Null	50.5% (51)	46.0% (126)	NS	47.2% (34)	NS
wt	49.5% (50)	54.0% (148)		52.8% (38)	
MnSOD					
Ala/Ala	2.0% (2)	0.7% (2)		0.0% (0)	
Ala/Val	14.8% (15)	21.2% (58)	NS	18.1% (13)	NS
Val/Val	83.2% (84)	78.1% (214)		81.9% (59)	
NQO1					
Pro/Pro	36.6% (37)	39.1% (107)		34.7% (25)	
Pro/Ser	45.6% (46)	44.5% (122)	NS	51.4% (37)	NS
Ser/Ser	17.8% (18)	16.4% (45)		13.9% (10)	

Abbreviations: Ala, alanine; *GSTM1*, glutathione S-transferase *M1; GSTT1*, glutathione S-transferase *T1; NQO1*, NAD(P)H:quinone oxidoreductase; NS, not significant; Pro, proline; Ser, serine; Val, valine.

the varicocele patients

Genotypes for four genes—*GSTM1, GSTT1, SOD2* and *NQO1*—were analysed using 101 male controls and 274 infertile patients, which included 72 patients with varicocele. Mean ages were 47.2, 33.2 and 34.9 years for controls, the total group of infertile patients and the infertile varicocele patients, respectively. As shown in Table 4, none of the frequencies of the genotypes were significantly different between either the controls and the overall group of infertile patients, or between the controls and the infertile varicocele patients.

4 Discussion

Varicocelectomy has become such a widely used treatment that by 1990 a series of 50 observational studies had been conducted that altogether showed an improvement in semen quality in 57% of men [33]. On the other hand, several reports have shown no benefit from varicocele surgery [34–36]. This subject is still controversial, but the current consensus seems to be that not all men with varicocele are infertile, and not all infertile patients with varicocele show an improvement in seminal findings after surgical repair. Thus, one clinically important issue regarding varicocele is preoperative recognition of who will respond to surgical treatment. Several studies have shown that ROS production is enhanced in varicocele patients [6, 7], and that oxidative stress is present in spermatic vein blood and seminal plasma [8–10]. Oxidative stress results from an imbalance between the production and reduction of ROS. Thus, the spermatogenesis in patients with an impaired ability to reduce ROS may be strongly influenced by varicocele. Our preliminary study found that a subset of *GSTT1*-wt patients with varicocele may show good responses to surgical repair. This report suggested that genetic susceptibility to ROS might be associated with varicocele-induced infertility [28].

In the earlier study, *GSTT1*-wt patients showed a good response to varicocelectomy, and response rates among groups with combinations of *GSTM1* and *GSTT1* genotypes were significantly different [28]. In this study, we extended the earlier study and confirmed these findings, although response rates among combined groups did not differ significantly. Genotyping of *GSTT1* may aid in the decision-making process while selecting treatment options for infertile varicocele patients. *GSTs* play an important role in detoxifying products of oxidative damage [12, 13]. No earlier study has examined the relationships between *GSTT1* genotypes and male infertility, and the molecular mechanisms and effects of *GSTT1* on testicular function is unknown. However, patients with a *GSTT1*-wt genotype have a greater ability to reduce ROS, and they may recover more effectively from accumulated ROS damage after varicocelectomy. Additionally, a poor response to varicocelectomy has been reported to be related to severe and irreversible testicular damage [37]. Thus, in our study, the poor response to varicocelectomy in patients with a *GSTT1*-null genotype may be because of the severe testicular damage that occurred before varicocele repair.

NQO1 has the potential to detoxify quinones that are found in cigarette smoke and in ambient air [38], acting as a phase II enzyme in the pathways of xenobiotic metabolism. Phase II enzymes catalyse the conversion of quinones to hydroquinones, which are readily excreted by the body. However, the Pro-to-Ser substitution in *NQO1* is associated with a loss of enzyme function because of protein instability [16, 17]. In our study, patients with an *NQO1*-Ser/Ser genotype showed a better response to surgical repair. Our findings suggest that quinone-containing substances may be related to the aetiology of varicocele-induced infertility. However, the *NQO1*-Ser/Ser genotype is rare, and only 17.8% of the control group showed this genotype in the present study. In the varicocele subgroups, only 10 patients were categorized in this genotype. Our findings regarding the effects of the *NQO1* genotype on varicocele-induced infertility thus require further investigation.

We showed that genotypes of *GSTT1* were significantly associated with postoperative improvement in a patient's concentration of motile sperm. Generally, two major end-points were used in earlier studies. One is the improvement of motile sperm concentrations, which we also used in this study. The other is that the improved levels of sperm parameters are sufficiently within the reference range defined by the WHO. In this study, the genotype of each gene was not significantly related to this end-point (data not shown), possibly because the number of patients in this study was too small to elicit statistically significant results regarding the frequency of patients whose seminal findings increased into the WHO reference range. However, it is still important to note that varicocelectomy improves motile sperm concentrations, because this factor influences patients' decisions regarding assisted reproductive techniques

(ART) options, and men with semen variables lower than the WHO reference range may still be fertile in this modern ART era.

Individuals with *GSTM1*-null, *GSTT1*-null, *SOD2*-Val/Val and *NQO1*-Ser/Ser genotypes may have altered tissue protection from exogenous and endogenous oxidants. The frequencies of these genotypes differ among races. The frequencies of the *GSTM1*-null, *GSTT1*-null, *SOD2*-Val/Val and *NQO1*-Ser/Ser genotypes in Asians have been reported to be 42.5%–51.3%, 51.0%–54.0%, 73.9%–75.1% and 12.7%–20.3%, respectively, whereas those in Caucasians are 45.0%–52.3%, 13%–15.7%, 21.4%–25.4% and 2.6%–4.4%, respectively [19–25, 38–41]. In our series, the *GSTM1*-null, *GSTT1*-null, *SOD2*-Val/Val and *NQO1*-Ser/Ser genotypes were found in 52.7%, 49.8%, 83.2% and 17.8% of the controls, respectively, which are consistent with those observed earlier in Asian populations. We checked for the existence of genetic polymorphisms in the *GSTM1*, *GSTT1*, *SOD2* and *NQO1* genes in the overall group of male infertile patients. In our study, the frequencies of these polymorphic genotypes in the overall group of infertile male patients were not different from those in male controls, suggesting that the *GSTM1*, *GSTT1*, *SOD2* and *NQO1* genes are not related to the risk of male infertility. Aydemir *et al*. [42] reported that the frequency of increased oxidative damage for sperm and seminal plasma in the male population with idiopathic infertility is higher in patients with a *GSTM1*-null genotype, although no significant differences were found in the distribution of the *GSTM1* variant genotype between idiopathic infertile subjects and fertile subjects. Chen *et al*. [27] reported that the frequency of sperm mitochondrial DNA damage in infertility patients with varicocele is higher in those with a *GSTM1*-null genotype, although the frequency of the *GSTM1*-null genotype did not differ between the varicocele group and the controls.

In conclusion, the rate of response to varicocelectomy was significantly higher in patients with a *GSTT1*-wt genotype. Characterization of the genetic profiles of patients is of great interest in defining optimal candidates for varicocelectomy. Further research with larger studies is needed to confirm the findings of our study.

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