ORIGINAL ARTICLE

Is thioredoxin reductase involved in the defense against DNA fragmentation in varicocele?

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We aimed to investigate the role of thioredoxin reductase (TR) and inducible heat shock protein 70 (iHsp70) and their relationship with sperm quality in varicocele (VAR) patients. Semen samples were obtained from 16 subfertile men diagnosed as VAR and 10 fertile men who applied to the Andrology Laboratory of Istanbul Medical Faculty of Istanbul University. The sperm TR and iHsp 70 expression levels were determined using Western blot analysis. The TR activity of the sperm was assayed spectrophometrically. The sperm quality was evaluated both by conventional sperm analysis and by a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique that assayed DNA-fragmented spermatozoa in semen samples. The percentage of TUNEL-positive spermatozoa in the VAR group (16.3%±5.6%) was higher than that in the fertile group (5.5%±1.9%). Significant inverse correlations were detected between the percentage of TUNEL-positive cells and both the concentration ($r=-0.609$; $P=0.001$) and motility ($r=-0.550$; $P=0.004$) of spermatozoa. Both the TR expression and activity were increased significantly in the VAR group ($U=22.0$; $P=0.001$ and $U=33.5$; $P=0.012$, respectively) as analyzed using the Mann–Whitney U Wilcoxon rank sum W test. Furthermore, significant positive correlations were found between TR expression and activity ($r=0.406$; $P=0.040$) and between TR expression and the percentage of TUNEL-positive cells ($r=0.665$; $P=0.001$). Sperm iHsp70 expression did not differ between the VAR and fertile groups. In conclusion, increased sperm TR expression might be a defense mechanism against apoptosis in the spermatozoa of men with VAR.
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INTRODUCTION

Reproduction is a basic process that living organisms need to produce progeny and to evolve by transmitting their genes. However, both the reproductive system and spermatozoa are continuously exposed to reactive oxygen species (ROS) due to the highly active metabolic process occurring during spermatogenesis. Although basal levels of ROS are required for normal sperm functions, the overproduction of ROS leads to lipid peroxidation in the spermatozoa membrane, which in turn can reduce sperm motility and viability and increase sperm DNA damage resulting in infertility.[1–6]

Human spermatozoa have evolved several mechanisms to defend against oxidative stress and minimize the damage caused by ROS. One mechanism is the thioredoxin system which is composed of thioredoxin, thioredoxin reductase (TR) and thioredoxin peroxidase. Thioredoxins are small proteins (12 kDa) that function in maintaining the redox state of oxidized proteins *via* the reversible oxidation of the cysteine residues in their conserved active site Trp-Cys-Gly-Pro-Cys.[7–11] In addition, several thioredoxin proteins, specifically Trx-1, Trx-2, TXL-1, ERDJ-5, TXL-1/TRP32 and sperm-specific thioredoxins, namely SPtrx-1, SPtrx-2 and Sptrx-3, have been recently reported.[12–17] Because these proteins are abundant in spermatogenic cells, attention has been paid to the relationship between infertility and the thioredoxin system.

However, such a relationship has not been clearly reported in the literature.

In contrast, heat shock protein 70 (Hsp70) plays a protective role in the cell, enabling the cell to withstand the negative effects of hyperthermia and other stress conditions by assisting protein folding, transportation and complex formation. They are also produced constitutively under normal conditions.[18–20] HspA2, which is a sperm-specific constitutive Hsp70, has been reported to assist in the transportation of proteins, the repair of fragmented DNA and the removal of cytoplasm during sperm maturation.[21–23] Therefore, Hsp70 has been proposed to be involved in the pathogenesis of male infertility. However, there are few studies investigating the association of sperm Hsp70 (both stress-inducible and constitutive) with male factor infertility in the literature.[24]

It has been suggested that varicocele (VAR) is associated with male infertility, but the pathophysiology remains unclear.[25–28] Several mechanisms have been proposed to explain the causes by which VAR impairs male fertility. Changes in testicular blood flow and venous pressure, Leydig cell dysfunction, oxidative stress and hyperthermia are some of the main suggested mechanisms.[27,29] In addition, some studies have reported that increased apoptosis has been associated with VAR.[25,26,30,31] However, for the initiation of apoptosis,

several physiological stimuli are required. One of these stimuli is elevated temperature of the testes. Spermatogenesis is well known to be worsened at elevated testis temperatures, implicating the high sensitivity of spermatogenetic cells to heat stress. Thus, heat stress has been observed to suppress sperm maturation subsequently decreasing sperm quality and resulting in infertility.[32,33] In light of this information, the objective of this study was to investigate the changes in sperm TR and in the inducible form of Hsp70 (iHsp70) in a group of men with VAR and the effects of these changes on sperm quality.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA)

Subjects, semen collection and quality evaluation

Semen samples were obtained from men who applied to the Andrology Laboratory of Istanbul Medical Faculty of Istanbul University after 72 h of sexual abstinence. The experimental procedure used in this study met the guidelines of the Ethical Committee of Istanbul University. After liquefaction for 15–30 min at room temperature, the semen samples were analyzed for sperm concentration and motility using a Neubauer hemocytometer superior (Marienfeld, Germany). The results were evaluated according to the guidelines of the WHO (World Health Organization).[34] Ten fertile donors were used as the control group (mean age: 31.8 ± 4.2 years). In the VAR group, 16 males diagnosed as being VAR were included (mean age: 35.0 ± 5.1 years). Four of the patients were classified as grade 1, six as grade 2 and six as grade 3.

Seminal leukocytes were considered to be normal at concentrations of $\leqslant 1 \times 10^6$ peroxidase-positive leukocytes per ml of semen.

Sperm extraction

After liquefaction, fresh semen was washed in 10 volumes of ice cold $0.15 \, \text{mol} \, \text{l}^{-1}$ NaCl and $30 \, \text{mmol} \, \text{l}^{-1}$ imidazole, pH 7.0, and centrifuged at $5000g$ for 20 min. The resulting pellet was lysed in ice cold solution of $30 \, \text{mmol} \, \text{l}^{-1}$ imidazole pH 7.0, $5 \, \text{mmol} \, \text{l}^{-1}$ DL-dithiothreitol, 0.1% Triton X-100 and 10% glycerol. The lysate was cleared by centrifugation at $5000g$ for 20 min. After removing the insoluble material, the supernatant was stored at $-70 \, ^\circ \text{C}$ until used for the detection of iHsp70 expression.[21]

Swim-up

The direct swim-up technique was performed without centrifugation. The semen portion was layered gently under the Pure-Sperm Wash solution (Nidacon International AB, Gothenburg, Sweden) and incubated for 1 h at $37 \, ^\circ \text{C}$ in a 5% CO_2 incubator (Thermo Electron Corporation Steri-Cult, San Jose, CA, USA). The swim-up portion was then fixed in 1% paraformaldehyde and used for the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay, as described below.

TR activity

The sperm TR activity was determined using the method of Holmgren and Björnstedt.[35] In this method, TR catalyses the redox reaction between 5,5′-dithio-bis-2-nitrobenzoic acid and nicotinamide adenine dinucleotide phosphate. At the end of this reaction, 5,5′-dithio-bis-2-nitrobenzoic acid is converted to a yellow product, 5-thio-2-nitrobenzoic acid. The decrease in the 5-thio-2-nitrobenzoic acid formation was monitored kinetically at 412 nm. The results were calculated using the extinction coefficient of $13\,600 \, \text{mol} \, \text{l}^{-1} \, \text{cm}^{-1}$ for

5-thio-2-nitrobenzoic acid and are expressed as mU per mg of sperm protein.

TR and iHsp70 determination by Western blot analysis

SDS polyacrylamide gel electrophoresis was performed using Bio Rad's mini gel system according to Laemmli's method.[36] The sperm samples containing 20 μg protein per lane were electrophoresed on a 7.5% gel in Tris-glycine buffer. After electrophoresis, the separated proteins were transferred onto an Immobilon-PVDF membrane (Millipore, Merck, MA, USA), at 20 V for 2 h 15 min.[37] The proteins on the membrane were blocked with 3% low-fat dried milk. After washing in phosphate-buffered saline (PBS) containing 1% Tween 20 three times, the blots were treated with a 1 : 1000 dilution of monoclonal primer antibodies that recognize iHsp70 (MAB3846; Chemicon International, Temecula, CA, USA) and TR (SC-28321, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and then washed three times with PBS containing 0.1% Tween 20. The resulting immunocomplexes were exposed to 1 : 10000 horseradish peroxidase-conjugated goat antimouse IgG (SAB-100; Stressgen Bioreagents Corporation, MI, USA) for 30 min. After washing with PBS containing 0.1% Tween 20, the labeled-antigen bands were visualized with an ECL Western blotting detection reagent (Amersham Pharmacia Biotech., Buckinghamshire, UK). The intensity of the bands was semiquantified densitometrically using the Vilber Lourmat gel documentation system and Bio–1D V.97 software, an image analysis program (Vilber Lourmat, Biotechnology Division, Torcy, France). The blots were stripped and subsequently reprobed with an α-tubulin monoclonal antibody (CBL270; Chemicon International, Temecula, CA, USA) as an internal standard. The band intensities were normalized to the α-tubulin content of individual samples. The value of a fertile donor which was run in the first lane in each assay as a control, was taken as 100 and compared with the other samples. The results were arbitrarily expressed as the percentage of the control value (%).

The protein content of the sperm samples was measured using bicinchoninic acid.[38] In this method, the peptide bonds reduce Cu^{2+} ions from the copper solution to Cu^+. The amount of reduced Cu^{2+} is proportional to the amount of protein present in the solution. The purple-colored product formed by the chelation of bicinchoninic acid with the Cu^+ ions was spectrophotometrically measured at 562 nm.

TUNEL assay

The DNA damage was identified using the TUNEL assay.[39] An *in situ* cell death detection kit (ApopTag Plus Fluorescein; Chemicon International, Temecula, CA, USA) was used to detect DNA fragmented spermatozoa according to the manufacturer's recommended protocol. Briefly, 3–4 h after the swim-up preparation, approximately 2×10^6 sperm were fixed with 1% paraformaldehyde for 10 min at room temperature. Following the fixation, the sample was permeabilized with ethanol/acetic acid. After washing with PBS and equilibration buffer, the TdT-labeled nucleotide mix was added to the slides, which were incubated at $37 \, ^\circ \text{C}$ for 1 h. After the incubation, the slides were washed with PBS, and an anti-digoxigenin fluorescent antibody was applied the samples, followed by incubation for 30 min at room temperature. After the antibody exposure, the slides were washed four times with PBS and analyzed under an Olympus fluorescence microscope (Ushio Inc., Tokyo, Japan). As a positive control, one sample was pre-treated with 0.1 U DNase I (Roche Diagnostics, Manheim, Germany) for 30 min at room temperature. As a negative control, the enzyme terminal transferase was omitted from the reaction mixture.

Table 1 The semen characteristics and sperm DNA fragmentation (TUNEL) of the fertile controls and VAR patients

	Fertile controls (n=10) (mean±s.d.) (median)	VAR (n=16) (mean±s.d.) (median)	Statistics	
Sperm concentration (×10⁶ ml⁻¹)	53.8±11.7 (55.5)	19.7±8.6 (17.0)	$r=-0.609$[b]	$P=0.001$
Sperm motility (%) (A+B)[a]	59.9±3.4 (60.5)	31.7±7.8 (31.5)	$r=-0.550$[b]	$P=0.004$
Morphology by WHO (%)	45.4±9.3 (45.0)	32.9±11.0 (33.5)	$r=-0.386$[b]	$P=0.052$
TUNEL (%)	5.5±1.9 (5.5)	16.3±5.6 (16.0)	—	$P=0.000$; U=0[c]

Abbreviations: TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VAR, varicocele.
[a] A+B: progressive motile sperm.
[b] Pearson's method was used for correlation analyses.
[c] Statistical analyses were performed using the Mann–Whitney U test. $P<0.05$ compared with the fertile controls (mean±s.d.).

Evaluations of DNA-damaged spermatozoa were performed by one investigator using unidentified (blinded) sperm preparations. First, the number of spermatozoa was counted by light microscope. Then, the number of damaged spermatozoa with green fluorescence was expressed as a percentage of the total spermatozoa. At least 200 cells were counted per field, and seven or eight random fields were used to determine the percentage.

Statistical analysis
The Statistical Package for the Social Sciences (SPSS Inc, Chicago, IL, USA) program was used for the statistical analyses. The data are expressed as the means±s.d. The statistical analyses were performed using the Mann–Whitney U Wilcoxon rank sum W test. The correlation coefficients were determined by Pearson's method.

RESULTS
The semen characteristics of the fertile and VAR groups are given in **Table 1**.

In our study with a relatively small number of VAR patients, the percentage of DNA-damaged spermatozoa as assayed by the TUNEL technique was higher in the VAR group (16.3%±5.6%) compared with that of the fertile group (5.5%±1.9%) (**Table 1**). In addition, significant inverse correlations were detected between the percentage of TUNEL-positive cells and both the concentration ($r=-0.609$; $P=0.001$) and motility ($r=-0.550$; $P=0.004$) of the spermatozoa.

For the Western blot analysis, the band intensities of TR and Hsp70 were normalized to α-tubulin as an internal standard. The values were expressed arbitrarily by comparison with one fertile sample (as a control) that was run in the first lane for each assay performed. A significant increase was found in the sperm TR expression in the VAR group compared with the fertile group (Mann–Whitney $U=22.0$; $P=0.001$) (**Table 2** and **Figure 1**). The sperm TR activity also increased significantly in the VAR group (**Table 2**) compared with the fertile group (Mann–Whitney $U=33.5$; $P=0.012$). Furthermore, a positive correlation was detected between the TR activity and TR expression in the spermatozoa ($r=0.406$; $P=0.040$) (**Figure 2**). In addition, we found a significant positive correlation between the percentage of TUNEL-positive cells and the TR expression in spermatozoa ($r=0.665$; $P=0.001$) (**Figure 3**).

In contrast, the iHsp70 expression in the sperm of the VAR patients did not differ from that in the fertile men (**Table 2** and **Figure 1**). Examples of TUNEL microphotographs from the fertile and VAR groups are shown in **Figure 4**.

DISCUSSION
Oxidative damage is considered to be one of the underlying mechanisms in the pathogenesis of male infertility. In fact, there are many reports describing reduced motility, abnormal morphology and decreased capacitation; implicating impaired sperm function due to oxidative damage.[40–44] In our previous studies, we have shown reduced sperm motility caused by treatment with peroxynitrite, which is a strong oxidizing and nitrating agent.[42,43] Increased oxidative stress parameters and decreased antioxidant capacity in patients with VAR have also been reported.[27,45–47] It has been suggested that the impact of oxidative stress on male infertility occurs through a change in the proliferation rate, induction of apoptosis or necrosis, modulation of gene expression and/or stimulation or inhibition of some cell signaling components.[5,48–50]

To the best of our knowledge, there are no studies investigating the thioredoxin system in VAR in the literature. Current studies on sperm-specific thioredoxins show that an association may exist between infertility and the SPtrxs which have functions related to spermatogenesis.[12–17] In this study, we found that both the expression and activity of TR were significantly increased in the VAR group compared with those in fertile men. The TR expression is thought to be increased as a consequence of a defense against the oxidative and/or apoptotic damage of spermatozoa and the repair of damaged proteins due to oxidative stress. In addition, the elevation of TR expression might have caused the increases in TR activity.

In addition, studies on the association of Hsp70 with infertility are limited in the literature and the reported results are not consistent. Related studies have mainly dealt with constitutive Hsp70 (Hsp70-2) and/or with iHsp70 in experimental animals,[33,51–55] as well as with HspA2, which is a human analogue of Hsp70-2,[21–23] mostly in the

Table 2 The expressions of TR and iHsp70 and the activity of TR in the spermatozoa of the fertile controls and VAR patients

	Fertile controls (n=10) (mean±s.d.)	VAR (n=16) (mean±s.d.)	Statistics[a]	
iHsp70 (%)	93.5±28.2	95.4±51.2	U=76.0	$P=0.856$
TR expression (%)	114.3±26.2	173.8±54.1	U=22.0	$P=0.001$
TR activity (mUTR mg⁻¹ protein)	184.0±55.8	283.1±100.4	U=33.5	$P=0.012$

Abbreviations: iHsp70, inducible heat shock protein 70; TR, thioredoxin reductase; VAR, varicocele.
[a] Statistical analyses were performed using the Mann–Whitney U test.
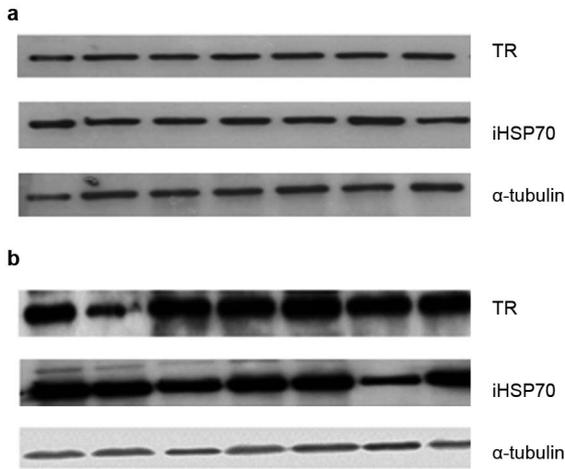$P<0.05$ compared with the fertile controls (mean±s.d.).

Figure 1 Examples of the expression profiles of TR, iHsp70 and α-tubulin of the spermatozoa from the fertile (**a**) and VAR (**b**) groups. The band intensities on the Western blots (TR and iHsp70) were normalized to α-tubulin as an internal standard. The values were expressed arbitrarily by comparison with a fertile sample (as a control) which was run in the first lane for each assay performed. iHsp70, inducible heat shock protein 70; TR, thioredoxin reductase; VAR, varicocele.

Figure 3 Positive correlation between the TUNEL results and TR expression. TR, thioredoxin reductase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling.

Figure 4 Examples of TUNEL microphotographs from the fertile (**a**) and VAR (**b**) groups. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VAR, varicocele.

testes, spermatids or spermatocyte cultures. However, there are few studies that explore the importance of Hsp70 in human [24,27,56,57] or animal spermatozoa.[58] Yesilli *et al.*[27] reported that sperm HspA2 activity was lower in infertile men with VAR than in the control group. In addition, sperm HspA2 gene expression was reported to be down-regulated in both oligozoospermic patients[56] and VAR patients.[57] In contrast, in our previous study, we demonstrated for the first time that Hsp70 was increased in both asthenospermic and oligoasthenospermic infertile men compared with fertile men.[24] We have assumed that increased Hsp70 acting as a chaperone prevented protein aggregation and participated in the refolding of damaged proteins following stress. In the present study, we found that iHsp70 remained unchanged in the VAR patients.

The importance of apoptosis or programmed cell death in human spermatozoa is currently a subject of great interest.[39,59–61] The final stage of programmed cell death is DNA fragmentation. One method to assay fragmented DNA in spermatozoa is the TUNEL technique. Conventional semen parameters are not always sufficient to determine sperm function and male infertility. Therefore, attempts have been made to link some apoptotic alterations, such as DNA fragmentation,
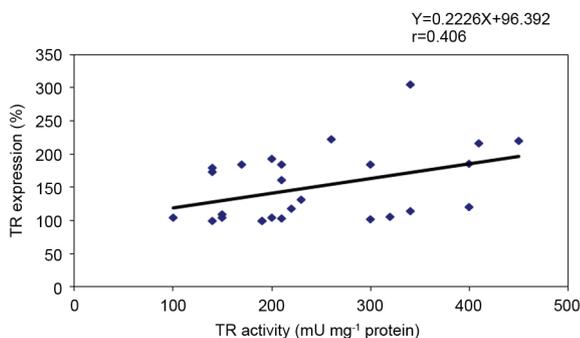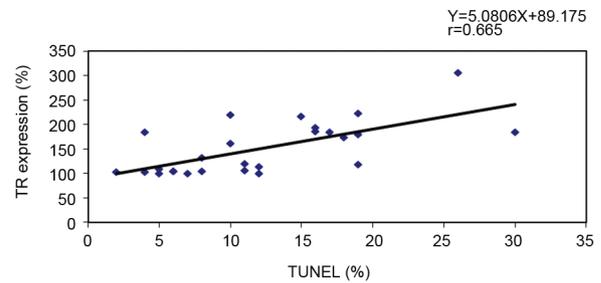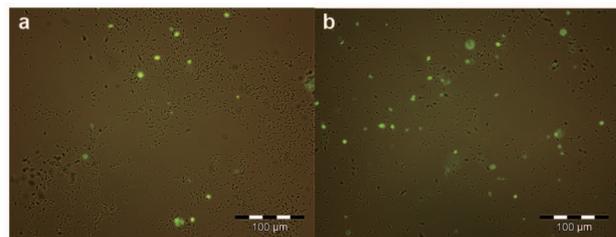

Figure 2 Positive correlation between TR activity and TR expression. TR, thioredoxin reductase.

in the spermatozoa with some conventional semen parameters to evaluate male infertility.[39,59,62] Indeed, there are a number of studies, including our previous study,[24] that have proposed the association between the presence of DNA-damaged spermatozoa and poor semen parameters in men.[31,48–50,59,61,63] In the present study, we found that the levels of DNA-damaged spermatozoa were higher in the VAR group compared with those of the fertile group. Significant inverse correlations between routine semen parameters (such as sperm concentration and motility) and the percentage of DNA-damaged spermatozoa were also observed. This increase in DNA-damaged spermatozoa confirmed the harmful effects of apoptosis on sperm quality.

In sperm exposed to stress, protein refolding and transportation are disrupted, and apoptosis is increased. The occurrence of these events in protein structures may stimulate increases in TR expression and activity. In conclusion, in our study, TR might be increased to assist in the repair of oxidized cell molecules, leading to the regeneration of essential thiol groups that protect proteins from oxidative stress and/or apoptosis in VAR. However, the limitations of this study include the relatively small overall number of subjects and the potential for unidentified confounding clinical parameters in these patients diagnosed with VAR.

AUTHOR CONTRIBUTIONS
CK and GA have done experimental work. AK has supplemented clinical material and MU, NKT, GÖE have spent effort in planning the work, evaluating of the results and writing the paper.

COMPETING FINANCIAL INTERESTS
All authors declare that there are no competing financial interests.

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