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The Effects of Thioredoxin and L-NAME on Hepatic Damage in the Experimental Liver Ischemia/Reperfusion Model: Can A Cumulative Effect Be Obtained?"

[Deneysel Karaciğer İskemi/Reperfüzyon Modelinde Tiyoredoksin ve L-Name'in Karaciğer Hasari Üzerine Etkilerinin İncelenmesi: Kümülatif Etki Elde Edilebilir mi?]

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ABSTRACT

Objectives: The aim of this study was to evaluate the effects of thioredoxin and N-nitro-L-arginine methyl ester (L-NAME) on ischemia/reperfusion injury after partial liver ischemia was induced by selective clamping of the portal veins and hepatic arteries of rats.

Material and Method: Rats were subjected to 1 hour of 70% hepatic ischemia, followed by reperfusion or sham. At the end of ischemia, phosphate-buffered saline solution, L-NAME and thioredoxin with or without L-NAME were infused. Analysis was performed at pre-ischemia, onset of ischemia and postreperfusion. Hepatic tissue blood flow was evaluated by laser Doppler. Afterwards, biochemical analyses were performed. Immunohistochemical method was used for apoptosis evaluation.

Results: Thioredoxin application increased hepatic tissue blood flow in reperfusion. After the inhibition of NOS enzyme with L-NAME, alanine aminotransferase, aspartate aminotransferase and P-selectin levels were significantly decreased when compared to ischemia/reperfusion group; this decrease was lower than the one in the thioredoxin group. Endothelins, nitric oxide, malondialdehyde levels and apoptosis ratio in the group in which L-NAME was applied had a considerabe extent of decrease when compared to ischemia/reperfusion group, and also it was significantly higher than the one observed in the thioredoxin group.

Conclusion: Thioredoxin is one of the important anti-oxidants in ischemia/reperfusion injuries for reducing the damages, and that excess amount of nitric oxide depression creates protective effects. No significant difference was detected between values of thioredoxin and L-NAME administration, which means that the co-use of thioredoxin and L-NAME did not create a cumulative effect.

Key Words: thioredoxin, nitric oxide, L-NAME, ischemia/reperfusion, reactive oxygen species, antioxidant system, apoptosis

ÖZET

Amaç: Bu çalışmanın amacı hepatik arter ve portal venin kısmi klemplenmesiyle kısmi karaciğer iskemi/reperfüzyon hasarı oluşturulan ratlarda, tiyoredoksin ve N-nitro-L-arginin metil esterinin (L-NAME) karaciğer hasarı üzerine olan etkilerini incelemektir.

Materyal ve Metod: Ratlar 1 saat boyunca %70 hepatik iskemiye maruz bırakılarak ardından reperfüzyon veya sham uygulandı. İskeminin sonunda, fosfat tamponlu salin solüsyonu, L-NAME ve L-NAME'li veya L-NAME'siz tiyoredoksin infüzyonu yapıldı. Analizler iskemi öncesi, iskemide ve reperfüzyon sonrasında gerçekleştirilmiştir. Hepatik doku kan akışı lazer Doppler ile değerlendirilmiştir. Biyokimyasal analizler gerçekleştirilmiştir. Apopitoz değerlendirmesi için immünohistokimyasal yöntem kullanılmıştır.

Bulgular: Tiyoredoksin uygulamasının reperfüzyonda hepatik doku kan akışını arttırdığı saptanmıştır. NOS enziminin L-NAME ile inhibisyonunun ardından, alanin aminotransferaz, aspartat aminotransferaz ve P-selektin seviyeleri iskemi/reperfüzyon gurubu ile kıyaslandığında önemli ölçüde azalmıştır; bu azalmanın tiyoredoksin gurubundaki azalmaya göre daha düşük seviyede olduğu saptanmıştır. L-NAME'nin uygulandığı guruptaki endotelin-1, nitrik oksit, malondialdehit seviyeleri ve apopitozis oranı iskemi/reperfüzyon gurubu ile kıyaslandığında önemli ölçüde azalmıştır ve buradaki azalmanın da tiyoredoksin gurubundan çok daha fazla olduğu gözlenmiştir.

Sonuç: Elde edilen bulgular doğrultusunda tiyoredoksinin iskemi/reperfüzyon hasarında, hasarı azaltmada önemli antioksidanlardan biri olduğunu ve fazla nitrik oksit miktarının baskılanmasının da koruyucu etkiler ortaya çıkaracağını düşünmekteyiz. Bunun yanında çalışmamızda tiyoredoksin ve L-NAME uygulama değerleri arasında önemli bir farklılık bulunmadığı ve tiyoredoksin ve L-NAME'nin bir arada kullanımının kümülatif bir etki yaratmadığı gösterilmiştir.

Anahtar Kelimeler: tiyoredoksin, nitrik oksit, L-NAME, iskemi/reperfüzyon, reaktif oksijen ürünleri, antioksidan sistemi, apopitoz

Introduction

Hepatic ischemia and reperfusion (IR) is a complex mechanism in which endothelial cells, complement systems, adhesion molecules, nitric oxide and antioxidative systems are all involved. Recent studies have shown that hepatic microcirculation failure, activation of neutrophils and endothelial cells, and apoptosis were the key steps in hepatic IR injury and resulted in energy imbalance, and dysregulation of oxidative system. Reactive oxygen species (ROS), inflammatory cytokines, vasoactive mediators; nitric oxide (NO) and endothelins (ET)] were generated from activated Kuppfer cells [1]. NO, which plays protective role in the experimental hepatic IR, is a potent gaseous vasodilator in the regulation of hepatic microcirculation especially in the reperfusion phase [2-3]. On the other hand, ET-1, the most powerful vasoconstrictor which was elevated especially in the reperfusion phase of IR, aggravated the injury. Derangement of ET-1 and NO is the crucial factor in microcirculatory failure and activation of adhesion molecules on endothelial cells, especially the P-selectin.

The cellular protein Thioredoxin (Trx) with molecular weight of 12 kDa has critical roles in the activation of transcription factors like nuclear factor-kappa B (NF-κB), and in the prevention of cells from apoptosis and oxidative stress, especially for excessive radical oxygen species [4-5]. Reduced Trx is the major cellular "protein disulfide reductase" and the major electron donor for many enzymes. While glutathione reductase only reduces the glutathione, Trx, which is the other component of thiol reductase system, it also reduces the other ROS [6]. The aim of this study is to evaluate the effect of Trx in the experimental liver ischemia/reperfusion with or without the influence of N-nitro-L-arginine methyl ester (L-NAME) which is the NO blocker in order to rule out the relation between NO and this redox system

Material and Method

Animals and Surgical Procedures

The study was performed according to the recommendations of the Animal Research Committee at Gazi University in Ankara, Turkey. A total of 50 rats weighing 235-275 g were used in the study. The animals were housed under cycles of 12 h of light and 12 h of dark in individual cages, and they were allowed free access to standard rat chow and water. All experiments were performed with rats that had fasted for 12 h before surgery. Rats were anaesthetized with intra-peritoneal ketamine (100 mg/kg body weight [BW]) and xylazine (20 mg/kg) and prepared for aseptic surgery. A midline incision extending from the xiphisternum to the pubis was made. A polyethylene catheter (PE-50, ID 0.28, OD 0.61; Portex, Hyte, UK) was inserted from the ileocecal vein to the portal vein to infuse the drugs. The liver was exposed with retractors placed in the flank, and a clamp was attached to the xiphisternum and elevated. The ligamentous attachments between the liver and the diaphragm were freed. In order to avoid splanchnic congestion, we used a model of partial liver ischemia. Partial liver ischemia was induced by selective clamping of the portal vein and hepatic artery, which supply the left lateral and median lobes of the liver (segments II–IV), using an atraumatic vascular clamp (Harvard Apparatus Inc., Hollinston, MA, USA) for 60 min; followed by 2 hours of reperfusion 10 minutes of which were performed with the studied solutions. To avoid the influences arising from major fluid loss or drying of the liver, the abdominal cavity was covered with wetted gauze.

Experimental Design

The rats were divided into five groups:

- *Sham group (n=10):* Animals were subjected to anaesthesia and laparotomy.
- *Control (I/R) group (n=10):* Reperfusion was performed after 60 min of partial ischemia, and 1 ml vehicle (phosphate-buffered saline solution) was infused into the portal vein for 10 minutes immediately upon perfusion.
- *Thioredoxin (Trx) group (n=10):* Reperfusion was carried out after 60 min of partial ischemia, and recombinant thioredoxin (10mg/kg) (Promega Corporation, WI, USA) was infused for 10 min via the portal vein immediately upon reperfusion.
- *N-Nitro-L-arginine methyl ester (L-NAME) group (n=10):* Reperfusion was conducted after 60 min of partial ischemia. L-NAME (10mg/kg) was infused for 10 min via the portal vein immediately upon reperfusion.
- *Trx and L-NAME group (n=10):* Reperfusion was performed after 60 min of partial ischemia. L-NAME (10mg/kg) and recombinant thioredoxin (10mg/kg) was infused for 10 min via the portal vein immediately upon reperfusion.

One hundred microgram L-NAME was dissolved in 40 ml phosphate–buffered saline solution. For Trx, 50 mmol/L Tris-HCl (pH=7.5) was dissolved in 40 ml solution including 1 mmol/L EDTA. Following I/R, 0.5 g tissue samples from left lobe and 4 ml blood samples were drawn from inferior vena cava during sacrification.

Hepatic tissue blood flow

Hepatic tissue blood flow (HTBF) was measured using a laser-Doppler flow meter (Periflux 5000; Perimed, Stockholm, Sweden) before ischemia, at 5, 15 and 45 min after the initiation of ischemia, and at 5, 15, 30 and 60 min after reperfusion. Succeeding time point, a mean of three values obtained at different lobes (segments II–IV) was calculated. The principle of the method is that light generated by a laser diode (780 nm wavelength with maximum emission energy of 1.0mW) penetrates the tissue, where it is reflected by circulating blood cells.

Analogue laser Doppler flow signals were digitalized. Blood flow was recorded for at least 30-s until a stable signal was obtained. Post-sampling data processing included pulse wave analysis with the integral under the curve. For integral estimation, the mean of the pulse waves within the 30-s sampling period was calculated. HTBF was expressed as an arbitrary unit (AU) [7].

Biochemical Studies

The rats were sacrificed at the 2nd hour following I/R. Immediately upon sacrification, 0.5 g tissue sample was taken from the left hepatic lobe and 4 ml blood sample was taken from the portal vein. Sham groups were simultaneously sacrificed.

Preparation of the Samples

Blood samples taken from the rats were transferred to 10 ml evacuated gel separator tubes and EDTA tubes. The blood samples were centrifuged at +4^{0}C and 1600 x g for 15 min. Approximately 500-1000 µl of the resulting serum samples were taken and used for biochemical tests. The remaining serum samples were separately transferred to tubes as 100 µl and 200 µl for P-selectin and nitric oxide studies, respectively. These serum samples were stored at -80^{0}C until the study started.

EDTA samples were transferred from EDTA tubes to centrifuge tubes and aprotinine was added so as to create 0.6 Trypsin inhibitor unit (TIU)/ml (1 TIU = 1025-1300 KIU (Kallikrein inhibitor unit) blood. The samples were centrifuged at +4^{0}C and 1600 x g for 15 min and the resulting plasma samples were stored at -80 ^{0}C until the study started.

AST, ALT, NO metabolites, P-selectin and Endothelin-1 study in serum and plasma samples:

Biochemical parameters, which were aspartate aminotransferase (AST) and alanine aminotransferase (ALT), were analyzed in serum sample with original kits, Thermo Fischer Scientific (Thermo Fischer Scientific, Vantaa, Finland) kits, in Konelab 60 auto-analyzer (Labsystems Clinical Laboratory Division, Espoo, Finland). The study was performed on the same day and on the same series in order to prevent the possible variations between days and studies.

Serum NO metabolites levels were measured with Stressgen's StressXpress® NO (total) kit (catalogue no: EKS-310). In this method, firstly, nitrate was transformed into nitrite with nitrate reductase enzyme, the resulting nitrite was dyed with azo dye (Griess Reaction) and its absorbance was measured at 540 nm. Concentrations of nitrite have been used as a quantitative measure of NO production.

We measured sP-selectin by means of a highly sensitive sandwich ELISA (enzyme-linked immunosorbant assay) technique and a commercially available test reagent set (Human sP-Selectin/ Diaclone Research, Codex, France) in line with the manufacturer's instructions. We carried out duplicate measurements with 10-µL aliquots of plasma and measured the absorbance at 450 nm with a microplate reader (ELx800; BioTek Instruments, Inc). A standard curve was prepared from seven sP-Selectin standard dilutions and sP-selectin sample concentration was determined.

ET-1 production was quantified using the protocol from the ET-1 ELISA kit (Phoenix Pharmaceutical, CA, USA). Samples for determination of tissue ET-1 levels were processed according to an established protocol for extraction of ET-1 prior to colorimetric ELISA of ET-1. The inter- and intraassay imprecision (as CV) was $\leq$ 14.5% and 5%, respectively. The lower detection limit was 0.1 ng/ml. Cross-reactivity of the ELISA was 100% to ET-1, 3.5% ET-2, 28% to ET-3, 50% to big ET1 and 70% big ET-2. Despite the presence of cross-reactivity to other ET isoforms, the assay measures primarily ET-1. We carried out duplicate measurements with 50 µL aliquots of sample and measured the absorbance at 450 nm with a microplate reader (ELx800; BioTek Instruments, Inc). A standard curve was prepared from seven ET-1 standard dilutions and ET-1 sample concentration was determined.

Preparation of Tissue Homogenate

Liver homogenates were prepared from the left hepatic lobe. The sample tissues extracted were then washed in % 0.9 NaCl solution with 0.16 mg/ml heparin. Excised liver was transferred to ice-cold homogenization medium (20mM phosphate buffer; pH 7.4), minced, and homogenized with a basic homogenizer (Ika Works ULTRA-TURRAX T 10 basic Disperser/Homogenizer). The homogenate was centrifuged at 3000 g at 4^{0}C for 10 min. And the sample was instantly frozen at -70^{0}C.

Measurement of the Tissue Protein:

Tissue protein measurement of the samples was performed with the Lowry method. The samples were vortexed and kept for 15 min. Then, 200 µl Folin reactive was added into the samples; they were properly vortexed and kept at dark for 1 h. The tubes were read against the blank at 750 nm and the results were calculated through the obtained absorbance values.

In our study, we expressed all the measured values by dividing them to tissue protein.

SOD, Lipid Peroxidation Product and Apoptosis Study in Tissue Samples:

Photometric kit, which belongs to Oxford Biomedical Research (Oxford, MI, USA) (Product No: FR10), was used for tissue SOD measurements. The kit was applied to Konelab 60i auto-analyzer and the analysis was conducted. The kinetic SOD measurement of the 525 nm absorbance change is performed after the addition of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo fluorine. The SOD activity is determined from the ratio of the autoxidation rates in presence (Vs) and in the absence (Vc) of SOD. The Vs/Vc ratio as a function of SOD activity is

independent of the type of SOD (Cu/Zn-SOD, Mn-SOD, Fe-SOD) being measured. One SOD activity unit is defined as the activity that doubles the autoxidation rate of the control blank (Vs/Vc=2). The obtained values were divided by tissue protein and expressed in U/mg tissue

Lipid Peroxidation Products (Malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) were analyzed as lipid peroxidation products in the tissue through Lipid Peroxidation kit, which was developed by Oxford Biomedical Research (Oxford, MI, USA) (Product no: FR 12). The resulting values were divided by tissue protein and expressed in nmol/mg tissue.

In rat liver tissues, Apo-BrdU-IHC in situ DNA fragmentation kit expression (TUNEL kit) was analysed through immunohistochemical method. A TUNEL kit (Wako, Osaka, Japan) TUNEL (Terminal transferase deoxy-UTP Nick End Labeling) the most commonly used in situ test for apoptosis where DNA strand breaks are detected by the binding of termi nal deoxynuclotidyl transferase to the 3′ ends of DNA fragments. DAPI (4′, 6-Diamidino-2-phenylindole dihydrochloride) is a blue fluorescent stain that binds to double stranded DNA, thus resulting in a 20-fold enhancement of fluorescence. Cells that have undergone apoptosis show pyknotic nuclei that can be visualized following DAPI staining.

Statistical Analyses

All data were presented as mean ± SEM (standard error of the mean). Statistical analyses were carried out using computer based the SPSS 15.00 programme. The Shapiro-Wilk test was used so as to determine the group distribution. Comparison between groups was done by Kruskal Wallis and comparison within the groups was conducted by using the Mann Whitney U test. Numerical parameters were compared by χ^2. Probability values below 0.05 were considered to be statistically significant.

Results

Levels of the measured parameters and SEM values were given in Table 1 and the p values between groups were also given in Table 1.

No mortality was seen during the experiment. Hepatic tissue blood flow results were given in Figure 1. The HTBF values were similar in all groups before ischemia. Partial hepatic ischemia decreased the HTBF levels to 20% of the original levels. Evaluation of the HTBF levels from 30 min of reperfusion until the end of the experiment showed that the HTBF levels of Trx-treated rats increased as much as those of the sham group (P>0.05). Treatment with Trx increased the level of HTBF when compared to the I/R, L-NAME and Trx-L-NAME groups (p<0.001). Adding L-NAME to this treatment, however, gave rise to a significant decrease in the HTBF levels (P<0.05).

It was found that partial ischemia increased the levels of ALT and AST significantly. The difference between the levels of AST in I/R group and sham group was significant (p<0.001), but the difference between the study groups and I/R was not significant. The difference was not significant between the AST levels of Trx, L-NAME, Trx-L-NAME and I/R groups (p=0.38, p=0,839, p=0.155, respectively). The increase of ALT was more noteworthy. Although the levels of ALT in the groups, treated with Trx, L-NAME and Trx-L-NAME were lower than those in the I/R group (p<0.001), they were still higher than the levels of the sham group.

Adhesion molecules like P-selectin and Endothelin-1 were found to be significantly elevated in the I/R group when compared to those found in the sham group (p<0.001). Although the levels of P-selectin and Endothelin-1 of Trx, L-NAME and Trx-L-NAME groups were significantly lower than the levels of I/R group (p<0.001), they were still higher than the sham group. The decrease was more prominent in Trx-L-NAME group.

Table 1. Data and SEM values of the measured parameters in the study groups.

	Sham (I)	Ischemia / reperfusion (I/R)	Thioredoxin (Trx)	L-NAME	Trx and L-NAME	Sham/ I/R	Trx/I/R	L-NAME/I/R	Trx-L-NAME/I/R
ALT (U/L)	206.7 ± 88.3	1113.4 ± 313.0**	426.8 ± 76.7*	507 ± 159**	413.8 ± 117.0	***<0.001***	***<0.001***	<0.001	<0.001
AST (U/L)	208.4 ± 76.8	468.5 ± 93.3**	360.1 ± 132.0*	479.5 ± 133.0**	397.2 ± 119.0*	***<0.001***	***0.038***	0.829	0.166
P-Selectin (ng/ml)	150.2 ± 65.4	448.5 ± 119.0**	237.9 ± 94.7*	279.4 ± 71.6*	173.5 ± 34.2	***<0.001***	***<0.001***	<0.001	<0.001
Endothelin-1 (ng/ml)	45.52 ± 12.70	125.46 ± 35.50**	94.99 ± 10.20**	84.85 ± 11.80**	72.24 ± 11.50*	***<0.001***	***0.001***	***<0.001***	***<0.001***
NO metabolites (μmol/L)	20.8 ± 5.5	42.1 ± 7.1**	23.8 ± 5.0	16.9 ± 6.1	12.3 ± 4.5*	***<0.001***	***<0.001***	***<0.001***	***<0.001***
SOD (U/mg protein)	423 ± 190	280 ± 102	537 ± 143	603 ± 221*	645 ± 154*	0.060	***0.001***	***<0.001***	***<0.001***
Lipid Peroxidation Product (nmol/mg protein)	1.15 ± 0.43	3.67 ± 1.87**	2.62 ± 0.75*	2.21 ± 1.05*	2.56 ± 0.46*	***<0.001***	***0.031***	***0.003***	***0.023***
Apoptosis (%)***	%4-6	%10-12	% 8-10	% 5-6	%2				

*Comparison between sham group and other groups (Mann Whitney U test, p<0.05)
** Comparison between sham group and other groups (Mann Whitney U test, p<0.001)
***Median apoptosis levels (%25-%75 percentile)

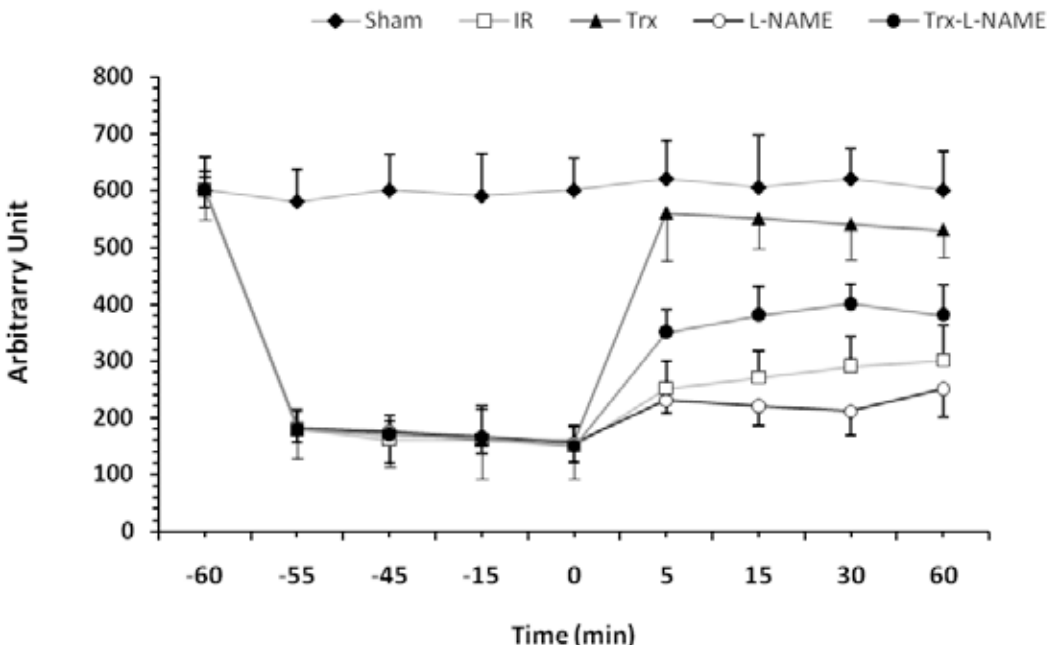

Figure 1. Time profile of hepatic tissue blood flow (HTBF) change before and after ischemia reperfusion. The levels of HTBF after partial hepatic ischemia significantly decreased to 20% (P<0.001). After 30 minutes of reperfusion, HTBF levels in the thioredoxin treatment group got closer to those of the sham group (P>0.05).

It was found that the levels significantly increased with the ischemia (p <0.001). However, the levels of NO metabolites in the groups L-NAME, and Trx-L-NAME decreased more slowly than the levels of the sham group. The levels of NO metabolites in the group Trx, L-NAME and Trx-L-NAME were significantly lower than the ones in the I/R group (p<0.001). When the ET-1/NO ratios of groups were calculated, they were found to be 0.21, 0.29, 0.4, 0.5, 0.58 in the groups sham, IIntracellular anti-oxidative enzymes (SOD) decreased insignificantly in I/R group when compared to the sham group (p=0.06). However, the levels of SOD increased in groups L-NAME and Trx-L-NAME and the difference between the levels of I/R group was statistically significant (p<0.001).

The level of lipid peroxidation products (MDA and HAE) significantly increased in the I/R group when compared to that of the sham group (p<0.001). The levels of lipid peroxidation products in groups Trx, L-NAME and Trx-L-NAME were higher than those in the sham group and lower than the ones in the I/R group. The difference was not statistically significant (p=0.32). Apoptosis increased more significantly in I/R group than it did in the other groups. However, the apoptosis in L-NAME group was lower than it was in the sham group. Apoptosis in Trx group was significantly higher than it was in the L-NAME group. The difference between the apoptosis ratios of I/R and Trx groups was statistically insignificant.

Discussion

The basic step in the ischemia/reperfusion injury is the lack of energy and disruption of oxidative phosphorylation. Certain protective mechanisms act for prevention like Trx and NO. The role of Trx in the experimental I/R model was evaluated in our study. One of the biochemical cellular injury markers is the measurement of cellular enzymes in the circulation. The increase in ALT and AST levels of I/R group decreased by means of Trx and L-NAME groups in our study. As expected, the cytoplasmic enzyme ALT was firstly affected by I/R rather than it was done by the mitochondrial enzyme AST. On the other hand, it was seen that the significant decrease in the levels of ALT and AST occurred in the Trx-L-NAME group. It was found that anti-oxidative Trx and NOS inhibitor L-NAME had protective roles in hepatocytes in I/R. Squadrito et al. showed that inhibition of NOS by L-NAME had decreased the formation of peroxynitrite [8]. Thiermann et al. found that excess vasodilatation was inhibited by L-NAME and decreased the injury of the hepatocytes [9]. As in our study, L-NAME significantly decreased the blood flow, but the protective effect was significant. On the other hand, although the NO levels of I/R were higher than the NO levels in Trx, the hepatic blood flow was found to be higher in the Trx group. Also, the protective role of Trx was seen as a result of the increased blood flow when compared with the roles in I/R and L-NAME groups. This showed that Trx eliminated the negative effects of increased blood flow during reperfusion. And the increased blood flow may be explained the presence of NO and the decreased ROS or peroxynitrites.

Endothelial derived adhesion molecule, P-selectin, is an important molecule for binding of activated leucocytes to the endothelium [10-11]. Experimental hepatic I/R studies showed that treatment with p-selectin antibodies

decreased the injury and increased the survival [12]. In our study, p-selectin levels were significantly increased in the I/R group when compared with those in the sham group. The results were found to be similar with the ones seen in the literature [11]. Also, the protective role of Trx and L-NAME was shown by the decreased p-selectin levels in these groups. In the circulation, Trx inhibited the invasion of neutrophils and prevented the extravasation of leucocytes [13]. In a recent study, IV administration of human recombinant Trx has decreased the I/R injury in rats, rabbits and dogs [13]. The effect of Trx can be explained by the activation of steroid receptors and NF-κB and AP transcription factors which led to increased p-selectin by Trx.

In the early stages of hepatic reperfusion, microcirculatory dysfunction with a loss of hepatic metabolism occurs as a result of an imbalance between vasoactive and vasodilative substances like ET-1 and NO [1, 14]. But, no correlation has been found between the ET-1/NO and the hepatic blood flow. This showed that blood flow not only depended on the ET/NO, but also on the other mechanism in which Trx took part like intracellular anti-oxidative factors. There have been conflicting data about the effects of NO. At the beginning of reperfusion, while the levels of NO were decreasing, the levels of ET-1 increased and vasoconstriction occurred in microcirculation [15]. It was shown that low levels of NO were physiologically important, but high levels of NO were toxic [16]. High levels of NO inhibited the cellular enzyme systems [16-17]. These enzymes took part in the energy, apoptosis, mutations, and several immunological systems. These studies showed that inhibition of iNOS was protective against the I/R injury. Although the peroxynitrites, which occurred after the reaction with NO, were not radicals, they were strongly oxidative. As they had longer half-lives when compared to the superoxide and nitric oxide, they could damage cellular macromolecules like DNA and all types of proteins. Also, peroxynitrite increase would lead to increased hydroxyl radical formation. The relation between NO and ET-1 is complex. While ET-1 increased the NO production by ET-B receptors, NO decreased the ET production via cGMP [18]. Several studies about NO and ET-1 like in our study showed that when the levels of ET were high, the levels of NO were found to be high, too. It was accepted that L-NAME, which was an NOS inhibitor, decreased the levels of NO. Hansen et al. showed that Trx-2 regulated the ROS production by TNF-α [19]. Welsh et al. showed that Trx-1 activated HIF-1 (hypoxia-induced factor-1) and led to iNOS gene inactivation [20]. But, the decrease of NO in Trx group was the result of the decrease in ROS and inactivation of TNF-α, which was the activator of iNOS.

It was observed that the levels of ET in Trx group decreased. There was no study about the relation between ET and Trx in the literature. This might be achieved by ROS inhibition and regulation of transcription factors of Trx. Cheng et al. showed that there was a relation between ET and ROS and also told that ROS played a role in the secondary messenger of the ET pathway [21].

It was shown that Trx cleared off H_2O_2 in the existence of methionine sulfoxide reductase or thioredoxin peroxidase [22]. This is the probable mechanism for protection against oxidative and nitrosative stress. Also, Trx activates the mitochondrial SOD and has protective role against NO-induced injury [23]. The data and our results supported the protective roles of Trx and L-NAME. On the other hand, Brown et al. showed that NO was bounded to the catalase with competition of H_2O_2 and degraded the enzyme catalase [24]. It was seen, in the study of Dobashi et al., that NO inhibited the catalase and SOD by decreasing the mRNA expression [25]. Our study supports the information taking place in the relevant literature.

MDA, which is a lipid peroxidation product, is used to show the level of oxidative stress. It was expected that lipid peroxidation products levels were found to be higher in the I/R group when compared with those in the sham group. Trx and L-NAME significantly decreased the MDA levels when compared with the ones in the I/R group. In the study of Wu et al., it was found that Trx, which was given in the second hour of I/R, significantly decreased the MDA levels when compared with the levels in the I/R with a ratio of 24.8% [26]. The similar protective role of Trx and L-NAME was observed in our study.

The results of apoptosis among groups were homogeneous, but the differences among groups were statistically significant. Trx has a potential for cell proliferation and cancer cell growth. Also, Trx had a protective role against the apoptosis which had been induced by ROS [27]. Reduced Trx formed a complex with signal regulatory kinase-1 (ASK-1) which protected the cell from apoptosis. In recent studies, it has been shown that NO acts as a radical and also forms peroxynitrite anion by reacting with ROS like superoxides and also increases the de novo transcription of p53 and stimulates apoptosis [28]. These findings showed us that Trx and L-NAME had anti-apoptotic roles in I/R.

When the results obtained are evaluated, it has been determined that with the application of recombinant Trx, hepatic (liver) cell injury, the formation of ROS and lipid peroxidation products, adhesion molecule release and apoptosis rate have all been declined. Simultaneously, it has been indicated that antioxidant enzymes and substances in hepatic cell level have increased. As a consequence of the inhibition of NOS with L-NAME, and prevention of excessive NO production, similar effects have been observed, too.

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