Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2012; 37 (4) ; 392–404.
doi: 10.5505/tjb.2012.68442

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Ekim, 2012 © TurkJBiochem.com

[Published online 30 October, 2012]

Effects of nitrite on hepatic ischemia-reperfusion injury

[Nitritin karaciğer iskemi-reperfüzyon hasarı üzerine etkileri]

Samiye Yabanoğlu Çiftçi[1],
Açelya Yalovaç[1],
Rumeysa Hekimoğlu[3],
Bülent Gümüşel[2],
Petek Korkusuz[3],
Gülberk Uçar[1]

Hacettepe University, Faculty of Pharmacy, Departments of [1]Biochemistry, [2]Pharmacology, [3]Faculty of Medicine, Department of Histology&Embryology, , Hacettepe University, 06100 Ankara, Türkiye

Yazışma Adresi
[Correspondence Address]

Prof. Dr. Gülberk UÇAR

Department of Biochemistry, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Türkiye
Tel. +903123052553
Fax. +903123114777
E-mail. gulberk@hacettepe.edu.tr

Registered: 2 May 2012; Accepted: 5 October 2012

[Kayıt Tarihi : 2 Mayıs 2012; Kabul Tarihi : 5 Ekim 2012]

ABSTRACT

Aim: Hepatic ischemia-reperfusion (IR) injury is a major complication associated with liver transplantation and surgery. The aim of this study is to investigate the cytoprotective effects of nitrite on hepatic IR injury.

Methods: Groups were arranged as follows: control, sham, IR protocol (45 minutes ischemia, 5 hours reperfusion) and study groups administered with 4 different concentrations of sodium nitrite given 12 and 24 hours prior to protocol. After IR protocol, serum transaminase and lactate dehydrogenase (LDH) enzyme activities were determined as quantitative indices of liver damage. Malondialdehyde (MDA) levels, glutathione levels and antioxidant enzyme activities were determined in liver tissues of all study groups. Additionally inflammatory and tissue remodeling processes were investigated. Histological evaluations were also performed.

Results: Serum transaminase and LDH activities were found to be increased in IR group compared to control group. Glutathione content and ratio of reduced glutathione to oxidized glutathione were found to be significantly decreased; oxidized glutathione and MDA content significantly increased in the IR group. All antioxidant enzyme activities were found to be lowered in IR group. TNF-α, IL-1β, IL-6, IL-8, IL-12, MMP-2 and MMP-9 levels were significantly increased in IR group compared to control group. In all nitrite administered groups, all parameters which were found to be changed with IR protocol were decreased to control levels depending on the concentration of nitrite. Total histological damage score at light microscope level was significantly higher in IR group. Nitrite improved histological damage scores by receiving similar scores with the control group.

Conclusion: Based on findings, nitrite administration was found to have cytoprotective effects on hepatic IR injury.

Key Words: Ischemia-reperfusion Injury, liver, mouse, nitrite.

Conflict of Interest: The authors declare that there was no conflict of interest in this work.

ÖZET

Amaç: Karaciğer iskemi-reperfüzyon (IR) hasarı, karaciğer transplantasyonu ve cerrahisiyle ilişkili önemli bir komplikasyondur. Bu çalışmanın amacı, nitritin karaciğer IR hasarındaki koyucu etkilerini araştırmaktır.

Yöntemler: Gruplar kontrol, sham, IR protokolü (45 dakika iskemi, 5 saat reperfüzyon) ve protokolden 12 ve 24 saat önce 4 farklı konsantrasyonda sodyum nitrit uygulanan çalışma grupları şeklinde belirlenmiştir. IR protoklünden sonra, karaciğer hasarının göstergeleri olan serum transaminaz ve laktat dehidrogenaz (LDH) enzim aktiviteleri belirlenmiştir. Tüm çalışma gruplarının karaciğer doku örneklerinde malondialdehit (MDA) seviyeleri, glutatyon seviyeleri ve antioksidan enzim aktiviteleri belirlenmiştir. Ek olarak enflamatuar ve doku yeniden düzenlenim süreçleri de araştırılmıştır. Sonuçlar histolojik bulgularla da desteklenmiştir.

Bulgular: Serum transaminaz ve LDH aktivitelerinin, kontrol grubuyla kıyaslandığında IR grubunda arttığı görülmüştür. IR grubunda redükte glutatyon içeriği ve redükte/okside glutatyon oranı belirgin biçimde azalmış; okside glutatyon ve MDA içeriklerinin belirgin biçimde arttığı bulunmuştur. IR grubunda tüm antioksidan enzim aktivitelerinin azaldığı gösterilmiştir. IR grubunda kontrol grubuna kıyasla TNF-α, IL-1β, IL-6, IL-8, IL-12, MMP-2 ve MMP-9 seviyeleri belirgin olarak artmıştır. Bütün nitrit uygulanan gruplarda, IR protokolüyle değiştiği belirlenen tüm parametreler, uygulanan nitrit konsantrasyonuna bağlı olarak azalmıştır. Işık mikroskobu seviyesinde toplam histolojik hasar skoru IR grubunda belirgin biçimde yüksekti. Nitrit, kontrol grubuyla benzer skorları göstererek, histolojik hasar skorunu iyileştirmiştir.

Sonuç: Bulgulara dayanarak, nitrit uygulamasının karaciğer IR hasarında koruyucu etkilerinin olduğu belirlenmiştir.

Anahtar Kelimeler: İskemi-reperfüzyon hasarı, karaciğer, fare, nitrit.

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Hepatic ischemia-reperfusion (IR) injury is a pathophysiological problem commonly observed in surgical procedures. Hepatic ischemia followed by reperfusion is resulted in tissue injury that contributes mainly to morbidity and mortality associated with shock, thermal injury, liver transplantation and resectional surgery [1]. The early phase of injury occurs in the absence of leukocyte infiltration and is thought to be initiated by a rapid change in the redox state of the tissue in favor of a more oxidative environment. The late phase of injury is dependent upon the production of several different cytokines and chemokines that promote the infiltration of large numbers of leukocytes into the tissue [3-5]. Extravasated polymorphonuclear leukocytes (PMNs) become metabolically activated and release large amounts of reactive oxygen species (ROS) together with extracellular matrix degrading enzymes such as collagenase and matrix metalloproteases [6]. The net result of this inflammatory infiltrate is an amplification of the acute injurious responses resulting in extensive inflammatory tissue injury.

Mitochondrial dysfunction is known to contribute to the progression of IR and a number of cytoprotective agents, including the protective ischemic preconditioning, mediate their action through mechanisms that converge at the level of the mitochondrion. NO is a well-characterized modulator of mitochondrial function and has been implicated in the signaling of ischemic preconditioning. Paradoxical reports have been published on effects of NO in IR injury. Some studies showed that NO worsens IR injury [7, 8] on the other hand some of them reported that NO has a protective role in IR. Various studies were performed concerning the exact cause of this paradoxical situation and it was reported that the concentration of released NO is important; high concentrations of NO may lead to the formation of reactive nitrogen species that enhance IR injury whereas lower concentrations of NO has protective effect [9-11]. The purpose of the present study was to evaluate the role of preconditioning with nitrite which is identified as an endocrine reservoir of NO [12, 13] in hepatic IR injury in mice and also to support findings with histological studies. Furthermore we aimed to determine effects of nitrite on antioxidant enzyme systems, cytokines and extracellular matrix degrading enzymes.

Material and Methods

Materials

All chemicals were purchased from Sigma Chemical Co. (Germany).

Liver IR Protocol in Mice

This study was approved by the Animal Ethics Committee of Hacettepe University (2006/15-13) and was performed in compliance with the Ethical Guidelines for Animal Studies. Study groups were randomly assigned as control, sham and IR protocol (Table 1). Each group consisted of 6 mice.

Male Albino mice (8–10 weeks) with body weights of 25–30 g were obtained from the Animal House of Hacettepe. The mice were maintained under conditions of a 12 hours light/dark cycle and had free access to food and water.

Partial mouse liver ischemia was performed according to the method of Abe *et al*. [14]. The median and left lateral lobes (approximately 70% of the liver) showed significant blanching after 45 minutes of ischemic period. Abdomen was closed and the animal was allowed to recover for 5 hours of reperfusion period**.** Immediately after the reperfusion period, the blood and tissues were collected from the anesthetized mouse. The right atrium was punctured and blood was aspirated with a syringe. Blood samples were centrifuged at 3500 x *g* for 10 min, and the obtained serum was stored at −20 °C. The hepatic lobes were promptly resected, weighted, washed in cold saline and kept at −80°C. Liver tissues were homogenized in cold potassium phosphate buffer (50 mM, pH 7.4); centrifuged at 3500 x *g* for 10 min at 4°C, and the supernatant was used for the determination of lipid peroxidation, glutathione content and antioxidant enzyme levels.

Determination of Serum Transaminase and LDH Activities

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined as

Table 1. Study groups of nitrite administration.

Group 1	Mice administered with 1.65 µg/kg sodium nitrite 12 hours prior to IR protocol
Group 2	Mice administered with 16.5 µg/kg sodium nitrite 12 hours prior to IR protocol
Group 3	Mice administered with 165 µg/kg sodium nitrite 12 hours prior to IR protocol
Group 4	Mice administered with 1650 µg/kg sodium nitrite 12 hours prior to IR protocol
Group 5	Mice administered with 1.65 µg/kg sodium nitrite 24 hours prior to IR protocol
Group 6	Mice administered with 16.5 µg/kg sodium nitrite 24 hours prior to IR protocol
Group 7	Mice administered with 165 µg/kg sodium nitrite 24 hours prior to IR protocol
Group 8	Mice administered with 1650 µg/kg sodium nitrite 24 hours prior to IR protocol

quantitative indices of liver damage according to the colorimetric method of Reitman [15]. Serum LDH activities were determined according to a method based on monitoring the conversion of NADH to NAD^+ at 340 nm [16]. Activities were expressed as $U.L^{-1}$.

Determination of Plasma and Tissue Nitrite and Nitrate Levels

Plasma and tissue nitrite and nitrate levels were determined according to a method based on Griess reaction [17]. Plasma nitrite and nitrate levels were expressed as $\mu mol.L^{-1}$ and tissue nitrite and nitrate levels were expressed as $nmol.g\ tissue^{-1}$.

Determination of Lipid Peroxidation in Liver Homogenates

Lipid peroxidation in liver tissues was determined according to the method of Ohkawa *et al*. [18] based on the reaction of MDA with thiobarbituric acid. Results were expressed as $nmol.mg^{-1}$.

Determination of Reduced and Oxidized Glutathione Levels

Reduced (GSH) and oxidized (GSSG) glutathione levels in liver tissues were determined according to the method of Sanchez-Alvarez *et al*. [19]. Glutathione levels were expressed as $nmol.mg^{-1}$.

Determination of Antioxidant Enzyme Activities in Liver Homogenates

Catalase (CAT) activities in liver tissues were determined according to the method of Ueda *et al*. [20] and activities were expressed as $nmol.mg^{-1}$. Glutathione S-transferase (GST) activities in liver tissues were determined spectrophotometrically by monitoring the formation of 1-chloro-2,4-dinitrobenzene (CDNB) and GSH conjugate at 340 nm [21] and activities were expressed as $U.mg^{-1}$. Glutathione reductase activities were determined according to the method of Carlberk and Mannevrik [22]; activities were expressed as $nmol.mg^{-1}$. Glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were determined by Glutathione Peroxidase (Cayman Chemicals, Ann Arbor, Mich.) and Superoxide Dismutase (Cayman Chemicals, Ann Arbor, Mich.) assay kits respectively and activities were expressed as $U.mg^{-1}$.

Determination of Myeloperoxidase Activities in Liver Homogenates

Myeloperoxidase (MPO) activities in liver tissues were determined according to the method of Ferreira *et al*. [23]. MPO activities were expressed as $U.mg^{-1}$.

Determination of Plasma Cytokine Levels

Plasma TNF-α and plasma IL-1β levels were determined by Mouse TNF-α ELISA Kit (BD BioSciences, USA) and Mouse IL-1β ELISA Kit (BD BioSciences, USA) respectively and expressed as $pg.ml^{-1}$. Plasma IL-6, IL-8 and IL-12 levels were determined by Mouse IL-6, IL-8 and IL-12 ELISA Kits (All from BD BioSciences, USA), respectively and expressed as $pg.ml^{-1}$.

Determination of Plasma MMP Levels

Plasma MMP-2 and MMP-9 levels were determined by MMP-2 and MMP-9 ELISA Kits (AbFrontier, Korea) and expressed as $ng.ml^{-1}$.

Light Microscopy on Paraffin Sections

Liver samples were fixed in phosphate buffered formalin and embedded in paraffin. Five to seven micrometer thick heamatoxylin & eosin and periodic acid schiff (PAS) stained sections were semi quantitatively evaluated with a Leica DMR microscope; DC500 digital camera by using Leica application suite (LAS) software (Westlar Germany). In brief 40 sections were examined in a blinded manner for each liver and a score from 0 to 3 or 4 was given to each liver section for the extent of sinusoidal congestion and hepatic cytoplasmic vacuolation infiltrating polymorphonuclear leukocytes, liver necrosis and

Table 2. Scoring System for Light Microscopy.

Score	Sinusoidal congestion and cytoplasmic vacuolation	Polymorphonuclear leukocyte infiltration	Liver necrosis and steatosis
0	No congestion and/or no cytoplasmic vacuolation in hepatocytes	None	Absence of necrosis and/or <5% steatosis
1	Mild (dilation of the centrolobular vein and/or cytoplasmic vacuolation in rare perivenular hepatocytes)	Rare cells	Spotty necrosis (scattered necrotic hepatocytes at zona 3) and/or mild (33%) steatosis
2	Moderate (dilation of the centrolobular vein plus sinusoidal dilation of zone 3 and/or cytoplasmic vacuolation of numerous perivenular hepatocytes)	Focal	Focal necrosis (periportal or perivenular or midacinar necrosis) and/or moderate (33% - 66%) steatosis
3	Severe (dilation of the centrolobular vein, sinusoidal dilation and/or cytoplasmic vacuolation of the hepatocytes of zone 3 and zone 2	Multi focal	Multifocal necrosis (necrosis in more than one acinar zone) and/or severe (>66%) steatosis
4	-	Diffuse and uniformly intense	-

steatosis according to Giovanardi et al [24] (Table 2).

Transmission Electron Microscopy on Plastic Sections

Liver samples were fixed in gluteraldehyde in Sorensons' phosphate buffer; post-fixed in osmium tetroxide and embedded in araldite (EMS, Germany). Semi-thin sections were stained with methylene blue-Azur II; thin sections were stained with uranyl acetate and lead citrate, analyzed on a transmission electron microscope (Jeol, JEM1400) attached Orius digital camera for acute tubular necrosis criteria. Ultrathin sections were examined in a blind manner (unaware of the groups). In order to check the presence of mitochondrial swelling, the width of the hepatocyte mitochondria was measured on at least 3 pictures, at five points on each picture, and the average values were taken. A total of minimum 100 mitochondria were measured for each sample [24, 25].

Statistical Analysis

Biochemical Data: All enzyme activities were expressed as mean ± SD of at least three independent experiments and analyzed by SPSS software for windows version 16.0. Mann-Whitney U test and Kruskal-Wallis were used for comparison of groups of the variables. Correlations between variables were assessed with Pearson's correlation coefficients (r), and $P<0.05$ was considered as statistically significant.

Histological Data: Independent variables were the groups and the dependent variables were the histology and biochemical blood test parameters for histological analysis. The normality of distribution was established using the Shapiro-Wilk test. All parameters were analyzed by nonoparametric tests Kruskal-Wallis was used for multiple comparison and Dunn as post-hoc test. Correlation between the histology and biochemical measurements was assessed by using Spearman test. Friedman test and its posthoc test were used to assess the difference in time. Descriptive statistical values were expressed as median, minimum and maximum. The difference was considered significant if $P<0.05$ in Dunn's test.

Results

Increased ALT, AST and LDH levels are typically considered as existence of acute hepatic injury. In our study deprivation of blood flow of liver in mice for 45 minutes followed by 5 hours of reperfusion caused a dramatic increase in liver injury as assessed by increases in serum ALT, AST and LDH levels. Serum ALT, AST and LDH levels were significantly increased in IR group compared to control and sham groups ($p<0.001$). Nitrite administration at 12 and 24 hours prior to IR protocol caused a dose dependent decrease in ALT, AST and LDH levels compared to IR group ($p<0.001$) (Table 3).

Plasma nitrite levels decreased in IR group compared to control and sham groups. Plasma nitrite levels of low dose nitrite administered study groups were similar with the physiological nitrite levels whereas in high doses of nitrite, plasma nitrite levels were increased (Table 4). Similarly, liver nitrite levels of IR groups decreased compared to control and sham groups while liver nitrite levels of sodium nitrite administered study groups, especially in high doses of nitrite, significantly increased (Table 4). Compared to control and sham groups, plasma and liver nitrate levels of IR group decreased while sodium nitrite administration at 12 and 24 hours prior to IR protocol caused a dose dependent significant increase in plasma and liver nitrate levels (Table 4).

MDA concentration, as an indice of lipid peroxidation, significantly increased in IR group when compared to the control and sham groups. Nitrite administration at 12 and 24 hours prior to IR protocol caused a dose dependent decrease in MDA levels compared to IR group (Table 5). Increased MDA levels following IR protocol indicate liver dysfunction. Nitrite administration decreased the MDA levels and liver functions were almost identical with control group.

GSH content and GSH/GSSG ratio significantly decreased whereas GSSG content significantly increased in IR group. As expected, with nitrite administration GSH content and GSH/GSSG ratio was found to be increased and GSSG content was found to be decreased compared to IR group (Table 5).

All antioxidant enzyme activities (GST, GR, GPx, CAT, SOD) significantly lowered in IR group compared to control and sham groups, possibly resulting from the depletion of the antioxidant pool, which is used to remove excess ROS produced during IR. Antioxidant enzyme activities of study groups, which were administered with sodium nitrite at 12 and 24 hours prior to IR protocol showed distinct increases, compared to IR group (Table 5).

MPO activities of liver tissue increased in IR group compared to control and sham groups. Besides, study groups of nitrite administration at 12 and 24 hours prior to IR protocol showed significant dose dependent decrease in MPO activity compared to IR group (Table 5).

Increased TNF-α, IL-1β, IL-6, IL-8 and IL-12 cytokine levels due to IR injury dose dependently decreased to control levels with nitrite administration. Plasma TNF-α, IL-1β, IL-6, IL-8 and IL-12 levels of IR group were found to be significantly increased compared to control and sham groups. Sodium nitrite administration at 12 and 24 hours prior to IR protocol caused a dose dependent decrease in plasma TNF-α, IL-1β, IL-6, IL-8 and IL-12 levels compared to IR group (Table 6).

According to our results, MMP-2 and MMP-9 protein levels are significantly increased following IR injury and they decreased to control levels with nitrite administration (Table 6).

Histology and Histomorphometry

At both light microscope (LM) and the electron micros-

Table 3: Serum ALT, AST and LDH activities as indices of liver damage in study groups (*** P<0.001 versus control, ΨΨΨ P<0.001 versus IR group). Each group consists of 6 mice and values represent the mean±SD of three independent experiments.

	ALT ($U.L^{-1}$)	AST ($U.L^{-1}$)	LDH ($U.L^{-1}$)
Control	45.40±0.52	51.53±0.50	486.70±20.01
SHAM	45.51±0.72	51.70±0.28	503.40±8.02
IR Group	5938.00±102.99 ***	6117.00±110.37 ***	6300.00±598.40 ***
Group 1	3477.00±146.06 □□□	5044.00±44.27 □□□	3627.00±900.90 □□□
Group 2	3004.11±107.15 □□□	3180.20±57.00 □□□	2054.00±98.53 □□□
Group 3	661.08±46.99 □□□	736.58±40.08 □□□	1093.00±176.10 □□□
Group 4	93.27±4.31 □□□	102.05±11.51 □□□	713.70±108.60 □□□
Group 5	1991.00±151.75 □□□	2204.00±116.87 □□□	2802.00±221.62 □□□
Group 6	1066.00±82.96 □□□	1194.00±88.60 □□□	1234.00±169.17 □□□
Group 7	295.90±8.86 □□□	314.30±9.48 □□□	725.50±138.00 □□□
Group 8	45.96±1.30 □□□	51.85±2.86 □□□	504.50±9.00 □□□

Table 4. Plasma and liver tissue nitrite and nitrate levels of control, sham, IR and study groups administered with sodium nitrite 12 and 24 hours prior to IR protocol (Values represent the mean±SD of three independent experiments; ***P<0.001, ** P<0.05, *P<0.01 versus control group; n=6).

	Plasma nitrite levels (µmol/L)	**Liver tissue nitrite levels (nmol/g tissue)**	**Plasma nitrate levels (µmol/L)**	**Liver tissue nitrate levels (nmol/mg tissue)**
Control	0.50±0.02	5.84±1.38	50.83±6.41	75.11±8.90
SHAM	0.50±0.13	5.82±2.01	53.10±12.00	74.27±9.67
IR Group	0.36±0.01**	3.52±0.56***	38.72±4.23**	42.84±9.01**
Group 1	0.46±0.05	4.72±0.18**	40.37±3.04**	54.76±10.08**
Group 2	0.53±0.06	4.82±0.27*	41.20±2.76**	77.58±9.26
Group 3	0.53±0.05	4.52±0.12**	42.18±3.02**	96.32±9.13**
Group 4	0.57±0.02**	4.19±0.22***	43.90±3.89**	144.3±10.07***
Group 5	0.52±0.03	5.32±0.59	53.10±4.05	74.83±12.48
Group 6	0.52±0.05	5.14±0.42	73.85±4.10***	91.91±16.01
Group 7	0.55±0.06	4.92±0.68*	87.99±6.61***	136.0±6.45***
Group 8	0.60±0.03***	4.81±0.42*	107.2±10.04***	194.5±18.23***

Table 5*. MDA levels, glutathione content, antioxidant enzyme activities and liver tissue MPO activities of control, sham, IR and study groups administered with sodium nitrite 12 hours prior to IR protocol (***P<0.001 versus control, ΨΨΨ P<0.001, ΨΨ P<0.01, Ψ P<0.05 versus IR, n=6)

Parameters	Control	SHAM	IR Group	Nitrite Administration 12 Hours Prior to IR Protocol				Nitrite Administration 24 Hours Prior to IR Protocol			
				Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7	Group 8
MDA (nmol.mg^{-1})	4.03±0.56	4.00±1.14	16.50±2.98***	14.00±2.35ΨΨ	10.00±2.20ΨΨΨ	8.34±2.12ΨΨΨ	6.25±1.12ΨΨΨ	12.00±2.50ΨΨ	8.45±1.02ΨΨΨ	7.00±1.12ΨΨΨ	5.80±0.86ΨΨΨ
GSH (nmol.mg^{-1})	73.00±12.01	72.80±11.32	30.26±14.86***	42.55±5.00ΨΨ	49.30±8.05ΨΨΨ	56.80±9.20ΨΨΨ	66.12±8.87ΨΨΨ	41.33±5.90ΨΨΨ	52.33±8.00ΨΨΨ	66.70±9.22ΨΨΨ	72.15±12.00ΨΨΨ
GSSG (nmol.mg^{-1})	1.30±0.13	1.31±0.11	8.94±12.01***	7.00±1.20Ψ	5.23±1.02ΨΨ	4.06±2.91 ΨΨ	1.40±0.54ΨΨΨ	6.50±1.20ΨΨΨ	4.20±0.70ΨΨΨ	2.90±0.21ΨΨΨ	1.32±0.15ΨΨΨ
GSH/GSSG	56.55±6.24	55.60±8.91	2.42±0.66***	6.20±0.81ΨΨΨ	9.23±1.51ΨΨΨ	14.20±3.01 ΨΨΨ	47.14±5.39 ΨΨΨ	6.36±1.07ΨΨΨ	12.44±2.80ΨΨΨ	22.80±5.10ΨΨΨ	55.40±9.21ΨΨΨ
CAT (nmol.mg^{-1})	69.90±8.74	69.00±12.00	30.12±6.00***	38.60±5.56ΨΨ	45.00±8.93 ΨΨΨ	55.22±5.88 ΨΨΨ	63.00±11.40 ΨΨΨ	37.00±5.80Ψ	44.00±9.30ΨΨΨ	58.08±9.40ΨΨΨ	68.70±11.75ΨΨΨ
GST (U.mg $^{-1}$)	3.78±0.53	3.80±0.57	1.10±0.23***	1.53±0.25Ψ	1.77±2.66ΨΨ	2.02±0.45 ΨΨ	2.29±0.31 ΨΨΨ	1.60±1.13Ψ	2.20±0.54ΨΨ	3.00±0.61ΨΨΨ	3.77±0.54 ΨΨΨ
GR (nmol.mg^{-1})	31.45±5.67	30.93±5.00	14.26±2.79***	18.50±2.70Ψ	21.88±6.00 ΨΨΨ	25.03±5.30ΨΨΨ	28.00±5.56ΨΨΨ	19.00±3.00ΨΨΨ	23.27±2.97ΨΨΨ	25.33±3.00ΨΨΨ	29.80±6.02ΨΨΨ
GPx (U.mg $^{-1}$)	1.31±0.23	1.30±0.11	0.35±0.06***	0.50±0.05Ψ	0.66±0.13 ΨΨ	0.87±0.05ΨΨ	1.26±0.16 ΨΨΨ	0.48±0.17ΨΨ	0.60±0.11ΨΨΨ	0.99±1.05ΨΨ	1.29±0.14 ΨΨΨ
SOD (U.mg $^{-1}$)	29.80±2.18	28.70±3.00	10.90±8.10***	14.22±3.00ΨΨ	18.00±2.88 ΨΨΨ	20.00±3.01ΨΨΨ	25.77±2.20 ΨΨΨ	11.40±3.00 Ψ	15.00±2.61ΨΨΨ	19.00±2.20ΨΨΨ	28.60±2.90ΨΨΨ
MPO (U.mg^{-1} protein)	0.57±0.03	0.61±0.15	5.21±0.24***	3.98±0.11ΨΨ	2.23±0.61ΨΨΨ	1.03±0.45 ΨΨΨ	0.84±0.11 ΨΨΨ	2.90±0.88 ΨΨΨ	1.93±0.16 ΨΨΨ	0.89±0.18ΨΨΨ	0.59±0.21 ΨΨΨ

* IR group was compared statistically with control group and each study group (Group 1-8) was compared with IR Group.

Table 6. TNF-α, IL-1β, IL-6, IL-8, IL-12, MMP-2 and MMP-9 levels of control, sham, IR and study groups administered with sodium nitrite 12 and 24 hours prior to IR protocol (*** P<0.001 versus control; ΨΨΨ P<0.001 versus IR, n=6).

	TNF-□ ($pg.ml^{-1}$)	IL-1□ ($pg.ml^{-1}$)	IL-6 ($pg.ml^{-1}$)	IL-8 ($pg.ml^{-1}$)	IL-12 ($pg.ml^{-1}$)	MMP-2 ($ng.ml^{-1}$)	MMP-9 ($ng.ml^{-1}$)
Control	0.46±0.18	0.3467±0.128	9.53±1.29	158.1±17.41	60.64±8.35	0.03±0.009	0.02±0.002
Sham	0.50±0.05	0.3000±0.11	9.22±2.93	155.1±13.59	59.61±25.71	0.03±0.02	0.02±0.005
IR Group	1028±301.30***	308.4±26.51***	687.3±167.23***	1593±152.69***	1134±204.38***	35.34±12.54***	33.70±5.31***
Group 1	465.8±87.43□□□	136.1±32.75□□□	379.7±35.47□□□	902.0±87.31□□□	735.0±104.27□□□	14.60±5.21□□□	10.40±2.85□□□
Group 2	192.0±65.23□□□	71.48±7.51□□□	190.6±57.21□□□	435.0±57.34□□□	454.8±54.62□□□	5.72±1.84□□□	3.59±0.59□□□
Group 3	16,55±2.87□□□	9,22±1.34□□□	99,13±15.34□□□	251.7±38.18□□□	229.5±113.28□□□	1.54±0.27□□□	1.33±0.12□□□
Group 4	1.27±0.42□□□	0.63±0.21□□□	19.51±7.49□□□	175.9±9.38□□□	70.35±8.35□□□	0.09±0.021□□□	13.54±28.53□□□
Group 5	383.1±27.36□□□	101.1±43.12□□□	370.7±147.87□□□	680.3±31.79□□□	643.7±149.17□□□	9.99±0.86□□□	7.26±1.26□□□
Group 6	90.54±19.34□□□	41.26±5.67□□□	115.1±28.51□□□	337.7±34.75□□□	273.4±62.73□□□	3.30±0.59□□□	2.37±0.49□□□
Group 7	8.70±1.85□□□	6.83±2.97□□□	64.00±16.41□□□	216.7±32.57□□□	109.3±15.32□□□	0.99±0.17□□□	0.95±0.13□□□
Group 8	0.59±0.12□□□	0.33±0.09□□□	12.11±2.95□□□	158.0±8.51□□□	56.33±7.93□□□	0.04±0.007□□□	0.02±0.002□□□

cope (EM) level, liver samples exhibited mild to moderate alterations in all experimental groups. IR injury consisted of mild to moderate sinusoidal congestion with the dilation of centrolobular vein and the sinusoids; the cytoplasmic vacuolization or blebbing of hepatocytes, focal polymorphonuclear leukocyte infiltration and mild spotty liver necrosis and steatosis. IR group received significantly (P<0.05) higher score (Median:3; min,max: 2,3) for sinusoidal congestion and the hepatocytic cytoplasmic vacuolization when compared to control/sham group (Median:0; min,max: 0,1). Individual cell necrosis with loss of nuclei was observed in perivenular hepatocytes of IR groups. Control and sham groups exhibited healthy morphology in all samples. High doses (165 μg/kg of 12 hours, 165 and 1650 μg/kg of 24 hours) of nitrite administered livers received similar histological scores regarding the sinusoidal congestion and the cytoplasmic vacuolization when compared to control. IR group received higher sinusoidal congestion (Median:3; min,max: 2,3) and hepatocyte cytoplasmic vacuolization scores comparing to that of the high doses (1650 μg/kg) of nitrite administered groups of both 12 and 24 hours (For 12 hours Median:1; min,max: 1,2; For 24 hours Median:1; min,max: 1,2).

The infiltration of polymorphonuclear leukocytes observed in the sinusoids was statistically (P<0.05) higher in IR group (Median:2; min,max: 2,3) compared to that of control/sham group (Median:0; min,max: 0,0). The leukocyte infiltration was significantly decreased in the high doses (1650 μg/kg of 12 hours, 165 and 1650 μg/kg of 24 hours) of nitrite administered group receiving statistically similar scores with control/sham group (For 1650 μg/kg of 12 hours Median:1; min,max: 1,2; For 165 μg/kg of 24 hours Median:1; min,max: 1,2; For 1650 μg/kg of 24 hours Median:1; min,max: 1,1). The nitrite administration improved PMNs infiltration score in a dose dependent manner (almost all doses) of 12 and 24 hours comparing to IR group (Median:2; min,max: 2,3).

Minimum spotty or focal necrosis and steatosis was observed in IR group (Median:1; min,max: 1,2) and nitrite administered IR groups. The necrosis and steatosis scores were improved within some nitrite administered groups 12 or 24 hours before IR protocol (For 1650 μg/kg nitrite of 12 hours Median:0; min,max: 0,1; For 1650 μg/kg nitrite at 24 hours Median:0; min,max: 0,1). Some nitrite administered groups received significantly (P<0.05) lower steatosis and necrosis scores compared to IR group (For 1.65 μg/kg nitrite of 12 hours Median:1; min,max: 0,1; For 1650 μg/kg nitrite at 12 hours Median:0; min,max: 0,1; For 165 μg/kg nitrite of 24 hours Median:0; min,max: 0,1; For 1650 μg/kg nitrite of 24 hours Median:0; min,max: 0,1).

The total histological damage score at LM level was significantly (P<0.05) higher in IR group (Median:6; min,max: 5,8) when compared to that of the control/sham (Median:0; min,max: 0,1). Nitrite improved histological damage scores by receiving statistically similar scores with the control/sham at both 12 and 24 hours. 165 and 1650 μg/kg of nitrite administered before 12 hours and all of the nitrite doses (from 1.65 to 1650 μg/kg) that were administered before 24 hours significantly (P<0.05) improved the total histological scores comparing to those of IR group (For 165 μg/kg nitrite of 12 hours Median:4; min,max: 3,5; For 1650 μg/kg nitrite of 12 hours Median:2; min,max: 2,5; For 1.65 μg/kg of nitrite of 24 hours Median:4; min,max: 3,5; For 16.5 μg/kg nitrite of 24 hours Median:4; min,max: 4,6; For 165 μg/kg of nitrite at 24 hours Median:3; min,max: 2,5; For 1650 μg/kg of nitrite

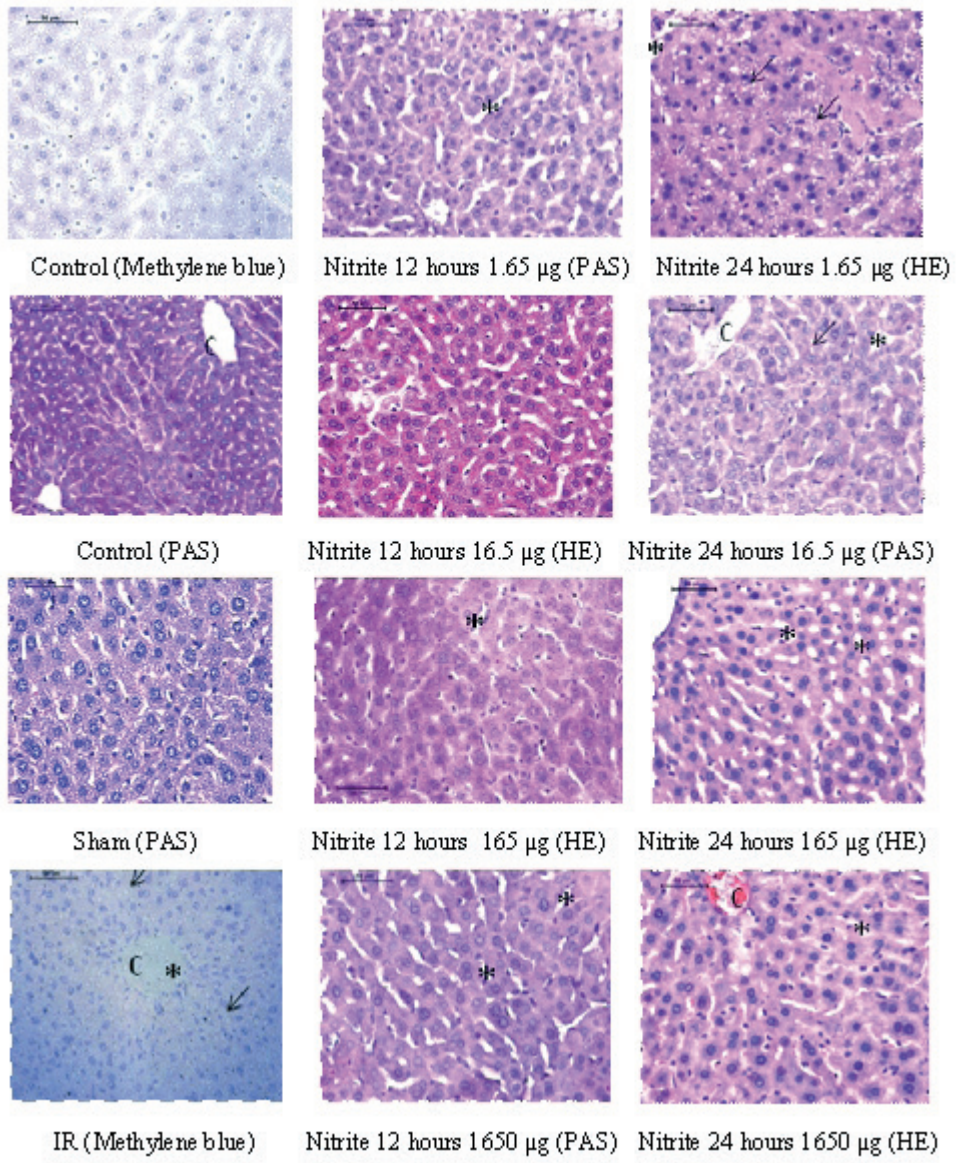

Figure 1. Light microscope images of liver tissues from control, sham, IR and nitrite administered study groups. Control and sham sections represented healthy images. In all IR protocol groups, mild
to moderate sinusoidal dilatation and congestion (asterisk) and hepatocyte vacuolization (arrow) were observed. It was detected that injury was improved with high dose nitrite administration (C: Central vein. HE: Hematoxylen eozin, PAS: Periodic asid schiff).

at 24 hours Median:2.5; min,max: 2,3) (Figure 1).

At the EM level, all of the study groups exhibited different ranges of mitochondrial swelling comparing to that of the control/sham group. Although the mitochondrial membrane was generally intact in all groups; loss of cristae and a decrease in matrix density were prominent in IR group. The lesser mitochondrial diameter observed in this study was significantly increased in IR group relative to control/sham group ($P<0.05$). The hepatocyte mitochondria exhibited a healthy ultra structure with an orderly mannered arrangement in control/sham groups. 1.65 and 1650 µg/kg of nitrite administration both before 12 and 24 hours and the 165 µg/kg of nitrite administration before 24 hours improved the mitochondrial ultra structure by receiving statistically similar lesser diameters relative to the control/sham group (For 1.65 µg/kg of nitrite of 12 hours Median:0.9; min,max: 0.6,1.1; For 1.65 µg/kg of nitrite 24 hours Median:0.9; min,max: 0.7,1.1; For 1650 µg/kg of nitrite of 12 hours Median:0.5; min,max: 0.1,0.8; For 1650 µg/kg of nitrite of 24 hours Median:0.6; min,max: 0.4,0.8; For 165 µg/kg nitrite of 24 hours Median:0.9; min,max: 0.5,1). Both the 165 and 1650 µg/kg nitrite administered groups 24 hours before and the 1650 µg/kg nitrite administered group 12 hours before received significantly better scores related to mitochondrial swelling when compared to IR group (Figure 2).

Individual cell shrinkage with nuclear fragmentation (apoptosis) and/or necrotic degenerative cellular changes

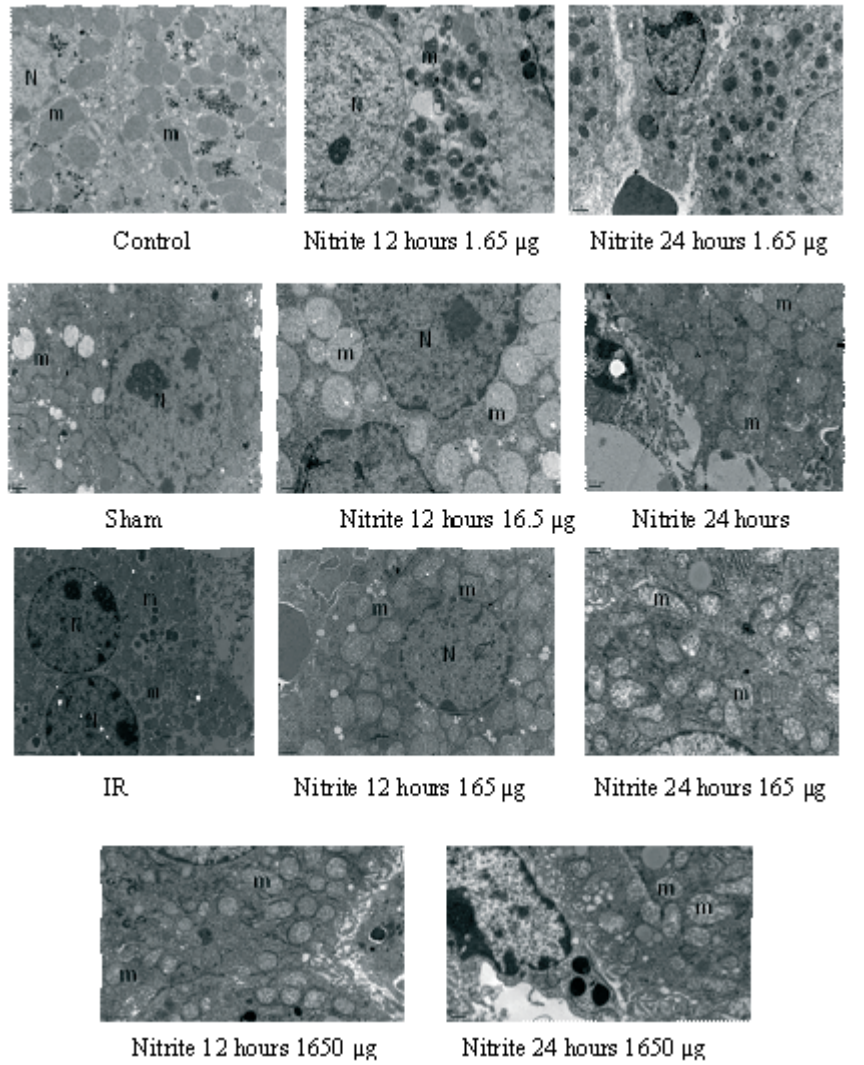

Figure 2. Electron microscope images of liver tissues from control, sham, IR and nitrite administered study groups. In all IR groups significant swelling in hepatocyte mitochondria (m) and deletion of mitochondria membranes are observed. Hepatocyte nucleus (N) seems healthy in all groups. In nitrite administered groups, less mitochondrial swelling and conserving mitochondria cristas are observed.

could be observed in some of the individual hepatocytes belonging to IR groups. However these degenerative changes did not uniformly involve all of the hepatic lobules and zones within the same lobule. Especially high doses of nitrite administered groups revealed several healthy lobules.

Evaluation of the Correlation Between Biochemical and Histological Measurements:

AST and ALT levels exhibited positive correlations with the sinus congestion and hepatocyte cytoplasmic vacuolization (r=0.583, P=0.00 for AST and r=0.592, P=0.00 for ALT), PMNs infiltration (r=0.486, P=0.00 for ALT and r=0.519, P=0.00 for AST respectively), hepatic steatosis and necrosis (r=0.351, p=0.00 for ALT and r=0.379, P=0.00 for AST). The total LM histological score that is received by making the sum of the three histological measurements gave again a positive correlation with the AST and ALT levels (r=0.557, P=0.00 for ALT and r=0.587, P=0.00 for AST). The mitochondrial width measurement at the EM level revealing the mitochondrial damage exhibited a positive correlation with the biochemical ALT and AST levels (r=0.477, P=0.00 for ALT and r=0.532, P=0.00 for AST).

Discussion

Hepatic IR injury is an important problem in clinical conditions such as liver surgery for tumor excision, transplantation, trauma and liver failure accompanying hemorrhagic shock [26, 27]. Blood flow needs to be cut off partially or completely during liver surgery [28]. During this period, secretion of intracellular enzymes, calcium uptake into cells, lipid peroxidation in biological membranes and finally necrosis occur [29]. Various mechanisms such as rapid formation of ROS, PMNL activation, endothelial and complement system activation take part in injury observed during reperfusion period [30]. Membrane lipids, proteins and nucleic acids are

shown to be the most sensitive components to reperfusion injury. But it is known that this process includes local and systemic inflammatory response and causes local and systemic permanent damage [31]. Ischemic preconditioning of an organ may induce protection against the injury caused by longer duration of ischemia and subsequent reperfusion. Since the cellular mechanisms of ischemic preconditioning have been elicited, researchers intend to mimic the effects of this phenomenon by chemical agents and generate a pharmacological conditioning [32].

NO is an important mediator, which can be synthesized by most of the cell types and takes part in several physiological and pathological processes in the body. It has been shown that NO, NO donors, NO activation or transgenic over expression of NO have protective effects on various IR models [33, 34]. NOS-independent NO resources are known to play a critical role in protection during ischemia or hypoxia and recent studies have reported that inorganic nitrite can be good NO resource [35, 36]. As nitrite reduces to NO enzymatically or non-enzymatically during hypoxia or acidosis it can be used as a new and efficient treatment to replace NO in disorders related to tissue ischemia. Studies supporting this idea have reported that administration of nitrite in physiological concentrations can protect tissues in vivo in hepatic and cardiac IR injury and nitrite-derived NO plays an important role in this cytoprotective effect [36]. Similarly, nitrite is reported to be efficient in hypoxia-induced pulmonary vasoconstriction and haemorrhagic shock model and hypochloric acid-induced chondrocyte toxicity in isolated cardiac IR injury model [37]. Despite the studies regarding the protective effects of nitrite administration before ischemia on hepatic IR injury in recent years [38, 39] the mechanism of this effect is still controversial. In this study, protective effects of nitrite treatment against hepatic IR injury, in 4 different concentrations administered 12 and 24 hours before ischemia, were evaluated.

Nitrite is a natural oxidation product of NO and can be reduced back to NO under hypoxic conditions [40]. Plasma concentration of nitrite varies between 300 nM and 500 nM in different species [41]. Tissue nitrite concentration varies between 0.5 μM and 20 μM [42]. Thus the nitrite dose that will be administered for increasing its plasma and tissue concentration should be carefully decided and it was determined that the administered dose may not be observed directly in plasma. Consequently, in this study plasma nitrite and nitrate levels were determined both in IR and nitrite administered groups.

Increased ALT and AST levels are accepted as indices of liver injury. In nitrite administered groups, ALT and AST levels were decreased to control group levels in a dose dependent manner. Furthermore, the effect of nitrite administration 24 hours before IR injury on ALT and AST levels were determined to be more effective compared to administration 12 hours before IR injury. These results were supported with histological analyzes and LDH enzyme level which is another indices of liver injury. Lipid peroxidation in liver tissue was determined by MDA level, which is a product of lipid peroxidation. Increased MDA levels following IR injury have indicated hepatic dysfunction. Nitrite administration caused a decrease almost to control levels. Reducing effect of nitrite administration on ALT, AST, LDH and MDA levels indicated that nitrite administration has a protective effect against ischemia in a dose dependent manner.

GSH depletion in IR is thought to be observed as a result of consumption of free radicals by GSH.

Neutrophils and monocytes contain MPO, a heme enzyme, in their primary lysosomal granules. This enzyme is in communication with oxygen dependent mechanisms. In presence of MPO, formed peroxide and chloride ions are converted into hypochloric acid. Leukocyte activation and inflammatory mediators that are triggered by IR injury cause tissue edema and increase vascular permeability. Our results show that nitrite administration prevents neutrophil activation and infiltration and reduce the inflammatory response. Possible mechanism for this effect may be the decrease in ROS production as a result of nitrite administration. Hence, H_2O_2 and ·OH will not be formed; chloride ions will not be converted into hypochloric acid and inflammation will not be triggered. Furthermore, antioxidant enzyme activities such as CAT, GST, GR, GPx and SOD and GSH levels were found to be decreased after hepatic IR protocol in mice compared to control group; and nitrite administration before ischemia were determined to cause an increase in antioxidant enzyme activities. In nitrite administered study groups, lipid peroxidation and GSSG levels were found to be decreased; GSH levels, GSH/GSSG ratio, CAT, GST, GR, GPx and SOD activities were found to be increased to control levels. Therefore the protective effect of nitrite can be thought to be related to ROS production mechanisms and antioxidant enzyme levels. Rapid increase in hepatic GSH levels during hepatic IR injury was suggested to be a result of neutralization effect of GSH on ROS.

Cytokines play important role in IR injury by inducing, mediating and determining severity of inflammatory response [43, 44]. These cytokines increase the expression of adhesion molecules and lead to leucocyte-sinusoidal endothelial cell interaction [45, 46], which result in increase in cytokine production. TNF-α is a cytokine, which is known to play a role in cellular injury in IR [6]. IL-12 mediates leucocyte activation [47] and acts as a primary trigger of cytokine cascade induced by hepatic IR injury. Increased IL-1 levels were reported following hepatic vascular occlusion performed for partial hepatechtomy in human [48]. Furthermore, isolated rat Kupffer cells are known to maintain IL-1 secretion spontaneously after reperfusion injury [49]. IL-6 induces generation of acute phase proteins such as serum amyloid A, C-reactive protein, complement C3, fibrinogen and

macroglobulin in hepatocytes [50]. There are inconsistent reports regarding the role of IL-6 during inflammatory process. Although some researchers have reported that liver regeneration is failed in IL-6 gene knock-out mice [51, 52], others have claimed that IL-6 has a protective effect on hepatic injury during liver regeneration. Pro-inflammatory mediators such as TNF-α, which appear during the early phase of liver injury, induce CXC chemokine formation [53-56]. IL-8 is a CXC chemokine and has a role on neutrophil recruitment to the injured tissue during IR [57]. All studies reporting the roles of cytokines on IR injury support that the inflammatory process before or after injury is complicated. Therefore various anti-inflammatory treatment strategies were developed for administration after liver transplantation to relieve the inflammatory process [58]. Effects of nitrite on these cytokine levels were determined in this study. Increased TNF-α, IL-1β, IL-6, IL-8 and IL-12 cytokine levels due to IR injury decreased dose dependently to control levels with nitrite administration. This result proved that the cytoprotective effect of nitrite is not only over antioxidant systems but also on inflammatory processes. Consistent with other results, response to nitrite is more effective when nitrite is administered before 24 hours of ischemia compared to 12 hours. On the basis of our results it is determined that nitrite has cytoprotective effect not only on antioxidant systems but also has an effect on inflammatory process.

Following the inflammatory process observed during tissue injury, fibrosis is observed. MMPs and their tissue inhibitors TIMPs are basic enzymes that have role in this fibrosis process. Studies showed that MPPs have important roles on IR injury [59]. Fibrosis is believed to occur as a result of imbalance between MMPs and TIMPs [60]. MMPs are zinc-dependent endopeptidases and belong to an enzyme family of proteases. They play a role in cell response to their microenvironment. The most important response is the ability of degrading extracellular matrix components [61]. In this study, MMP levels in hepatic IR injury performed in mice and effects of nitrite administration on these levels were determined. MMP-2 and MMP-9 are important for liver due to their degradation ability of collagen IV and fibronectin, two important components of space of disuse [62-64].

MMP-2 and MMP-9 are expressed not only by non-parenchymal liver cells such as Kupffer cells or sinusoidal endothelial cells but also by hepatocytes [65, 66]. Our findings suggest that nitrite administration before ischemia has a protective effect also via extracellular matrix components.

In summary, our results clarified that a significant ROS-mediated tissue injury occurs during hepatic IR and nitrite administration either 12 or 24 hours before ischemia has a protective effect against injury. This is the only study that considers effects of nitrite preconditioning on antioxidant enzyme activities and at the same time on cytokine and MMP levels in the same group of mice.

Acknowledgement

The study was supported by Hacettepe University, Scientific-Research Foundation (Project number: HUBAB 06-01301-003)

Conflict of Interest: The authors declare that there was no conflict of interest in this work.

References

[1] Carden DL, Granger DN. Pathophysiology of ischemia-reperfusion injury. J Pathol 2000; 190:255-266.

[2] Fan C, Zwacka RM, Engelhardt JF. Therapeutic approaches for ischemia/reperfusion injury in the liver. J Mol Med 1999; 77:577–592.

[3] Jaeschke J. Mechanisms of reperfusion injury after warm ischemia of the liver. J Hepatobiliary Pancreat Surg 1998; 5:402–408.

[4] Zwacka RM, Zhou W, Zhang Y, Darby CJ, Dudus L, *et al*. Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-kappaB activation. Nat Med 1998; 4:698–704.

[5] Husted TL, Lentsch AB. The role of cytokines in pharmacological modulation of hepatic ischemia/reperfusion injury. Curr Pharm Des 2006; 12:2867–2873.

[6] Jaeschke H, Farhood A. Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 1991; 260:G355-362.

[7] Glantzounis GK, Rocks SA, Sheth H, Knight I, Salacinski HJ, *et al.* Formation and role of plasma S-Nitrosothiols in liver ischemia-reperfusion injury. Free Radic Biol Med 2007; 42:882-892.

[8] Suzuki Y, Deitch EA, Mishima S, Lu Q, Xu D. Inducible nitric oxide synthase gene knockout mice have increased resistance to gut injury and bacterial translocation after an intestinal ischemia-reperfusion injury. Crit Care Med 2000; 28:3692-3696.

[9] Chen T, Zamora R, Zuckerbraun B, Biliar TR. Role of nitric oxide in liver injury. Curr Mol Med 2003; 3:519-526.

[10] Ferdinandy P, Schulz R. Nitric oxide, superoxide and peroxynitrite in myocardial ischemia-reperfusion inury and preconditioning. Br J Pharmacol 2003; 138:532-543.

[11] Hon WM, Lee KH, Khoo HE. Nitric oxide in liver diseases: friend, foe or just passerby? Ann N Y Acad Scie 2002; 962:275-295.

[12] Siriussawakul A, Zaky A, Lang JD. Role of nitric oxide in hepatic ischemia-reperfusion injury. World J Gastroenterol 2010; 16(48):6079-86.

[13] Jensen FB. The role of nitrite in nitric oxide homeostasis: A comparative perspective. Biochim Biophys Acta 2009; 1787(7):841-848.

[14] Abe Y, Hines IN, Zibari G, Pavlick K, Gray L, *et al*. Mouse model of liver ischemia and reperfusion injury: method for studying reactive oxygen and nitrogen metabolites in vivo. Free Radic Biol Med 2009; 46(1):1-7.

[15] Reitman S, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. Am J Clin Pathol 1957; 28:56-63.

[16] Bauer JD. Clinical laboratory methods. 1982; 576, Ninth ed. St Louis: The C.V. Mosby Company.

[17] Moshage H, Kok B, Huizenga JR, Jansen PLM. Nitrite and Nitrate Determinations in Plasma: A Critical Evaluation. Clin Chem 1995; 41(6):892-896.

[18] Okhawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95:351–8.

[19] Sanchez-Alvarez R, Almeida A, Medina JM. Oxidative

stress in preterm rat brain is due to mitochondrial dysfunction. Pediatr Res 2002; 51: 34-39.

[20] Ueda M, Mozaffar S, Tanaka A. Catalase from Candida boidinii 2201, Methods Enzymol 1990; 188:463-465.

[21] Jakoby WB. Glutathione transferases: An overview. Methods Enzymol 1980; 113:495-504.

[22] Carlberg I, Mannervik B. Glutathione reductase. Methods Enzymol. 1985; 113:484-490.

[23] Ferreira M, Barcelos LS, Teixeira MM, Bakhle YS, Andrade SP. Tumor growth, angiogenesis andinflammation in mice lacking receptors for platelet activating factor (PAF). Life Sci 2007; 81(3):210–217.

[24] Giovanardi RO, Rhoden EL, Cerski CT, Salvador M, Kalil AN. Pharmacological preconditioning using intraportal infusion of L-arginine protects against hepatic ischemia reperfusion injury. J Surg Res 2009; 155:244-253.

[25] Jin C, Zhang PJ, Wu XM, Zhou B, Li Y, *et al*. Impact of hypoxic preconditioning on apoptosis and its possible mechanism in orthotopic liver autotransplantation in rats. Hepatobiliary Pancreat Dis Int 2009; 8:40-45.

[26] Lemasters JJ, Thurman RG. Reperfusion injury after liver preservation for transplantation. Annu Rev Pharmacol Toxicol 1997; 37:327-338.

[27] Olthoff KM. Can reperfusion injury of the liver be prevented? Trying to improve on a good thing. Pediatr Transpl 2001; 5:390-393.

[28] Hasselgren PO. Prevention and treatment of ischemia of the liver. Surg gynecol obstet 1987; 164:187-196.

[29] Serracino-Inglott F, Habib NA, Mathie RT. Hepatic Ischemia-reperfusion injury. Am J Surg 2001; 8:160-166.

[30] Zimmerman BJ, Granger DN. Reperfusion injury. Surl Clin North Am 1992; 72:65-83.

[31] Wilhelm J. Metabolic aspects of membrane lipid peroxidation. Acta Univ Carol Med Monogr 1990; 137:1-53.

[32] Hardy KJ, McClure DN, Subwongcharoen S. Ischemic preconditioning of the liver: a preliminary study. ANZ J Surg 1996; 66:707-710.

[33] Jones SP, Greer JJM, Haperen R, Duncker DJ, Crom R, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. Proc Natl Acad Sci U S A 2003; 100(8): 4891-4896.

[34] Kanno S, Lee PC, Zhang Y, Ho C, Griffith BP, *et al*. Attenuation of myocardial ischemia/reperfusion injury by superinduction of inducible nitric oxide synthase. Circulation 2000; 101:2742-2748.

[35] Bryan NS, Calvert JW, Gundewar S, Ji SY, Lefer DJ. Dietary nitrite supplementation protects against myocardial ischemia-reperfusion injury. Proc Natl Acad Sci 2007; 104(48):19144-19149.

[36] Duranski MR, Greer JJ, Dejam A, Jaganmohan S, Hogg N, *et al*. Cytoprotective effects of nitrite during in vivo ischemia–reperfusion of the heart and liver. J Clin Invest 2005; 115(5):1232-1240.

[37] Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia–reperfusion damage. Proc Natl Acad Sci U S A 2004; 101:13683-13688.

[38] Phillips L, Lopez-Neblina F, Toledo-Pereyra LH. Nitric Oxide Mechanism of Protection in Ischemia and Reperfusion Injury. J Invest Surg 2009; 22:46-55.

[39] Elrod JW, Calvert JW, Gundewar S, Bryan NS, Lefer DJ. Nitric oxide promotes distant organ protection: evidence for an endocrine role of nitric oxide. Proc Natl Acad Sci 2008; 105(32):11430-11435.

[40] Lauer T, Preik M, Rassaf T, Strauer BE, Deussen A, *et al*. Plasma nitrite rather than nitrate refletcs regional endothelial nitric oxide synthase activity but lacks intrinsic vasodilator action. Proc Natl Acad Sci U S A 2001; 98:12814-12819.

[41] Kleinbongard P, Dejam A, Lauer T, Rassaf T, Schindler A, *et al*. Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. Free Radic Biol Med 2003; 35(7):790-796.

[42] Bryan NS, Rassaf T, Maloney RE, Rodriguez CM, Saijo F, *et al*. Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics in vivo. Proc Natl Acad Sci 2004; 101(12):4308-4313.

[43] Fong Y, Moldawer LL, Shires GT, Lowry SF. The biologic characteristics of cytokines and their implication in surgical injury. Surg Gynecol Obstet 1990; 170:363-378.

[44] Strieter RM, Kunkel SL, Bone RC. Role of tumor necrosis factor-alpha in disease states and inflammation. Crit Care Med 1993; 21:447-463.

[45] Pober JS, Warner-Lambert/Parke-Davis award lecture. Cytokine-mediated activation of vascular endothelium. Physiology and pathology. Am J Pathol 1988; 133:426-433.

[46] Thornton AJ, Strieter RM, Lindley I. Cytokine-induced gene expression of a neutrophil chemotactic factor/IL-8 in human hepatocytes. J Immunol 1990; 144:2609-2613.

[47] Harada N, Okajima K, Uchiba M. Dalteparin, a low molecular weight heparin, attenuates inflammatory responses and reduces ischemia-reperfusion-induced liver injury in rats. Crit Care Med 2006; 34(7):1883-1891.

[48] Clavien PA, Camargo CA, Gorczynski R. Acute reactant cytokines and neutrophil adhesion after warm ischemia in cirrhotic and non-cirrhotic human livers. Hepatology 1996; 23:1456-1463.

[49] Teoh NC, Farrell GC. Hepatic ischemia reperfusion injury: Pathogenic mechanisms and basis for hepatoprotection. J Gastroenterol Hepatol 2003; 18:891–902.

[50] Ramadori G, Christ B. Cytokines and the hepatic acute-phase response. Semin liver dis 1999; 19:141-155.

[51] Cressman DE, Greenbaum LE, DeAngelis RA, Ciliberto G, Furth EE, *et al*. Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. Science 1996; 274:1379-1383.

[52] Sakamoto T, Liu Z, Murase N, Ezure T, Yokomuro S, *et al*. Mitosis and apoptosis in the liver of interleukin-6-deficient mice after partial hepatectomy. Hepatology 1999; 29:403-411.

[53] Colletti LM, Kunkel SL, Walz A, Burdick MD, Kunkel RG, *et al*. Chemokine expression during hepatic ischemia/reperfusion-induced lung injury in the rat. The role of epithelial neutrophil activating protein. J Clin Invest 1995; 95:134-141.

[54] Lentsch AB, Yoshidome H, Cheadle WG, Miller FN, Edwards MJ. Chemokine involvement in hepatic ischemia/reperfusion injury in mice: roles for macrophage inflammatory protein-2 and KC. Hepatology 1998; 127:1172-1177.

[55] Yoshidome H, Lentsch AB, Cheadle WG, Miller FN, Edwards MJ. Enhanced pulmonary expression of CXC chemokines during hepatic ischemia/reperfusion-induced lung injury in mice. J Surg Res 1999; 81:33-37.

[56] Hisama N, Yamaguchi Y, Miyanari N, Ichiguchi O, Goto M, *et al*. Ischemia-reperfusion injury: the role of Kupffer cells in the production of cytokine-induced neutrophil chemoattractant, a member of the interleukin-8 family. Transplant Proceed 1995; 27:1604-1606.

[57] Luster AD. Chemokines, chemotactic cytokines that mediate inflammation. N Engl J Med 1998; 338:436-445.

[58] Lutz J, Thürmel K, Heemann U. Anti-inflammatory treatment strategies for ischemia/reperfusion injury in transplantation. J Inflamm 2010; 27(7):2-8.

[59] Fowell AJ, Benyon RC. Can matrix metalloproteinases be targeted in hepatic ischemia/reperfusion injury? Hepatology

2008; 47(1):14-16.

[60] Viappiani S, Sariahmetoglu M, Schulz R. The role of matrix metalloproteinase inhibitors in ischemia-reperfusion injury in the liver. Curr Pharml Des 2006; 12(23):2923-2934.

[61] Lee MH, Murphy G. Matrix metalloproteinases at a glance. J Cell Sci 2004; 117(18):4015-4016.

[62] Lehmann TG, Wheeler MD, Froh M, Schwabe RF, Bunzendahl H, *et al*. Effects of three superoxide dismutase genes delivered with an adenovirus on graft function after transplantation of fatty livers in the rat. Transplantation 2003; 76(1):28-37.

[63] Hiranuma S, Ito K, Noda Y, Ozasa H, Koike Y, Horikawa S. Amelioration of hepatic ischemia/reperfusion injury in the remnant liver after partial hepatectomy in rats. J Gastroenterol Hepatol 2007; 22(12):2167-2172.

[64] Nakamoto N, Tada S, Kameyama K, Kitamura K, Kurita S, *et al*. A free radical scavenger, edaravone, attenuates steatosis and cell death via reducing inflammatory cytokine production in rat acute liver injury. Free Rad Res 2003; 37(8):849-859.

[65] Taniguchi M, Uchinami M, Doi K, Yoshida M, Sasaki H, *et al*. Edaravone reduces ischemia-reperfusion injury mediators in rat liver. J Surgl Res 2007; 137(1):69-74.

[66] Ninomiya M, Shimada M, Harada N, Shiotani S, Hiroshige S, *et al*. Beneficial effect of MCI-186 on hepatic warm ischemia-reperfusion in the rat. Transplantation 2002; 74(10):1470-1472.