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Full Length Research Paper

Preventing organ injury with carvacrol after renal ischemia/reperfusion

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In this study, antioxidant enzyme activities and the histopathologic structure of rats were examined in order to determine the injury that occurs in the liver after renal I/R. There were 5 groups: Group I (Control), Group II (Right nephrectomy + untreated control), Group III (Right nephrectomy + I/R and olive oil treated), Group IV (Right nephrectomy + I/R and 25 mg.kg^{-1} carvacrol + olive oil treated group), Group V (Right nephrectomy + I/R and 50 mg.kg^{-1} carvacrol + olive oil treated group). 45 min of Ischemia and 24 h of Reperfusion were applied to all groups of rats except Group I. Liver CAT, SOD, Gpx enzyme activity and AST, ALT levels were evaluated at the end of the experiment and values close to the control group were obtained for rats to which 50 mg.kg^{-1} of carvacrol was administered. Significant injuries such as cellular degenerative changes, sinusoidal congestion and cytoplasmic vacuolation were determined for Group II in light microscopic examinations of liver sections. 25 mg.kg^{-1} of carvacrol dose was found to be protective in Group IV.

Key words: Renal ischemia/reperfusion, antioxidant, carvacrol, liver, remote organ, oxidative stress.

INTRODUCTION

Ischemia is the insufficiency or stopping of blood flow to an organ due to various reasons. Reperfusion is the restoration of blood flow to the tissue by eliminating the factor, which causes ischemia (Bilzer and Gerbes, 2000; Serracino-Inglott et al., 2001; Montalvo-Jave et al., 2008). Ischemia/Reperfusion (I/R) induced local response in kidney tissue has been well documented in various studies (Kadkhodaee et al., 2009). It is known that I/R also have a destructive effect on organs which are not initially affected by ischemic damage (Thurman, 2007). It has been shown that remote hepatic parenchyma injury occurs early and progresses after the induction of a systemic inflammatory response due to I/R injury. Moreover, microvascular perfusion deficits do not appear to be essential for the initiation of hepatic injury, but may be a complement dependent procedure (Gulec et al., 2008). Renal I/R injury may cause liver oxidative stress and increase lipid peroxidation in liver tissue (Fadillioglu at al., 2008).

Endogenous and exogenous antioxidants try to prevent the radical injury that occurs. While endogenous antioxidants are superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (Gpx), Vitamin C and Vitamin E are exogenous antioxidants. Several studies have demonstrated the beneficial effect of antioxidants in protecting the liver against ischemia/reperfusion injury (Rhee et al., 2002; Giakoustidis et al., 2006; Zhang et al., 2006). Sizlan et al. (2009), demonstrated that proanthocyanidins (PA) has a significant effect in the protection of the intestine and the remote organs against mesenteric I/R injury.

Carvacrol is a predominant constituent of essential oils, and is of the *Origanum* species. It is a monoterpenic phenol which has $C_{10}H_{14}O$ closed chemical formula. Synonyms: isopropyl-*o*-cresol, *p*-cymen-2-ol, 2-hydroxy-*p*-cymene, 5-isopropyl-2-methylphenol, iso-thymol (Figure 1). Carvacrol is considered to be an antioxidant because a large number of its antioxidant characteristics have been determined through its various activities and it

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Abbreviations: SOD, Superoxide dismutase; **CAT,** catalase; **Gpx,** glutathione peroxidase.

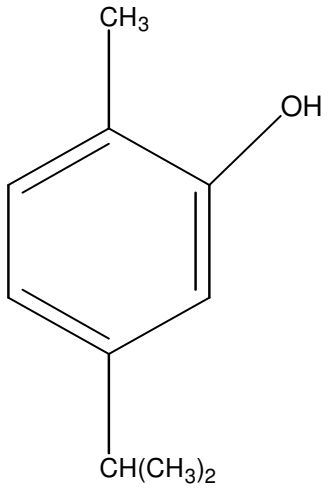


Figure 1. Carvacrol (13).

contains hydroxyl (-OH) groups in its chemical structure (Baser, 2008; De Vincenzi et al., 2004). Therefore, in our study the possible protective effect of carvacrol was researched by evaluating antioxidant enzyme activity and liver histology resulting from renal I/R.

MATERIALS AND METHODS

The experimental protocols were approved by the Institutional Ethical Committee for Animal Care and Use at Eskisehir Osmangazi University, Eskisehir, Turkey. Animals were obtained from the institute's medical and surgical experimental research center and all experiments were carried out at the same center (protocol number: 2009/135).

Plant extract

The plant substance tested in this study, carvacrol, was isolated from steam distillated essential oil of *Origanum onites* L. collected from West Anatolia. For the isolation, fractional distillation was performed using a lab-size glass fractional distillation unit containing a column packed with S/S Knit Mesh packing material (2.8 cm × 1.35 m). The reflux ratio was adjusted at 10/1 to 20/1 and the medium pressure was 8 to 10 mm Hg. Carvacrol-rich fractions were bulked to obtain carvacrol with 99% purity (GCMS).

Animals

Male Wistar albino rats, weighing between 230±30 g, were used in the experiment. The experiment was performed following a stabilization period in the laboratory. They were used after 2 weeks of adaptation. They were housed in polycarbonate cages in an air-conditioned room (12 Light/12 Dark, 22 ± 2ºC, 50 ± 5% humidity). They were fed with laboratory pellet chows and water was given *ad libitum*.

Experimental protocols

The rats were randomly divided into five groups, each consisting of 7 animals:

Group I: The animals were designated as the control group.
Group II: Right nephrectomy + I/R and saline solution (SF) treated control.
Group III: Right nephrectomy + I/R and olive oil treated.
Group IV: Right nephrectomy + I/R and olive oil + 25 $mg.kg^{-1}$ carvacrol treated.
Group V: Right nephrectomy + I/R and olive oil + 50 $mg.kg^{-1}$ carvacrol treated.

Animals from Groups II, III, IV and V' under xylazine (10 $mg.kg^{-1}$) and ketamine (70 $mg.kg^{-1}$) anesthesia (Kulisic et al., 2004) had laparotomy performed to make the right renal artery and vein visible by clearing the tissues around them. By the help of sterile surgical silk suture liquid passage was blocked through renal artery and vein. The right kidney was extracted from the animal by cutting the vein, thus achieving the nephrectomy. Antiseptic solution and surgical stitching were applied to the laparatomy zone. Each one of these rats was cured by being treated and kept in individual cages for 15 days.

Group II animals received 1 mL SF per os 60 min prior to renal I/R. In the experiments, Carvacrol was administered together with olive oil in order to prevent lesions which occur during oral application. Animals in Group III received only olive oil and animals in Group IV and Group V received carvacrol and olive oil, respectively, followed by xylazine (10 $mg.kg^{-1}$) and ketamine (70 $mg.kg^{-1}$) anesthesia and laparotomy to make the left renal artery and vein visible by clearing the tissues around them. With the help of sterile microvascular bulldog clamps, liquid passage was blocked through renal artery and vein for 45 min to cause ischemia. Oxygenation and feeding of the tissue was thus prevented. By removing the clamps at the end of the ischemia period, the blood flow was restored and oxygenation of the tissue was achieved over a 24 hreperfusion period (Sokmen et al., 2004). After the reperfusion period, all the animals were intracardially killed/destroyed by bleeding. Blood samples and the liver tissue samples that were collected from all the rats and stored in deepfreeze (-80°C) conditions in order to examine aspartate aminotransferase (AST) and alanine aminotransferase (ALT); CAT, SOD and Gpx enzyme activities (Korkmaz and Kolankaya, 2009).

Biochemical assay

The blood concentration of AST and ALT were determined using commercially available kits.

Determining isozyme activity in liver tissue

Preparation of liver homogenate

Prior to electrophoretical analysis, each liver sample (100 mg/ml buffer) was homogenized in 50 mM phospate buffer (pH: 7.0). The homogenate was then centrifuged at 10.000 rpm for 25 min and the supernatant obtained was used for electrophoretical analysis. All liver parameters were expressed as activity per mg protein. The protein concentration in each fraction was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumine as a standard.

Electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (native-PAGE) was performed on samples of liver, essentially as described by Laemmli (1970) except that SDS was omitted from all buffers and the samples were not boiled before electrophoresis. The protein content of supernatants was determined using Lowry's method (1951). The enzymes were run on the basis of equal amounts of protein (70 µg) in a 10% gel for SOD and Gpx and 8% gel for CAT. Electrophoretic separation was performed at 4°C with a constant power supply of 50 V for stacking gel and 100 V for separating gel. Staining for the activity of each enzyme was performed separately as follows:

(i) CAT activity was detected using the method of Woodbury et al. (1971). Here, the gel was soaked in 5 mM H_2O_2 solution for 10 min and then washed with water and stained with a reaction mixture containing 1% potassium ferricyanide (w/v) and 1% ferric chloride. The enzyme appeared as a yellow band superimposed on a dark green background. The reaction was terminated by adding water and the gel was photographed at once.
(ii) SOD activity was identified using the method of Beauchamp and Fridovich (1971). The gel was soaked in 50 mM Tris–HCl buffer (pH: 8.0) containing 10 mg nitroblue tetrazolium (NBT), 1 mg ethylene diamine tetraacetic acid (EDTA) and 2 mg riboflavin (50 mL final volume), and kept in the dark for 30 min. The gel was then placed on an illuminated light box to locate the area of SOD activity, which appeared as a clear zone on a bluish-violet background.
(iii) Gpx isozymes were separated using the method of Lin et al. (2002). The gel was soaked in 50 mL of 50 mM Tris–HCl buffer (pH 8.0) containing 200 mg reduced glutathione and 8 µL of 30% H_2O_2 for 20 min. The gel was then transferred to 50 mL of 50 mM Tris HCl buffer (pH 8.0) containing 25 mg NBT and 25 mg phenazine methosulphate (PMS). The appearance of white bands in the gel was taken to indicate the presence of Gpx isozymes. Band areas, which were created by each isozyme activity, were measured by using the Kodak Gel Logic 1500 Imaging System.

Histopathological evaluation

Liver was fixed in 10% neutral formalin for histological investigations. 5 µm thick sections in paraffin taken from each liver sample were stained with standard Hematoxylin and Eosin (H and E). Samples were investigated by light microscopy by using the Spot Advanced Software (V. 3.2.4; Diagnostic Instruments, Sterling Heights, USA). Sections were digitally photographed using a Spot Insight Color 3.2.0 diagnostic camera. The morphological changes were scored (1; No evidence of injury, 2; mild, 3; moderate, 4; severe) in order to perform comparison between the groups.

Statistical analysis

The results were expressed as the mean ± SE of seven animals per group. The One way analysis of variance (ANOVA) and TUKEY tests were used for the analysis and comparison of data within and between groups (SPSS 12.0 for windows). The Mann-Whitney *U* test was used to compare the scores of morphological changes for the groups. Differences were considered significant at $P < .05$.

RESULTS

Biochemical investigation

The results of AST and ALT levels in the blood of Group I, II, III, IV and V animals are shown in Figure 2. Serum AST and ALT levels in Group II are statistically different from Groups IV and V. There is no statistical difference between Group IV and V.

Isozyme activity in liver

In the liver tissue samples, CAT isozyme was seen as single band (Figure 3), SOD was seen as two bands (Figure 4) and Gpx was seen as five bands (Figure 5).

Liver histopathology

The histopathological evaluations of the liver sections from all groups are shown in Table 1. The liver sections of Group I animals were shown to have normal morphological structure. In the sections of Group II cellular degenerative changes were intense, sinusoidal congestion and cytoplasmic vacuolation were observed (Figure 6). Enlarged sinusoids appeared in liver sections of Group III (Figure 7). Although, the sinusoidal congestion and cytoplasmic vacuolation of liver sections of Group IV was not exactly as the ones of Group I, it was observed that the histological structure was preserved (Figure 8). In Group V, the cytoplasmic vacuolation and enlarged sinusoids were low and the histological structure was partly preserved (Figure 9).

DISCUSSION

Injuries in various degrees might occur in remote organs along with I/R which results from the reduction or stopping of blood flow in the kidney (Gulec et al., 2008). Liver injury is one of the remote organ injuries induced by kidney I/R. Acute renal defects are associated with the aetiology of liver disease (Serteser et al., 2002). Fadillioglu et al. demonstrated that melatonin treatment may prevent liver oxidant stress induced by distant injury of kidney I/R (Fadillioglu et al., 2008). In other studies, it was shown that 45 min of hepatic ischemia and 1 h of reperfusion may alter renal functions and may cause oxidative stress on renal tissue (Polat et al., 2006). Renal

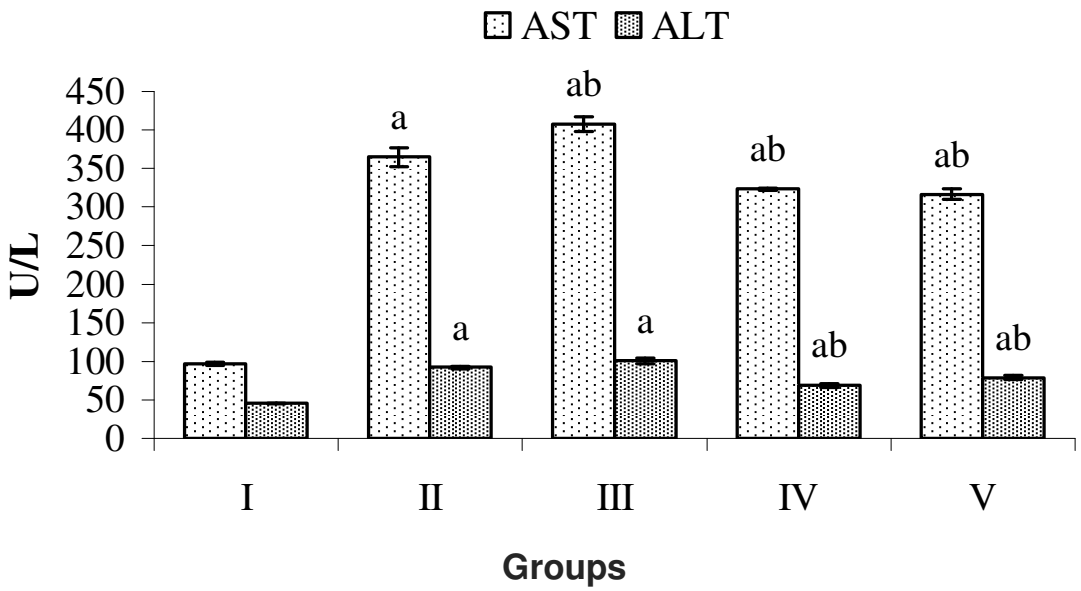


Figure 2. AST and ALT serum levels for Group I, II, III, IV and V mean ± standard error (SE). According to $p < 0.05$, a: Different from Group I; b: Different from Group II.

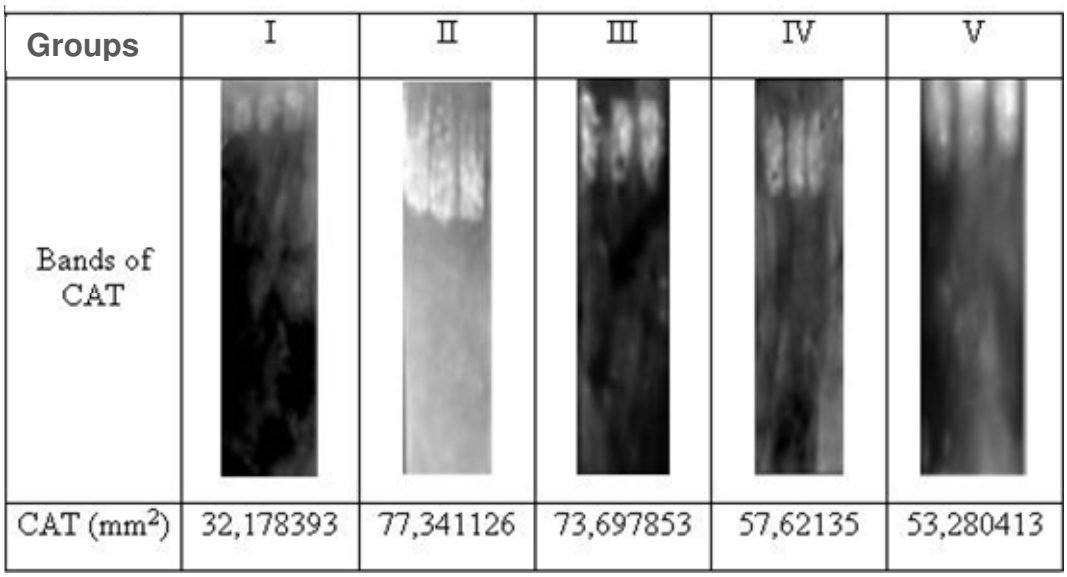


Figure 3. Single band images of CAT isozyme in liver tissue and average values of band areas.

Table 1. Histopathologic evalutions for each group.

Group		Sinusoidal congestion	Enlarged sinusoids	Cytoplasmic vacuolation
I (n = 7)	Control	1	1	1
II (n = 7)	Right nephrectomy + I/R + SF treated	3.7	2.7	3.7
III (n = 7)	Right nephrectomy + I/R + olive oil treated	3.4	3.7	2.7
IV (n = 7)	Right nephrectomy + I/R + olive oil + 25 $mg.kg^{-1}$ carvacrol treated	2	1.2	2
V (n = 7)	Right nephrectomy + I/R + olive oil + 50 $mg.kg^{-1}$ carvacrol treated	2.5	2.7	2.2

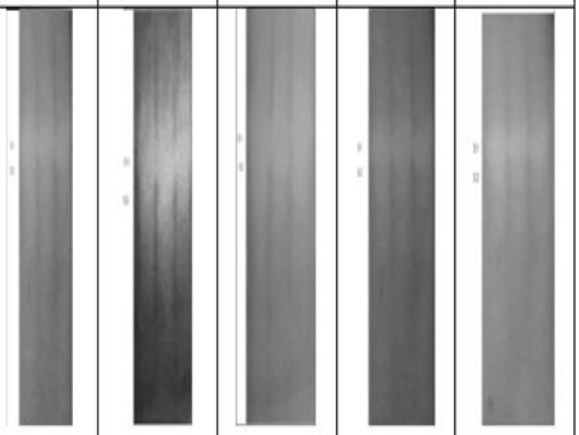
Groups	I	II	III	IV	V
Bands of SOD					
SOD1(mm^2)	14,70393	37,81421	36,24432	34,96162	23,89623
SOD2(mm^2)	13,79846	35,85624	35,12625	32,84352	22,55681

Figure 4. Band images of two isoforms of SOD isozyme in liver tissue and average values of band areas.

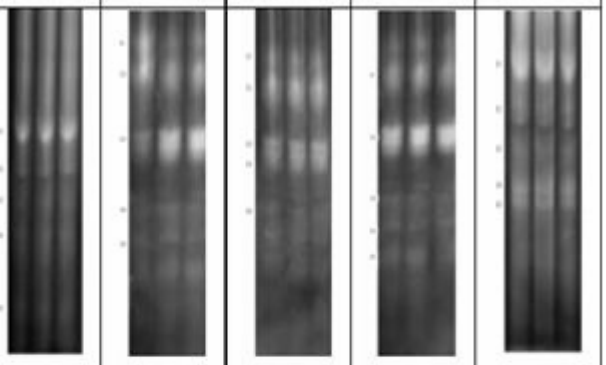
Groups	I	II	III	IV	V
Bands of Gpx					
Gpx1(mm^2)	15.22423	50.84036	46.25424	38.492	30.82642
Gpx2 (mm^2)	13.22142	47.25055	44.3663	36.82425	25.95634
Gpx3 (mm^2)	11.32465	45.18213	43.62896	35.32629	23.8553
Gpx4 (mm^2)	10.62632	44.64294	42.6421	34.69632	22.7423
Gpx5 (mm^2)	9.84517	43.7254	41.78893	33.25484	21.34258

Figure 5. Band images of five isoforms of Gpx isozyme in liver tissue and average values of band areas.

injury associated with liver disease is an extensively encountered clinical problem of varied etiology and high mortality (Kadkhodaee et al., 2009).

Serum AST and ALT levels were measured mainly as a marker of hepatic parenchymal cell injury. In the study reported here, the serum, AST and ALT levels increased

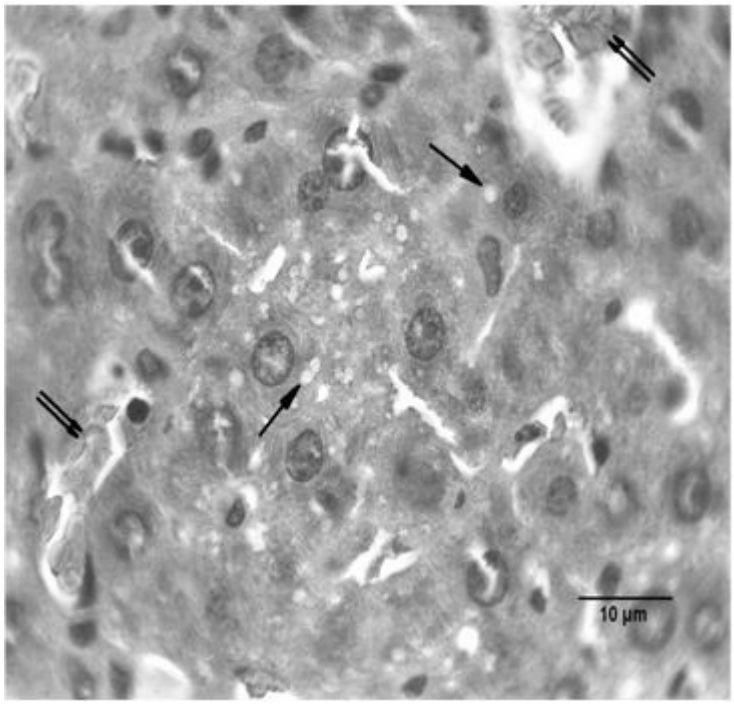


Figure 6. In Group II, cellular degenerative changes, cytoplasmic vacuolization (↗) and sinusoidal congestion (↗).

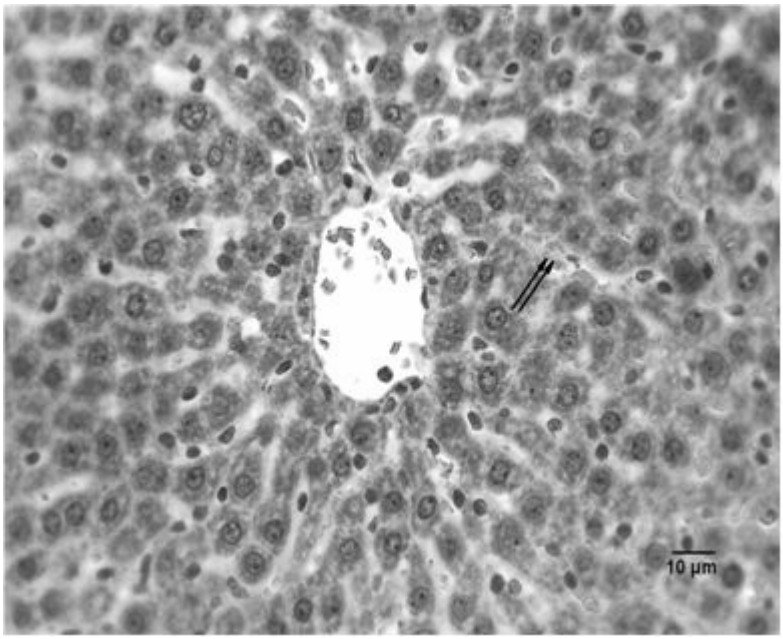


Figure 7. In Group III, enlarged sinusoids appeared in liver sections (↗).

significantly after renal I/R (45 min /24 h). In the present study, carvacrol treatment (50 mg.kg^{-1}) prevented the alterations in the ALT and AST enzyme activities. In the present study, antioxidant enzymes and histological structure were examined in order to determine the injury, which occurred in the liver of rats after renal I/R. Living

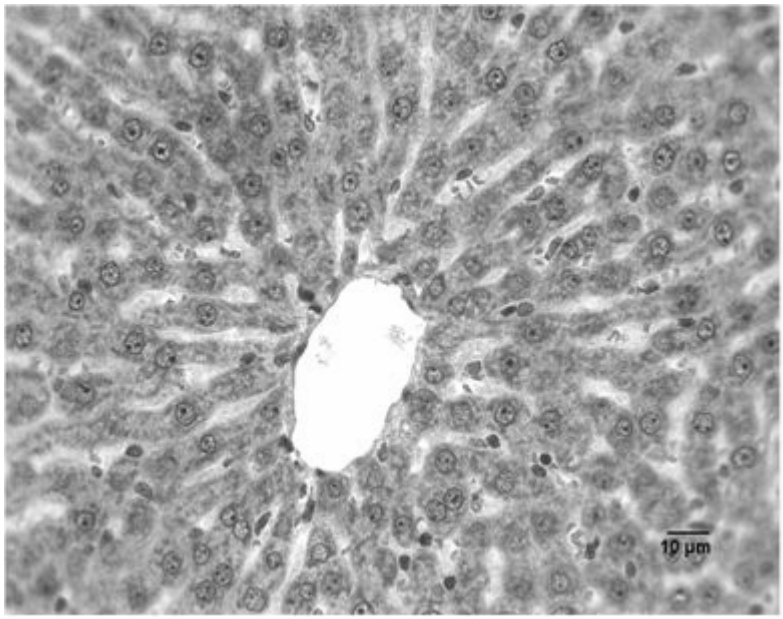


Figure 8. In Group IV, histological organization was protected.

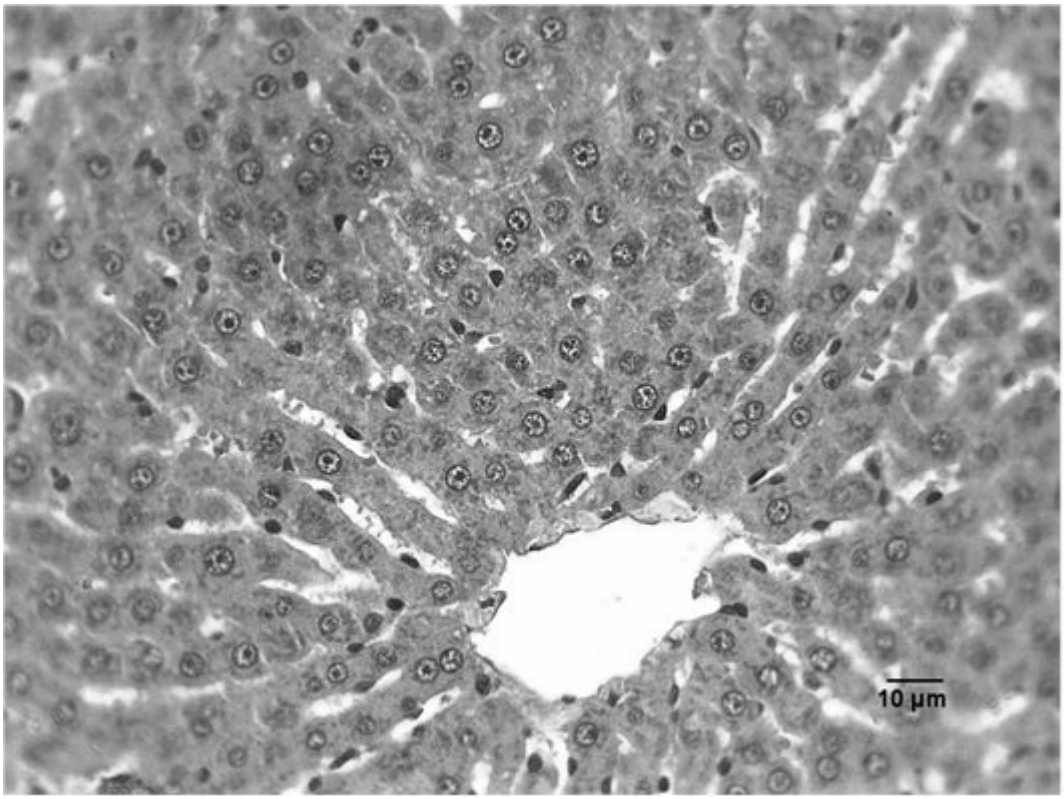


Figure 9. In Group V, partial protection was observed in liver sections.

tissues were endowed with innate antioxidant defense mechanisms, including the enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (Gpx) (Jayakumar et al., 2006).

Our studies on the electrophoretic pattern of hepatic catalase isozymes revealed one band of low intensity in control rats (Group I). However, it was observed as markedly elevated intensity in right nephrectomy + untreated control group (Group II). Right nephrectomy + I/R and olive oil treated group (Group III) was observed to be in proximity with Group II by means of catalase intensity. A reduction in dose dependent band intensity was observed in the carvacrol (25 $mg.kg^{-1}$) treatment group (Group IV) and the carvacrol (50 $mg.kg^{-1}$) treatment group (Group V). While, SOD1 and SOD2 isoforms of SOD isozyme showed high band intensity in Group II, they slightly decreased in Group IV. However, the decrease in band intensities of both isoforms in Group V is remarkable. When we examined the band intensities of isoforms of Gpx in the liver, we came across results similar to SOD. Although, band intensity was high for all isoforms of Group II and III, it was lower in Group IV and V.

The results of the study performed by Jayakumar et al. (2006) and Ramesh et al. (2009) show similarities to our study. These researchers reported that exogenous antioxidants had positive effects on CAT, SOD and Gpx enzyme activity in the liver tissue. The histopathologic results of our study for the liver sections of Group I, II and III also support our antioxidant enzyme findings. Kadkhodaee et al. (2009) have demonstrated that negative changes occur in liver histology, such as necrotic cell death and/or apoptosis, function and oxidative stress after renal I/R. They stated that 45 or 60 min of ischemia caused these changes and emphasized that remote organs should be protected. We also made similar findings. In our histopathologic evaluation, sinusoidal congestion and vacuolization were seen in Group II. Our histological findings demonstrated a 25 $mg.kg^{-1}$ carvacrol dose to be protective and a 50 $mg.kg^{-1}$ carvacrol dose to be partially protective for liver injury occurring after renal I/R.

We concluded that carvacrol treatment may prevent liver oxidant stress induced by remote injury of kidney I/R. In our study we saw that while the protective oral dose for preventing remote organ injury as a result of I/R was 50 mg.kg-1 for antioxidant enzymes, it was 25 mg.kg-1 in accordance with our histological findings. In the light of these findings we suggest our dose ranges, which we have used for the hepatoprotective effect of carvacrol, to be taken as a reference for similar studies in the future.

Some authors have shown that natural products protect I/R injury in the organs (Sizlan et al., 2009; Emre et al., 2006).The carvacrol used in our study is known to have antioxidant, antimicrobial, antitumor, antimutagenic, antigenotoxic, analgesic, antismasmodic, anti-inflammatory, angiogenic, antiparasitic and antiplatelet effects (Baser, 2008). Uyanoglu et al. (2008) have reported that carvacrol increased the regeneration in the liver and had a protective effect. Canbek et al. (2008) also demonstrated that carvacrol is protective after liver I/R.

Our study suggested that carvacrol is effective on remote organ damages due to renal I/R injury. Several useful attributes of carvacrol have been scientifically proven *in vivo* studies, however, studies on the protective effects in I/R model are limited. Most of such medicines are plant based. Many plants found in nature have been used to treat various illnesses. One such plant is oregano (Kekik in Turkish). The health beneficial effects of carvacrol obtained from oregano oil have been shown scientifically. This way, people have been various reasons consist of reduce to oxidative damage or end of damage that we think that economic and social benefits occur to contribute.

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