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Protective Effect of Carvacrol Against Oxidative Stress Injury in Rats Following Renal Ischemia/Reperfusion

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Abstract

In this study, possible protective effects of carvacrol were investigated against experimental renal ischemia/reperfusion (I/R) injury in rats. Groups are determined as Group I (Control), Group II (I/R+saline), Group III (I/R+olive oil), Group IV (I/R+olive oil+25 mg/kg carvacrol) and Group V (I/R+ olive oil +50 mg/kg carvacrol). After right nephrectomies, 45 minutes of ischemia and 24 hours of reperfusion were applied to Group II, III, IV. SOD, CAT and Gpx activities were determined by electrophoresis. BUN, CRE MDA, MPO levels were determined spectrophotometrically. Classical HE staining of renal tissues was performed. Compared with control; SOD, CAT, Gpx activities were increased in all groups. Compared with Group II; SOD, CAT, Gpx activities were decreased gradually and enzyme activities is the lowest in Group V among all groups. Compared to Group IV with Group V; BUN, CRE, MDA, and MPO enzyme activity lowest than in Group V. In the Group V histopathological analyses, It was observed that the injury was significantly decreased. The results of this study have demonstrated that 50 mg/kg dose of carvacrol is more effective than 25 mg/kg dose of carvacrol against renal I/R injury prevention.

Key words: Renal ischemia/reperfusion, Antioxidant, Carvacrol, Kidney, Reactive oxygen species

INTRODUCTION

Renal ischemia/reperfusion (I/R) injury occurs in many clinical applications (such as transplantation, partial nephrectomy, shock and vascular surgery) and this injury constitutes the main reason for acute renal failure [1, 2]. During ischemia, blood flow reduction and decreased oxygen levels lead to adenosine triphosphate, (ATP) depletion, impaired oxidative metabolism, generation of reactive oxygen species (ROS), Na/K pumps inhibition [3]. And Reperfusion characterized with leukocytes influx, endothelial cell activation, neutrophil infiltration, inflammation and generation of vasoactive mediators and apoptosis. Especially ROS, NO species are released in this phase [3–5]. The increased ROS can be regulated by endogenous or externally supplemented antioxidants I/R injury can limited by cell protective enzymes (SOD, CAT, Gpx etc.) and antioxidants [6].

Carvacrol, which is available in essential oil of the thyme, is a phenolic compound with anti-oxidant characteristics [7] and carvacrol is a monoterpenic phenol. It is known that carvacrol is responsible for biological activity of the thyme [8]. Studies demonstrated that carvacrol has anti-diabetic [9] anti-inflammatory [10] antioxidant, anti-microbial, neuroprotective [11], cancer anti-proliferative [12], hepatoprotective [13] and liver regenerative effects [8]. Limited studies have been found effects of carvacrol on the I/R injury [14, 15]. One of these studies, the effect of carvacrol was investigated against experimental double-sided renal I/R injury. And 75 mg/kg i.p injected carvacrol is effective against experimental double-sided renal I/R injury [15]. In Potočnjak and Domitrović study, carvacrol is effective against cisplatin triggered renal injury [14]. But 25mg/kg and 50 mg/kg carvacrol (by gavage) effects not known yet.

In our study, the effects of 25 mg / kg and 50 mg / kg doses carvacrol administered (by gavage) single sided renal I/R injury were examined by biochemical, histopathological and electrophoresis (Native Page) method.

MATERIALS AND METHODS

The Institutional Ethical Committee approved the experimental protocols for Animal Care and Use (protocol number: 2009135). Animals were taken from the medical and surgical experimental research centre of the institute and all experiments were carried out in the same centre.

Animals

Male Sprague dawley 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±2 ºC, 50±5% humidity). They were fed with laboratory pellet chows and water was given ad libitum.

Plant Extraction and Carvacrol Application

Essential oil obtained from Origanum onites L. by using steam distillation (fractional distillation) unit was analyzed with gas chromatography/mass spectrometry and its fraction, which was rich for carvacrol, was separated (8). Carvacrol with a purity of 99%, which was obtained in this way and provided by Prof. Dr. K. H. C. Başer, was used in our experimental studies.

Carvacrol were administered with a single dose of gavage every day during a week, Carvacrol was administered together with olive oil in order to prevent lesions which occurred during oral gavage.

Experimental Protocols

The rats were randomly divided into five groups (n=7). In Group I (Control group) animals, any surgical procedure was not performed. Group II (I/R + saline) was determined as a negative control group and I/R injury created but neither treatment substance (carvacrol) nor solvent (olive oil) was given. Group III (olive oil) was determined as the olive oil group in which the carvacrol was dissolved and I/R injury created. Group IV (25mg/kg Carvacrol + olive oil) and V (50 mg/kg Carvacrol + olive oil) was determined as carvacrol treatment groups. In Group IV, V 25mg/kg, 50 mg/kg Carvacrol was dissolved in olive oil and was given animals for a week via gavage, respectively and I/R injury was created.

Under anaesthesia, right nephrectomies were performed in Group-II, III, IV and V. As a result of healing for two weeks, IR injury was created at Group-II, III, IV and V. For this purpose; Under xylazine (10 mg/kg) and ketamine (70 mg/kg) anaesthesia [16], laparotomy was done for make renal artery and vein visible. With the help of sterile micro-vascular bulldog clamps, renal artery and vein were blocked for 45 min to cause ischemia and 24 h of reperfusion.

The renal tissue samples and blood serums were collected from all of the rats and stored under deep freeze (-80 oC). CAT, SOD, Gpx, MDA enzyme activities measured in kidney and CRE, BUN in serum. Also for histological analysis renal tissue samples were fixed in 10% neutral formalin.

Biochemical Analyses

Renal function was assessed by serum CRE and BUN concentration [17]. The blood samples were centrifuged and separated into serums for 10 min at 3000 rpm through an Eppendorf 5804 R model device. CRE and BUN measurements were performed by using commercial kit through Crony auto-analyzer.

Measurement of renal MDA and MPO activity, MDA was determined using Ohkawa method [18].

Determination of Isozyme Activity in Kidney Tissue

Activities of CAT, SOD and Gpx isozymes were determined in homogenized kidney. The protein content of supernatants was determined using Lowry's method [19] and bovine serum albumin as standard. Native-PAGE electrophoresis was performed at +4°C 120 V (for SOD and GPx) and 140 V (for CAT) as described by Laemmli (1970) ([20]. 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.

Determination of CAT activity was determined by Woodbury et al (1971) method [21]. The enzyme appeared as a clear zone on a dark blue background.

Determination of SOD activity was determined by [22]. The enzyme appeared as a clear zone s on a green background.

The method of Lin et al. (2002) was used to measure Gpx isozyme activity [23]. The enzyme appeared as a clear zone on a violet background. The bands that showed the Gpx isozyme activity were observed as transparent colour on a violet background.

CAT, SOD, Gpx band areas were measured by using the Kodak Gel Logic Imaging System.

Histological Analyses

Standard procedures were applied to tissue processing. 4-5 µm thick sections were taken on microtome and, H&E staining was performed. All tissue sections were evaluated histologically with the help of the 3.2.0. model digital camera Spot Insight brand, of the CH40 model light microscope Olympus brand, and Spot advanced 4.0.6 version software program.

Statistical Analysis

"SPSS 20 for Windows" package program was used for evaluation of the obtained data. MDA values were analyzed according to One Way Analysis of Variance. "Fisher LSD Method" was used in relation to the multiple comparison. MPO results were analysed according to Kruskal-Wallis One Way Analysis of Variance on Ranks.

Differences between the experimental groups were accepted to be significant if $P<0.05$. Results were expressed as the mean ± SE

RESULTS

Biochemical Findings

In all groups, BUN, CRE levels were measured in blood serum samples and MDA, MPO values were measured in kidney tissues. Obtained BUN, CRE, MDA MPO values were compared statistically, between groups.

Groups	BUN (mg/dL)	CRE (mg/dL)	MDA (nmol/mg protein)	MPO (U/mg protein)
I	29,67 ± 0,55	0,47 ± 0,03	3,414±0,424	0,00448±0,00078
II	89,90 ± 2,30^{a}	1,49 ± 0,05^{a}	8,037±0,778^{a}	0,01392±0,00762
III	188,26±3,78^{ab}	3,49±0,13^{ab}	4,479±0,328^{b}	0,00401±0,00067^{b}
IV	176,57±1,36^{ab}	2,86±0,14^{ab}	5,112±0,942	0,00479±0,00110
V	157,87±2,77^{ab}	2,39±0,10^{ab}	4,079±0,330^{b}	0,00391±0,00094^{b}

Table 1. Mean values ± SE values of BUN and Creatinine quantities in the blood serum samples and MDA and MPO quantities in the kidney tissue samples (n=7).

BUN and CRE values were examined and similar statistical results were found. I / R group was found significantly higher than the control group ($P<0.05$). I/R group BUN, CRE values compared to Group III (olive oil), Group IV(25 mg/kg carvacrol+ olive oil), Group V (50 mg/kg carvacrol+olive oil) values and found to be BUN, CRE values increased. But it was observed that BUN and CRE values of Group IV, V which are carvacrol treatment groups, decreased gradually towards Group III (olive oil) (Table 1).

MDA and MPO parameter values for all experimental groups, which were biochemically determined for the kidney tissue samples, are given in Table 1.

According to Table 1, It was observed that I/R group MDA values were significantly higher than Group I, III, V ($P<0.001$). And this difference was a little less in Group IV (25 mg/kg carvacrol+ olive oil) ($P<0.01$). Group III (olive oil) and Group V(50mg/kg carvacrol+olive oil) compared to itself, there was a decrease of MDA values in Group V.

MPO values examined and it was observed that I/R group MPO values were higher than all groups. And difference between Group II and Group III/V values is statistically significant ($P<0.05$) on the other hand MPO values of Group I, III, IV (25 mg/kg carvacrol+ olive oil) and V (50mg/kg carvacrol+olive oil) were close to each other. From these groups Group V MPO values most closer to control group.

Isozyme Activity

In the kidney tissue samples, CAT isozyme was seen as a single band, SOD was seen as two bands (SOD1 and SOD2) and Gpx was seen as four bands (Gpx1, Gpx2, Gpx3, Gpx4). (Table 2)

According to the electrophoretic analysis results of the CAT enzyme, the band area (19,43713 mm2) for the tissue sample in the Group I had low density while the band area (62,36910 mm2) in the Group II was found to be highly dense. When group III(olive oil), IV(25mg/kg carvacrol+olive oil), V(50mg/kg

carvacrol+olive oil) were evaluated between each other, a gradual decrease was observed between the groups in terms of cat enzyme activity. And this value was the lowest in Group V (48,86971 mm2) (Table 2, Figure 1).

Groups	CAT mm^2	SOD mm^2		Gpx mm^2			
		1	2	1	2	3	4
I	19,43713	6,397003	5,469937	5,468969	2,993433	2,640663	2,521442
II	62,36910	42,142200	40,28180	32,32640	27,85400	20,91121	18,3245
III	58,40621	35,273229	32,630164	17,16123	16,69059	13,84828	13,88878
IV	52,40039	31,768320	21,518624	15,10823	13,04535	12,67494	11,94538
V	48,86971	24,413960	19,941696	10,77926	9,427753	9,679773	10,24683

Table 2. CAT, SOD and Gpx enzyme activity with band area

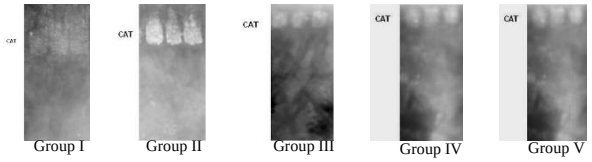

Figure 1. Electrophoretic bands, which were created as a result of CAT enzyme activity in the kidney tissue samples of rats.

Two isoforms of SOD were seen in kidney tissue (SOD1 and SOD2). The SOD1 isoenzymes of the kidney tissue, the SOD1 band area in the Group I had low density. In the SOD1 band area of the Group II, an increase was observed. A gradual decrease was observed in Group III, IV and V band areas and SOD1 band area of Group V was lowest among all groups. SOD2 band density also showed similar results in all groups. SOD2 band density of Group II was the highest; SOD2 band density of Group V is the lowest among all groups (Table 2, Figure 2)

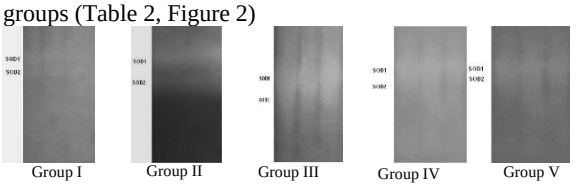

Figure 2. Electrophoretic bands, which were created as a result of SOD enzyme activity in the kidney tissue samples of rats.

Four isoforms of Gpx were seen in kidney tissue (Gpx1, Gpx2, Gpx3, Gpx4). In all isoforms of the Gpx enzyme, a quite low band area was observed for the Group I. Highest band areas were observed in the Group II (32,3264 mm2-27,854 mm2-20,91121 mm2-18,32452 mm2). The Group V had lowest band density (10,77926 mm2-9,427753 mm2-9,679773 mm2-10,24683 mm2) like SOD and CAT bant density of Group V (Figure 3).

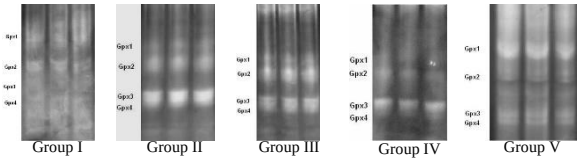

Figure 3. Electrophoretic bands, which were created as a result of Gpx enzyme activity in the kidney tissue samples of rats

Histopathology

Bowman capsule and normal tubular structure were observed in H&E kidney sections of the Group I (Figure 4). Expansions were observed between glomerulus and bowman capsule in the kidney sections of the Group II. Tubular degeneration, cell fractionation and brush border losses were detected in Group II (Figure 5). It was observed that the injury was significantly decreased in the Group V when compared to the other groups. Tubule cells and glomerular structure, which were similar to the control (Figure 6).

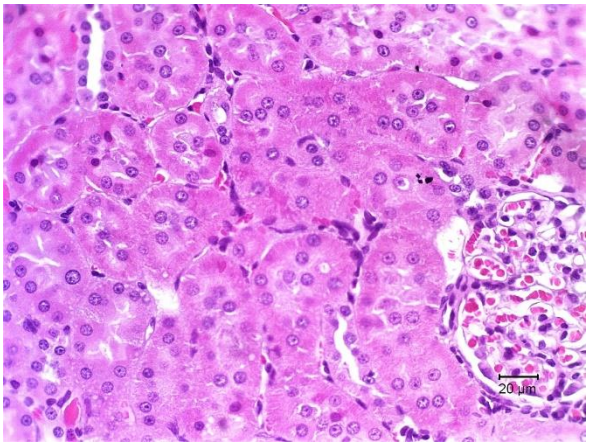

Figure 4. Glomerular structures, bowman capsule and tubules in the kidney tissue sections of the Group I.

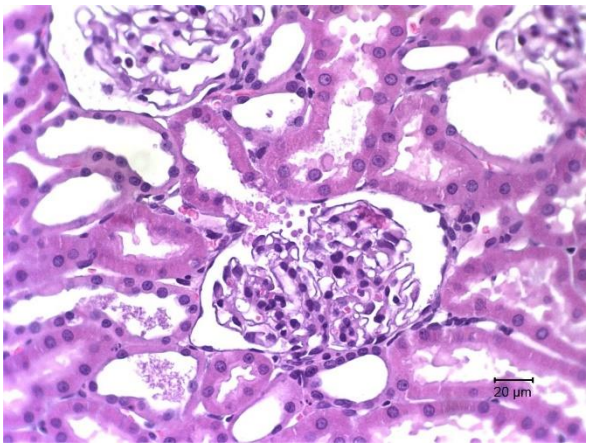

Figure 5. Tubular deformation in the kidney tissue section of the Group II. Change in the glomerulus and bowman capsule.

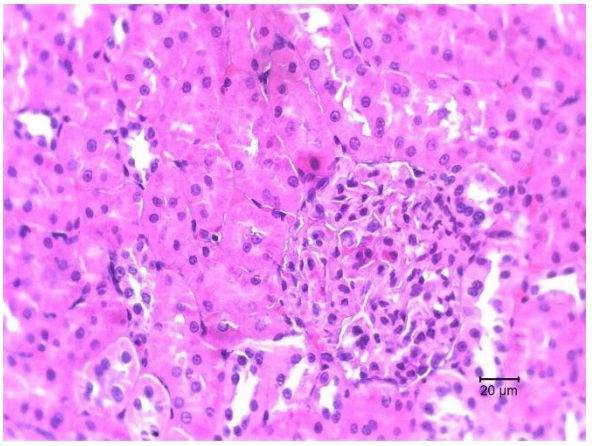

Figure 6. Tubule cells and glomerule structure, which are similar to the control, in the kidney tissue sections of the Group V.

DISCUSSION

I/R injury is a series of complex events which include various pathological processes. Several studies have suggested that ROS initiates lipid peroxidation in I/R injury and plays an important role in formation of this injury [24, 25].

It is known that herbal anti-oxidants also provide efficient protection against oxidative injury although they are not as effective as the enzymatic anti-oxidants [26]. Today, many studies show that I/R injury in heart, liver, brain, intestines and kidneys may be prevented to a certain extent through some anti-oxidants [27].

Urea, which is the protein metabolism product synthesized in the liver, is excreted through kidneys. BUN level can be used as a marker of renal function [28]. Serum BUN level

following I/R were found to be higher than in all groups when compared to the group I. This suggested that I/R negatively affect the renal functions. While there was a significant improvement in other test parameters for the Group V, it was observed that BUN value was less than other treatment groups.

An increase in the serum creatinine level following I/R is a condition, which indicates a dysfunction in proximal tubule cells of the kidney. A statistical difference was observed between all groups in terms of creatinine. It was determined that the difference level of the Group V was positive. Similar studies showed parallelism with our findings [17].

As for the MDA levels, which show the level of lipid peroxidation, a significant increase was observed in the Group II while a significant decrease was recorded with regard to the Group V [17]. A statistically significant difference was observed between the Group I and the Group V and a value, which was similar to the control value, was determined for the Group V. Besides, the significant decrease in the MDA results of the Group III suggested that olive oil had protective effects.

In our study, data of the MPO, which is a neutrophil infiltration and activation parameter, is analysed. Post-ischemic PMNL results in formation of ROS that plays a role in I/R injury through its MPO enzyme content [17] The kidney tissue in our study showed an increase in the Group II as a result of MPO and it was observed that this value was statistically different from the Group III and the Group V. In terms of this parameter, it was thought that the substances used in the Group III and the Group V had protective effects.

The electrophoretic analysis results of the CAT enzyme, which was seen as a single band in the kidney tissue, a significant increase was observed in the band area of the CAT enzyme in the Group II and a significant decrease was recorded in the band area of the Group V. Similar band areas were detected in the Group III and IV. When the SOD enzyme was evaluated, it was observed that SOD1 and SOD2 isoforms were significantly increased in the Group II while a low band density was detected in the Group V. In the Group II, isoenzyme band areas were found to be dense in all forms of Gpx. In Group V, a less dense band area was recorded when compared to the Group II.

In our study an increase was determined in I/R group. In some studies like our study. Increased antioxidant enzyme activities were determined in I/R group [29, 30]. But in some studies, there was a decrease in I/R groups. In Dobashi et al. (2000) , there was a decrease in the amount of anti-oxidant enzyme as a result of I/R injury. Dobashi et al. (2000) reported that; in all I/R groups which have different time points have significant decrease of SOD, CAT and Gpx activities compared to control [31]. In another study, renal SOD activity was decreased and CAT was increased after 45 min of ischemia and 24 hours of reperfusion group [32].

A significant increase with regard to anti-oxidant enzyme activation areas in the Group II and decrease in Group IV-V and suggested that carvacrol decreases oxidative stress. Among the related studies [30] reported that SOD, CAT, Gpx values of the I/R group were significantly higher and that significant decrease was recorded in the treatment group, in which rats were administered with Olea europaea l. (olıve) leaf extract agaınst, in renal I/R injury as a result of 45 min of ischemia and 24 hour of reperfusion. In another study [29], CAT and SOD activities were decreased in treatment group. In similar study the effect of carvacrol was investigated against experimental double-sided renal I/R injury. It has been reported that carvacrol (75g/kg, i.p) decreased SOD, CAT, Gpx activities [15]. However in rats exposed to restraint stress, SOD, CAT, Gpx were deacrease in I/R group and an increase was observed in treatment groups or only carvacrol administered groups [33].

These differences in the results suggests that there is a possible difference with regard to the animal species, methods and models used in these studies. The reason for the decrease in the enzyme activity in our results may carvacrol have a protective effect against I / R damage prior to the antioxidant defense system activation.

The histopathological analysis of the kidney tissue, it was observed that tubule and glomerule structure of the kidney had a normal appearance and that there was no damage in relation to the tissue samples of the Group I. Significant changes with regard to the glomerulus and bowman capsule, expansion in the capsule interval as well as brush border loss and intertubular hemorrage were detected in the Group II. As for the histological assessments for the studies on the renal I/R injury, it was reported that vacuolization, tubular dilatation, necrosis areas, intercellular hemorrage occurred in the tubule cells of the kidney following I/R [17, 34]. These findings are coherent with our histological data. Partial improvement was observed in the Group III and IV as a result of the histological analysis. The Group V, parallel results were obtained with the Group I. These results showed that dose of carvacrol applied in the Group V may prevent the damage that occurred with the effect of ROS.

Canbek et al. (2008) reported that carvacrol had protective effects on the rats, which were subjected to total liver I/R (45 min of ischemia/60 min of reperfusion). In the study, it was reported that 73 mg/kg dose of carvacrol, which was applied on the Wistar albino rats in an intraperitoneal way, significantly decreased serum AST, ALT, GSH, MDA and CAT values and protected the liver tissue [35]. In another study, ıt was shown that preventive role of carvacrol against methotrexate induced renal injury [36].

This study showed that carvacrol, as a free radical sweeper agent, had preventive effects against I/R injury. Consequently, uptake of natural phenols through diet (fruit, vegetable etc.) is very important. According to the findings obtained from our study, we can state that carvacrol significantly decreases the damage arising out of renal I/R depending on the dose. But higher doses of carvacrol should be investigated against I / R damage

Conflict of Interest Statement

No financial contributions or conflicts of interest to declare in this study.

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