Antioxidant effect of rosmarinic acid against renal ischemia reperfusion injury in rat; a histopathological study

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Abstract

Introduction: Renal ischemia reperfusion (I/R) occurs during renal surgical procedure that leads to renal injuries and can progress to acute renal failure. Objectives: Due to central role of reactive oxygen species (ROS) in pathogenesis of renal I/R, rosmarinic acid (RA) an antioxidant agent were used against renal I/R injuries. Materials and Methods: Forty male mature Sprague Dawley rats (180–200 g) were divided into five groups (8 rats per group) as follows; group 1 control; group 2 I/R, without treatment; group 3 I/R + RA (50 mg/kg); group 4 I/R + RA (100 mg/kg) and group 5 I/R + RA (200 mg/kg). RA was given orally by gavage in groups 3, 4 and 5 one hours before surgery. I/R induced through ligation of renal pedicles bilaterally for 45 minutes and then 24-hour reperfusion. After 24 hours, blood were sampled, Left kidney fixed in formal saline solution and then tissue section (5 µ thickness) prepared and were stained with PAS method. Serum malondialdehyde (MDA), urea and creatinine were measured. Histological variables were assessed morphometrically and semiquantitativly. The data compared by SPSS 13 and Mann-Whitney U test at P < 0.05.

Results: Histological injuries (tubular necrosis, casts, diameter, tubular volume density, mean glomerular volume) and serum creatinine, urea, MDA were improved in RA treated groups in comparison with group 1 (P<0.05), but the treatment cannot be kept variables at the same level as that of control group.

Conclusion: Histopathological injuries of renal I/R, were reduced by RA via inhibition of lipid peroxidation and finally led to amelioration of serum urea and creatinine.

Introduction

Renal ischemia reperfusion (I/R) occurs in kidney surgeries such as partial nephrectomy, renal artery angioplasty, transplantation and elective urological operations. Owing to renal vessels ligation during the procedures ischemia (tissue hypoxia) occurs and leads to renal damages especially proximal convoluted tubules (PCT) injuries (1-3). Following surgical procedures, acute renal failure can progress, resulting in tubular necrosis, decreasing of glomerular filtration and increase in renal resistance (4). Severe damages also occur during reperfusion. After ischemia and restoration of blood flow, production of reactive oxygen species (ROS) such as superoxide, hydroxyl, H2O2 and activation of leukocytes and endothelial cells contribute to reperfusion injury (5). Decrease of innate antioxidant enzymes, increase of intracellular sodium and calcium, stimulation of nitric oxide (NO) synthetize, endonucleases and phospholipases also occur during reperfusion phase (6).

Ischemic proximal tubule cells also produce mediators as proinflammatory cytokines (e.g., TNF-α, IL-6, IL-1β and TGF-β) and chemotactic cytokines such as monocyte chemo attractant protein-1 (MCP-1) and IL-8 (7).

There are many studies that administration of antioxidant agents improved renal I/R injury (1-3,8,9). In this research for the first time...
time, rosmarinic acid (RA) an antioxidant phenolic compound was used for inhibiting of renal ischemia/reperfusion injuries. Antioxidant property of RA was shown in our last researches (10-13). Other beneficial effects of RA include anti-inflamatory (12,14), reducing NF-κB, increasing glutathione transferase, anti Bcl-2 activity (15) and potent scavenger of peroxynitrite (16).

**Objectives**

Because of fundamental role of ROS in pathogenesis of renal I/R, RA an antioxidant agent for the first time was used in combat with renal I/R injuries. No detailed research has been carried out on the efficacy of RA in the modulation of oxidative stress associated with histopathological injuries via renal I/R in experimental animals.

**Materials and Methods**

Forty male mature Sprague Dawley rats (180–200 g) were selected. They were kept at room temperature of 22°C and a humidity of 50±10% with 12 hours light/dark cycle. Animals were divided into five equal groups randomly including 8 rats per each as follows: Group 1. Control, surgery without I/R; Group 2. I/R, without treatment; Group 3. I/R + RA (50 mg/kg); Group 4. I/R + RA (100 mg/kg) and group 5. I/R + RA (200 mg/kg). RA was administrated orally by gavage in groups 3, 4 and 5 with doses 50, 100 and 200 mg/kg (13,17) respectively one hour before surgery (RA was prepared from Sigma–Aldrich USA Company (97% C18H16O8 Mw 360,131 – p number 536954).

**Surgery method**

After 8 hours fasting, animal weighted and then anesthetized by injection of chloral hydrate (400 mg/kg) intraperitonealy. Abdominal skin were shaved and disinfected with povidone iodine solution. The abdominal cavity was exposed through a midline incision, and the renal pedicles were carefully isolated. Bilateral renal pedicles were occluded by using no traumatic vascular clamps for 45 minutes. Occlusion of renal artery was confirmed by observing the color changing of the entire kidney surface and some increasing in kidney size. After removing the renal clamps, kidneys were observed for 5 minutes to ensure return of renal blood circulation. Afterwards 1 mL of warmed saline was injected intraperitoneally, and the incision was sutured in two layers. During 45 minutes of ischemia, both intestines and kidneys were conserved with humid, hot and sterilized gauze (6,18).

**Sampling**

Twenty-four hours after surgery animal were anesthetized (Nesdonal 50 mg/kg ip), then blood sample were obtained from animals’ hearts and allowed to clot for 20 minutes in laboratory temperature and then centrifuged at 2000 rpm for 10 minutes for serum separation. After blood sampling, kidneys were removed. The left kidney weighted and was fixed in formal saline solution and then was cut to slices approximately 1 mm thickness. At least 48 hours after fixation renal slices were processed; paraffin sections (5-µ thickness) were prepared from all slices and were stained by PAS method.

**Histopathological assessments**

Kidney sections was used for assessment of histopathological changes and random microscopical fields were selected and studied at 400 time magnification.

**Proximal convoluted tubule necrosis** was assessed as a blinded manner by an expert histologist. Tubular necrosis was assessed on a score described as follows: zero, no cell necrosis; 1, mild usually single-cell necrosis in sparse tubules; 2, moderate, more than one cell involved in sparse tubules; 3, marked tubules exhibiting total necrosis in almost every power field; 4, massive total necrosis (19).

**Tubular cast and leukocyte infiltration** degree was determined using a semi-quantitative graded scale. The renal injury was semi-quantitatively: 0 , normal kidney; 1 , minimal damage (less than 5% area of the cortex or outer medulla); 2 , mild damage (5%-25% area of the cortex or outer medulla); 3 , moderate damage (25%-75% involvement of the cortex or outer medulla); 4 , severe damage (more than 75% involvement of the cortex or outer medulla). Mean scores were presented per group (20,21).

**Severity of glomerulosclerosis** was assessed semi-quantitatively. This part of the study was performed by an experienced histologist in a blinded fashion. Severity in tissue sections was assessed by assigning a score 0-4 to each glomerulus according to the glomerular tuft demonstrating sclerosis: normal glomerulus = 0; up to 25% involvement = 1; 25% to 50% involvement = 2; 50 to 75% involvement = 3 and more than 75% involvement = 4. The glomeruli were selected for assessment that appeared randomly in microscopical fields. At least 150 glomeruli were assessed in kidney sections of each animal (17,22). Average glomerulosclerosis score was calculated from total evaluated glomeruli in sections of each kidney and used as an estimation of glomerulosclerosis in each animal.

The volume density of proximal convoluted tubule per cortex was estimated by the point counting rule as described in our previously researches (13,17,20). Briefly microscopical image from each section superimposed on a point probe (frame 13 × 14-cm square with 360+). At total magnification of ×300, points that hit epithelium of proximal tubules (positive periodic acid Schiff brush border) were counted. The tubular profiles that fell inside the probe and did not cross the lower and left lines of the probe were selected for point counting. From each kidney, at least 60 microscopical fields were assessed. The volume density of proximal convoluted tubule per cortex was estimated by below equation (23).

\[
P C T \text{ volume density}=\Sigma \text{ Pp/} \Sigma \text{ Pt}
\]

where \(\Sigma \text{ Pp}\) is the sum of points hitting PCT epithelium and \(\Sigma \text{ Pt}\) is points falling on reference space (probe). If 10 fields are assessed then \(\Sigma \text{ Pt}\) is 10 × 360.

**Mean glomerular volume** were estimated from glomerular area assessment. The surface areas of 100 glomeruli images per animal kidney were measured with the Motic image plus (Version 2) software program on PAS stained tissue.
sections at 400 time magnification via motic microscope equipped by motic camera (Figure 1). The mean glomerular volume (VG) was calculated according to the formula of Weibel and Gomez.

\[ VG = \text{Area} \times 1.38/1.01 \]

where 1.38 represents the shape coefficient, and 1.01 represents the size distribution coefficient (24).

Tubular diameter measured on microscopical image via Motic image plus (version 2) software program on PAS stained tissue sections at 400 time magnification via motic microscope equipped by motic camera (Figure 1).

**Ethical issues**

Prior to the experiment the Animal Ethics Committee of the Medical University of Lorestan approved the protocol and confirmed that it is in accordance with the national health and medical research council guidelines. The principles of Animal Ethics Committee of the Medical University of Lorestan founded on the tenets of the Declaration of Helsinki.

**Statistical analysis**

All values were expressed as mean ± SEM. The histopathological and biochemical variables were compared between groups by nonparametric Mann-Whitney U test. Differences between the animal kidneys were analyzed through one-way analysis of variance (ANOVA) followed by Tukey test. Statistical analyses were performed using the SPSS 13 software and P value less than 0.05 was considered significant.

**Results**

**Effect of RA on kidney weight in renal I/R**

Kidney weight was significantly increased in I/R group (I/R without treatment) in comparison with the control group (P<0.05). Treatment by RA (100 and 200 mg/kg) ameliorated increase of kidney weight significantly when compared with I/R group and restore its level to the level of the control group (Figure 2).

**Effect of RA on histopathological parameters against renal I/R**

Tubular necrosis in PCT significantly increased after renal I/R compared with control group (P<0.05). Treatment with RA in groups 3, 4 and 5 significantly attenuated the PCT necrosis when compared with control group (P<0.05) but treatments could not keep tubular necrosis at its level in the normal group (Table 1).

Tubular cast in control group was significantly increased compared with control group. Treatment with RA in groups 4 and 5 (100 and 200 mg/kg) resulted in significant decrease in tubular cast against I/R group (P<0.05), but could not maintain tubular cast at its level in the control group (P<0.05, Table 1).

Tubular diameter in I/R group increased significantly in comparison with control group (P<0.05). The increased tubular diameter inhibited in groups 3, 4 and 5 when compared with I/R group, but only in group 5 (RA 200 mg/kg) tubular diameter saved at the same level as control group (P<0.05, Table 1).

PCT volume density showed a significant decrease in I/R group compared to control group (P<0.05). In groups 3, 4 and 5, treatment with RA showed significant increase in PCT volume density when compared with I/R group (P<0.05), but could not maintain the PCT volume density at its level in the control group (Table 1).

Mean glomerular volume increased significantly in I/R group in contrast control group (P<0.05), and saved as the same level as control with every dose of RA (P<0.05, Table 1).

There was no significant difference between groups in glomerulosclerosis assessment (Table 1). Increased leukocyte infiltration in I/R group in comparison with control group (P<0.05) were attenuated with RA treatment, but showed significant difference to control group (P<0.05, Table 1).

Serum creatinine and urea increased significantly in I/R group when compared with control group. Treatment with RA significantly decreased in comparison with control group (P<0.05, Table 1).

**Effect of RA on serum factors in combat with renal I/R**

Serum creatinine and urea increased significantly in I/R group when compared with control group. Treatment with RA significantly decreased in comparison with control group (P<0.05, Table 1).

![Figure 1. Measurement of glomerular surface area and tubular diameter on microscopical image via Motic system and software (x400, PAS).](image)

![Figure 2. Effect of RA against kidney weight in renal I/R. Group 1. Control, Group 2. I/R without treatment. Group 3. I/R surgery + RA (50 mg/kg). Group 4. I/R surgery + RA (100 mg/kg). Group 5. I/R surgery + RA (200 mg/kg). * Significant change in comparison with control group at P < 0.05. # Significant change in comparison with I/R group at P < 0.05.](image)
Table 1. Effect of RA against histopathological parameters in renal I/R.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tubular necrosis (0-4)</th>
<th>Tubular cast (0-4)</th>
<th>Tubular diameter(µm)</th>
<th>PCT volume density (%)</th>
<th>Mean glomerular volume (µm³×10)</th>
<th>Glomerular sclerosis (0-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0.41±0.15</td>
<td>0.15±0.04</td>
<td>52.91±4.03</td>
<td>0.247±0.008</td>
<td>0.485±0.22</td>
<td>0.077±0.007</td>
</tr>
<tr>
<td>2. I/R without treatment</td>
<td>2.035±0.31*</td>
<td>1.25±0.13*</td>
<td>94.86±6.58*</td>
<td>0.135±0.007*</td>
<td>0.523±0.14*</td>
<td>0.082±0.023</td>
</tr>
<tr>
<td>3. I/R + RA (50 mg/kg)</td>
<td>1.182±0.13*</td>
<td>1.041±0.08*</td>
<td>74.7±2.33*</td>
<td>0.173±0.007*</td>
<td>0.489±0.27</td>
<td>0.067±0.009</td>
</tr>
<tr>
<td>4. I/R + RA (100 mg/kg)</td>
<td>0.93±0.11*</td>
<td>0.946±0.06*</td>
<td>60.7±2.84*</td>
<td>0.203±0.012*</td>
<td>0.505±0.24</td>
<td>0.061±0.012</td>
</tr>
<tr>
<td>5. I/R + RA (200 mg/kg)</td>
<td>0.87±0.09*</td>
<td>0.626±0.15*</td>
<td>49.56±3.65*</td>
<td>0.211±0.11*</td>
<td>0.474±0.27*</td>
<td>0.065±0.008</td>
</tr>
</tbody>
</table>

Abbreviations: RA, rosmarinic acid; I/R, ischemia reperfusion; PCT, proximal convoluted tubule.
Values represented as mean ± SEM. *Significant change in comparison with control group at P < 0.05. #Significant change in comparison with I/R group at P < 0.05.

Table 2. Effect of RA against serum parameters changes in renal I/R.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum creatinine (mg/dl)</th>
<th>Serum urea (mg/dl)</th>
<th>Serum MDA (nmol/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control</td>
<td>0.61±0.04</td>
<td>39.43±1.39</td>
<td>5.77±0.25</td>
</tr>
<tr>
<td>2. I/R without treatment</td>
<td>1.397±0.23*</td>
<td>96.42±18.39*</td>
<td>9.44±0.79*</td>
</tr>
<tr>
<td>3. I/R + RA (50 mg/kg)</td>
<td>0.84±0.04*</td>
<td>58.4±5.21*</td>
<td>8.01±0.61*</td>
</tr>
<tr>
<td>4. I/R + RA (100 mg/kg)</td>
<td>0.75±0.0342*</td>
<td>54.33±4.772*</td>
<td>6.45±0.622*</td>
</tr>
<tr>
<td>5. I/R + RA (200 mg/kg)</td>
<td>0.71±0.0472*</td>
<td>49.1±1.912*</td>
<td>5.51±0.432*</td>
</tr>
</tbody>
</table>

Abbreviations: RA, rosmarinic acid; I/R, ischemia reperfusion; MDA, malodialdehyde; NO, nitric oxide; PON, paraoxanase.
Values represented as mean ± SEM. *Significant change in comparison with control group at P < 0.05. #Significant change in comparison with I/R group at P < 0.05.

Figure 3. Effect of RA on leukocyte infiltration in renal I/R. Group 1. Control, Group 2. I/R without treatment. Group 3. I/R surgery + RA (50 mg/kg). Group 4. I/R surgery + RA (100 mg/kg). Group 5. I/R surgery + RA (200 mg/kg).
* Significant change in comparison with control group at P < 0.05.
# Significant change in comparison with I/R group at P < 0.05.

Discussion
Acute kidney injury caused by renal IR due to vessels ligation during renal surgeries is often associated with high morbidity and mortality. Oxidative stress via ROS production plays a pivotal role in renal I/R injuries (25). Thus because of critical role of ROS in I/R pathogenesis, today agents that can reduce ROS have received considerable attention. Although many experimental studies show a decreased injury and preserved renal function after ROS inhibition, efficient treatments are still limited. Currently the therapy for I/R injury is mainly based on supportive care and fluid administration and I/R injury remains a major cause of morbidity and mortality (26).

Our present results for the first time showed that RA treatment significantly ameliorates renal I/R injury by improving biochemical renal function tests and histopathological parameters via reduction of lipid peroxidation.

In this study, serum MDA increased by renal I/R and ameliorated by RA treatment significantly in comparison with I/R group. Such results also reported by using other antioxidants by researchers (4,27–30). MDA is an oxidative stress marker and a major product of lipid peroxidation. Increased serum MDA resulted from increasing of free radicals or endogenous antioxidants imbalance following renal I/R (31).

Despite vital role of reperfusion for tissue revitalization, reperfusion enhances the generation of ROS. When the ROS exceed the neutralization capacity of innate antioxidants, they may attack to cell membrane polyunsaturated lipids, proteins and DNA that lead to alteration in cell functions and initiating a cascade of inflammation, apoptosis and cell death (32).
As is already known, increases in serum urea and creatinine levels are important indicators of renal dysfunction, which is characterized by changes in glomerular filtration and epithelial injury. In our study serum urea and creatinine levels increased in I/R group significantly as compared with control group. With RA treatment, serum urea and creatinine ameliorated in comparison with I/R group.

Same results were reported with use of different antioxidants by other researchers (27,33–35). Amelioration of serum urea and creatinine shows that RA probably reduces epithelial and glomerular injuries against I/R pathogenesis. In our study, histological changes (tubular necrosis, tubular cast, tubular diameter, PCT volume density, leukocyte infiltration) and kidney weight were improved by RA treating against I/R injury, but the variables could not
be saved as that of normal group. The same results were reported by using other antioxidant (27,28,33,36). Mean glomerular volume increased by I/R and ameliorated by RA treatment. There is no significance alteration between groups about glomerulosclerosis, but ozgur reported glomerulosclerosis in renal I/R injuries (37).

Ischemia decreases ATP production and finally leads to tissue ATP depletion. ATP depletion leads to Rho GTPase inactivation that makes activation of ADF (Actin depolymerizing factor) or coflin in the apical brush border of proximal tubules (38,39). Activated coflin (ADF) rapidly depolymerizes apical actin cytoskeleton and redistribution. Deterioration of microvillar structure leads to formation of membrane blebs, which may be either internalized or shed into the tubular lumen. Brush border membrane components that are released into the lumen give to cast formation and tubular occlusion (40). Activation of coflin also can induce apoptosis in PCT cells by inducing release of cytochrome C from mitochondria to cytoplasm (41).

Interruption of the apical cytoskeleton by ADF depletion also results in loss of tight junctions and adherents junctions between tubular cells and leads to tubular cells disconnection (42). Ischemia leads to relocalization of integrins to the apical membrane, and then makes detachment of PCT cells from the basement membrane (43). Ischemic proximal tubule cells also generate mediators such as proinflammatory cytokines (e.g., TNF-α, IL-6, IL-1β, and TGF-β) and chemotactic cytokines such as monocyte chemo attractant protein-1 (MCP-1) and IL-8 (44).

After ischemia and restoration of blood flow, production of ROS, such as superoxide, hydroxyl, H2O2 and activation of leukocytes and endothelial cells (5), decrease of innate antioxidant enzymes, increase of intracellular sodium and calcium, stimulation of NO synthetize, endonucleases and phospholipases occur during reperfusion (6) and contribute to reperfusion injury. Mechanism involved PCT changes by renal I/R reported by author elsewhere (45,46). Improvement of tubular cell injuries and other histological variables in I/R showed that RA may be inhibited or diminished the mechanisms involved in histological injuries.

In summary, RA ameliorated renal histopathological variables and improved serum MDA, in against renal I/R. Beside RA improved histopathological changes and renal functional test (serum creatinine and urea) probably via inhibition of lipid peroxidation but cannot save serum creatinine and urea at the same level as that of control group. The detailed protective molecular mechanisms of RA against renal I/R cannot be fully explained by our results, and needs to more investigations in future. Nevertheless histological results by RA in combat with renal I/R are satisfactory, although RA treatment cannot totally normalize renal tissue structures, serum urea and creatinine.

**Conclusion**

Our results suggest that histopathological injuries induced by renal I/R, were reduced by RA through inhibition of lipid peroxidation and finally leads to amelioration of serum urea and creatinine.

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**Conflicts of interest**

The authors declared no competing interests.

**Authors’ contribution**

MT and HRS done histopathological and morphometrical studies. AH and HA wrote the primary draft and AT edited the final paper.

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