

Protective effect of chlorogenic acid on renal ischemia/reperfusion injury in rats

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Summary Objectives: Ischemia/reperfusion (I/R) injury is a common cause of renal injury and to date, many pharmacological agents have been identified to decrease I/R injury. One of the potential compound that can target I/R injury is chlorogenic acid (CGA). It has potent anti-inflammatory, antibacterial, anti-oxidant, analgesic and antipyretic activities in *in vitro* experiments and *in vivo* animal models. The aim of the study was to investigate the protective characteristic of CGA on renal I/R injury.

Material and Methods: 24 rats were randomly allocated to three groups (n = 8): Sham, I/R+CGA and I/R groups. CGA was administered intraperitoneally at a dose of 20 mg/kg, 10 min before reperfusion. I/R injury was achieved by clamping the left renal artery for 45 minutes, followed by reperfusion for 4 hours. The left kidneys of the rats were examined for tissue damage by histopathological and biochemical examination. For histological evaluation, EGTI scoring system was used. For biochemical examination total oxidant status, total antioxidant status and oxidative stress index were used. The power analysis indicated that 8 subjects per group would be required to produce 80% chance of achieving statistical significance at p < 0.05 level. The results are expressed as mean ± SD. Mann-Whitney U was performed for statistical analysis.

Results: Histopathological examination of the tissue damage revealed that all kidneys in the sham group were normal. I-R group had significantly higher histopathological scores than other groups. Histopathological improvement was seen after CGA treatment. TAS, TOS and OSI values of I-R group were significantly higher than sham group (0.88 vs 0.76 (p: 0.004), 13.8 vs 7.04 (p: 0.021) and 0.15 vs 0.09 (p: 0.034), respectively). In CGA treated group TAS, TOS and OSI levels were 0.84, 6.47 and 0.07, respectively. CGA treatment resulted in significant improvement in TOS and OSI parameters.

Conclusions: CGA treatment provided marked improvement in renal histology and suppressed oxidative stress. Thus, CGA may have a protective effect in renal tissue against I/R injury.

KEY WORDS: Renal ischemia; Oxidative stress; Chlorogenic acid; Rat.

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INTRODUCTION

Ischemia/reperfusion (I/R) injury is a common cause of renal injury arising from a variety of clinical circumstances, including partial nephrectomy, renal transplan-

tation, iatrogenic trauma, sepsis and shock (1, 2). It is characterized by restriction of the blood flow, followed by restoring the blood flow and oxygenation. Cessation of blood flow causes ischemia and tissue damage. Restoration of blood flow during reperfusion period, often leads to exacerbation of these harmful events instead of improving (3). The pathologic processes underlying this injury are complex and include interactions between the endothelium, cell death programs and immune system (4). Reactive oxygen radicals (ROR), necrosis, apoptosis, and inflammation plays a role in this process but the exact mechanisms remain unclear (5, 6). The production of ROR is considered a key reason for oxidative stress during the reperfusion period (6). Thus, targeting oxidative stress' processes is an ideal therapeutic approach. I/R injury in renal transplantation often leads to allograft dysfunction and increased rejection (7). In partial nephrectomy, renal tissue damage may occur due to clamping of renal arteries (8). In the clinical scenario, renal I/R models generated in animals are important to understand the pathophysiology of renal injury and the potential treatment options. To date, many pharmacological agents such as N-acetylcysteine (9), Allopurinol (10) or Mannitol (11) have been identified to decrease I/R injury after nephron sparing surgery. To prevent the kidney damage due to I/R injury, several anti-inflammatories and antioxidants have been used in experimental studies (12-14). Another potential compound that can target I/R injury is chlorogenic acid (CGA). It is formed by esterification of quinic and caffeic acids and it is one of the polyphenols abundant in the human diet (15). It has potent anti-inflammatory, antibacterial, anti-oxidant, analgesic and antipyretic activities in *in vitro* experiments and *in vivo* animal models (16-18). Thus, we investigate protective effects of CGA against renal I/R injury in an *in vivo* rat model which may potentially help us in urological surgeries such as partial nephrectomy and renal transplantation where clamping is required. To examine this, we evaluated histopathological findings and biochemical analyses (including

total oxidant (TOS) status, total antioxidant (TAS) assays). In our knowledge, there have been no studies concerning the protective effect of CGA against renal I/R injury.

MATERIALS AND METHODS

The experimental and surgical procedures were conducted according to routine animal care guidelines, and the *Guide for the Care and Use of Laboratory Animals* (19). The approval was obtained from *Institutional Animal Care and Use Committee of Karadeniz Technical University (Trabzon, Turkey)* (Approval Number/ID: 2019/5). 24 male Sprague-Dawley rats (8 weeks old, weight 230-300 g) were purchased from the *Karadeniz Technical University Laboratory Animals Research Centre (Trabzon, Turkey)*. All animals were kept in captivity under the same nutritional and environmental conditions. Rats were entrained under a 12:12 h dark: light cycle (lights on 6 am-6 pm) with stable temperature (21 ± 2°C) and humidity (60 ± 5%). The rats had sterile water and food available ad libitum.

Experimental design

- Rats were randomly and equally divided into 3 groups;
1. *Vehicle-treated ischemic (I/R)*: After sterile conditions were obtained, a midline laparotomy was performed. Isotonic saline (1 mg/kg) was applied intraperitoneally 10 min before the beginning of reperfusion. The left kidney pedicle was clamped with an artery clamp for 45 minutes. After 45 minutes of left renal ischemia, the occlusion clamp was removed for reperfusion for 4 hours and the incision was closed.
 2. *Vehicle-treated sham (Sham)*: Rats underwent the same surgical procedures except unilateral renal occlusion. During the experiment, they were kept under anesthesia with gauze, soaked in saline in the abdominal cavities.
 3. *CGA-treated ischemic (I/R+CGA)*: After sterile conditions were obtained, a midline laparotomy was performed. CGA (*Sigma-Aldrich*) (20 mg/kg) was applied intraperitoneally 10 min before the beginning of reperfusion. The left kidney pedicle was clamped with an artery clamp for 45 minutes. After 45 minutes of left renal ischemia, the occlusion clamp was removed and the incision was closed.

Administration of CGA

CGA was dissolved in saline (vehicle) and administered intraperitoneally at a total dose of 20 mg/kg 10 minutes before reperfusion.

Surgical procedure

For anesthetic ketamine hydrochloride (100 mg/kg, *Ketalar, Eczacıbası, Turkey*) and xylazine (10 mg/kg) were used intraperitoneally. Following fluid replacement with 3 mL.kg⁻¹.h⁻¹ lactated Ringer's solution, the surgical area was prepared for sterilization. Then a midline laparotomy incision was performed and the left kidney pedicle was dissected. Left renal ischemia was induced by clamping the left renal artery for 45 min for the I/R and I/R-CGA groups. For reperfusion the clamp was removed and the pulsation of renal artery was verified visually. After controlling the bleeding, the skin layers

were sutured. The rats were sacrificed 4 h after completion of the reperfusion and the left kidneys were removed and stored for biochemical and histopathological examination under favorable conditions.

Histological analysis

Removed kidney was fixed with 10% formalin and embedded in paraffin. 5 µm tissue sections obtained for *Hematoxylin* and *Eosin* staining. An experienced, independent pathologist, who was blinded to the groups, analyzed three different tissue sections in each group, using a *Zeiss Axio Imager A2 microscope (Carl Zeiss AG, Germany)*. The histological evaluations of the renal tissue were graded as described in the study of *Medeiros et al.* (20) (Table 1). The scores were applied to microscopic changes consistent with tubular necrosis: vacuolization of tubular cells, tubular lumen dilation, intra-tubular cylinders, interstitial fibrosis and tubular cell necrosis. For histological evaluation, EGTI scoring system, which was developed especially for animal studies in kidney tissues in the context of injury, was also used (21), (Table 2). This system consists of histological damage in 4 separate components: *Endothelial, Glomerular, Tubular, and Interstitial*.

TAS and TOS assays

The serum TAS and TOS levels were determined with a

Table 1.
Scoring system for renal histopathology.

Score	Histopathological pattern
0	Normal
0.5	Small focal damaged areas
1	< 10% Cortical damaged zone
2	10-25% Cortical damaged zone
3	25-75% Cortical damaged zone
4	> 75% Cortical damaged zone

Table 2.
The EGTI histology scoring system.

Tissue type	Damage	Score
Tubular	No damage	0
	Loss of Brush Border (BB) in less than 25% of tubular cells. Integrity of basal membrane	1
	Loss of BB in more than 25% of tubular cells, Thickened basal membrane	2
	(Plus) Inflammation, cast formation, necrosis up to 60% of tubular cells	3
	(Plus) Necrosis in more than 60% of tubular cells	4
Endothelial	No damage	0
	Endothelial swelling	1
	Endothelial disruption	2
	Endothelial loss	3
Glomerular	No damage	0
	Thickening of Bowman capsule	1
	Retraction of glomerular tuft	2
	Glomerular fibrosis	3
Tubulo/Interstitial	No damage	0
	Inflammation, haemorrhage in less than 25% of tissue	1
	(Plus) necrosis in less than 25% of tissue	2
	Necrosis up to 60%	3
	Necrosis more than 60%	4

novel automatic method, developed by Erel (22, 23). The ratio of TAS to TOS is defined as *oxidative stress index* (OSI), expressed as percentage.

Statistical analysis

IBM SPSS 22 version (SPSS IBM, Turkey) program was used for analysis. Before starting to study, we performed power analysis. The power analysis indicated that 8 subjects per group would be required to produce 80% chance of achieving statistical significance at $p < 0.05$ level. The Kolmogorov-Smirnov test was performed to determine the normality of data. The results are expressed as mean \pm SD. Mann-Whitney U was performed for statistical analysis, as appropriate. A p value below 0.05 was considered statistically significant.

Table 3.
Histopathology scoring of cortical damage of the groups.

Rats	Sham group	I/R group	I/R + CGA group
1	0	0.5	0.5
2	0	1	0
3	0	1	1
4	0	1	0.5
5	0	0.5	0.5
6	0	0.5	0.5
7	0	1	1
8	0	0.5	0.5

Table 4.
Comparison of rats in terms of EGTI scoring.

Rats	Sham group	I/R group	I/R + CGA group
1	0	8	5
2	0	7	3
3	0	8	6
4	0	8	3
5	0	5	3
6	0	5	4
7	0	8	7
8	0	4	6

Figure 1.
Histological images of rat renal cortex sections. a; Normal renal cortex (sham group), b; tubular necrosis (I/R), c; tubular injury (I/R+CGA group).

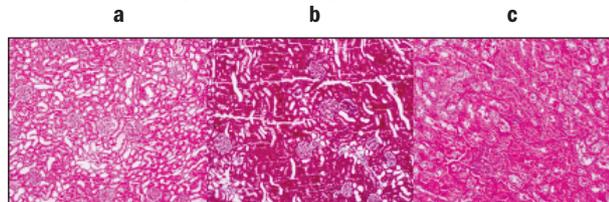


Table 5.
Comparison of groups in terms of biochemical parameters.

	TAS median (min-max)	P	TOS median (min-max)	P	OSI median (min-max)	P
Group 1-2	0.84 (0.76-1)-0.76 (0.66-0.80)	0.021	6.47 (2.1-23.7)-7.04 (4.7-13.9)	0.83	0.07 (0.03-0.26)-0.09 (0.06-0.21)	0.52
Group 2-3	0.76 (0.66-0.80)-0.88 (0.75-0.98)	0.0048	7.04 (4.7-13.9)-13.8 (6.4-18.5)	0.021	0.09 (0.06-0.21)-0.15 (0.09-0.19)	0.034
Group 1-3	0.84 (0.76-1)-0.88 (0.75-0.98)	0.49	6.47 (2.1-23.7)-13.8 (6.4-18.5)	0.046	0.07 (0.03-0.26)-0.15 (0.09-0.19)	0.040

(1) I-R +CGA, (2) sham, (3) I-R. Mann Whitney U test

RESULTS

CGA showed histopathologic improvement in ischemia reperfusion injury as shown in Tables 3 and 4. All rats in the sham group had normal histopathological findings. By contrast, as shown in table 3, 4 (50%) rats in the I/R group had small focal damaged areas and 4 (50%) had $< 10\%$ cortical damage. In I/R+CGA group, 1 (12.5%) rat had normal kidney, 5 (62.5%) had small focal damaged areas and 2 (25%) had $< 10\%$ cortical damage. EGTI scores of the rats in each group are shown in Table 4, separately. The pathological figures were shown in Figure 1. As shown in Table 5, CGA improved biochemical values. TAS, TOS and OSI values of the sham group was significantly lower than I/R group ($P: 0.004, 0.021, 0.034$, respectively). There was no significant difference between the sham and I/R + CGA groups in terms of TOS and OSI values ($P: 0.83, 0.52$, respectively). TOS and OSI values of the I/R group were significantly higher than the other groups ($P: 0.021, 0.034$, respectively for comparison of sham and I/R groups and $P: 0.046, 0.040$, respectively for comparison of I/R+CGA and I/R groups).

DISCUSSION

Renal I/R injury is a major reason for renal dysfunction. It induces an inflammatory response and oxidative stress. At the site of inflammation, leukocytes infiltration occurs and results in the secretion of pro-inflammatory cytokines, including TNF- α , HMGB1, IL-6, and IL-1 β (24). ROR, produced during reperfusion is considered to play a central role in I/R injury by direct attack on multiple molecule sequences. In living organisms, ROR arise as a result of normal biological metabolism and they can distort the structures of DNA, fats, proteins and carbohydrates. To ensure I/R experimentally, the left renal artery was occluded for 45 min. It was shown that the 45 min model of IR injury used here provides reproducible and robust assessment of treatment effects against IR injury (25, 26). Oxidative stress and antioxidant status can be assessed by several markers and various methods. However, it is both time-consuming and costly to measure these markers separately (27). For this reason, in this study we used TOS, TAS and OSI levels to measure the oxidative stress status. In recent years it has become more common to measure these values (23, 28, 29). In this study TAS, TOS and OSI levels were found to be significantly higher in I/R group compared to sham group and CGA alleviated these parameters. The histopathological classification system presented in Table 1 was used for histological diagnosis. However, since this system shows only cortical damage. Renal IR injury is a complex process which effects the glomerular, tubulo-interstitial and endothelial cells. Acute tubular necrosis, loss of endothelial cell integrity, glomerular

ischemic damage and tubulo-interstitial damage are the hallmarks of renal IR injury which is important for complete and comprehensive documentation. For this reason, EGTI scoring system was used together with other system. Because it is reliable, simple, more informative and more detailed scoring system about the degree of tissue damage of the kidney (21). The histological study showed tubular dilation, tubular necrosis, cellular edema and inflammatory cell infiltration in the tubular interstitium. These lesions were less intense in CGA treated rats compared to untreated animals.

In order to block inflammatory response and oxidative stress, several drugs have been used to prevent renal I/R injury in several experimental studies (13, 14). However, the new experimental studies will help us to find the most appropriate feasible treatment. In the present study, CGA was examined for its potential effects on regulating renal I/R injury. CGA is a polyphenol, which is abundantly found in coffee, fruits and vegetables. It has been used as an antioxidant, analgesic and anti-inflammatory. It has a certain number of R-OH radicals that are capable of forming the hydrogen free radical, thereby protecting tissue cells from oxidative damage (30). It has been shown to act as a scavenger of hydroxyl radicals, peroxynitrite and superoxide radicals in a concentration-dependent manner *in vitro* (31). In the study of Yun *et al.* (32) CGA given at 10 mg/kg intraperitoneally, 10 min before ischemia and reperfusion was chosen as the most effective dose for histology evaluation for I/R- induced hepatic injury.

In our study, it was administered intraperitoneally at a total dose of 20 mg/kg 10 minutes before reperfusion. Previous studies in rat models have shown that CGA is protective against hepatic and focal cerebral I/R injury (32, 33). We have observed that CGA has a protective effect against renal I/R injury in our study. We considered that CGA may serve a protective role in the rat model of renal I/R injury. To the best of our knowledge, there is no data showing the effect of CGA on I/R kidney injury and evaluating TAS, TOS levels and histopathology together.

As a limitation of our study, since we did not perform a right nephrectomy, we did not measure plasma creatinine, the most commonly used marker as a measure of renal excretory function (34).

CONCLUSIONS

CGA treatment provided marked improvement in renal histology and suppressed oxidative stress. Thus, CGA may have a protective effect in renal tissue against I/R injury.

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