Cardiovascular Topics

Investigation of the effects of ellagic, vanillic and rosmarinic acid on reperfusion-induced renal injury

Alper Gurmen, Orkut Guclu, Serhat Huseyin, Nuray Can, Eray Ozgun, Mursel Buyukadali, Adem Reyhancan, Suat Canbaz

Abstract

Introduction: The aim of this study was to investigate the effects of ellagic, vanillic and rosmarinic acid on reperfusion-related kidney damage, developed in an experimental lower-extremity ischaemia/reperfusion (I/R) model.

Methods: Forty-eight female Sprague-Dawley rats were divided into six groups. A median laparotomy and dissection were performed. In the I/R group, 60 minutes of ischaemia followed by 120 minutes of reperfusion was achieved. In addition one group was given 100 mg/kg ellagic acid, one group was given 12 mg/kg vanillic acid, one group was given 50 mg/kg rosmarinic acid and one group was given all three drugs 15 minutes before clamp removal. Bilateral kidney and blood samples were taken in all groups.

Results: Tubular epithelial degeneration, necrosis of the tubule epithelium and vessel wall thickening were significantly higher in the I/R group. Some parameters in the groups that were given drugs were found to be lower than in the I/R group and close to that of the control group. Total oxidant status (TOS) and oxidative stress index (OSI) were significantly higher and total antioxidant status (TAS) was significantly lower in the I/R group. Although not statistically significant in the groups given drugs, TAS was higher, and TOS and OSI were lower than in the I/R group.

Conclusion: The antioxidant effect of ellagic, vanillic and rosmarinic acid administration may have beneficial effects on renal damage after reperfusion in acute lower-extremity ischaemia. This study is expected to provide information for future clinical trials.

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Ischaemia is the inability to remove waste products produced by metabolism as a result of decreased or interrupted blood flow to a tissue or organ. Reperfusion, on the other hand, is the process of restoring blood flow in order to provide the necessary energy requirement to the tissue or organ after ischaemia and to remove harmful metabolic end products formed after ischaemia. With the restoration of blood flow, the end products formed by oxidation of these metabolites accumulate in the tissue and spread to the system.[1,2]

Neutrophil activation by reperfusion, release of pro-inflammatory cytokines, complement activation, formation of free oxygen radicals and proteases, and release of vasoconstrictor agents such as endothelin, angiotensin and catecholamines are responsible for the local and systemic effects of ischaemia/reperfusion (I/R) injury.[3]

Although it is most commonly seen in temporary cross-clamp applications to the abdominal aorta in aortic surgery, lower-extremity I/R injury occurs in unilateral or bilateral acute femoral artery occlusions, and traumatic or iatrogenic arterial injuries.[4,5] In addition, other causes of reperfusion injury include thrombolytic treatments applied in cerebrovascular events, myocardial infarction, mesenteric and peripheral arterial embolisms, correction of ischaemia and hypovolaemia occurring in surgical and traumatic events such as sepsis, shock, burns, pancreatitis, organ transplantation, and tourniquets applied to the extremities during surgical intervention.[4]

I/R injury causes important pathologies in many organs, especially the kidney, lung, liver and heart. I/R can occur with many clinical manifestations, from transient reperfusion arrhythmias to multiple organ failure syndrome, which can be fatal.[6]

Reactive oxygen species (ROS) attack membrane lipids, resulting in lipid peroxidation. They can also affect cellular proteins, lipids, nucleic acids and other potentially sensitive substances. This process eventually results in excessive free radical production and organ dysfunction, and ROS can lead to many diseases. They are mainly responsible for I/R injury.

Various treatment strategies applied to reduce I/R damage include several antioxidant vitamins, bioflavonoids and drugs.[7]

Ellagic acid (EA) is a polyphenolic compound found in plants in fruit and nuts, such as strawberries, walnuts, hazelnuts and blueberries. It has been reported that EA exhibits different pharmacological effects, including anti-inflammatory, antioxidant and inhibition of tumour formation.[7] Various studies have shown that vanillic acid (VA) is associated with multiple pharmacological activities, including inhibition of snake venom activity, antimicrobial, analgesic, anti-inflammatory and antioxidant activities.[8]

Rosmarinic acid (RA) is a naturally occurring polyphenolic antioxidant found in many common plants. RA is isolated from lemon balm and peppermint plants, including *Melissa officinalis*, *Rosmarinus officinalis* and *Prunella vulgaris*. It has been shown that RA has antioxidant, anticarcinogenic, anti-inflammatory, antidepressant and antimicrobial effects.[9]

In this study, we planned to investigate the histopathological and biochemical effects of EA, VA and RA on kidney damage due to reperfusion in rats exposed to lower-extremity ischaemia by application of a cross-clamp to the abdominal aorta.

Methods

This study was carried out in the local experimental animals unit laboratory after the approval of the animal experiments local ethics committee's decision. Forty-eight female Sprague-Dawley rats, 3.5–4 months old and weighing 190–250 g, were used in the study. The rats were randomly divided into six groups of equal numbers ($n = 8$).

All rats were anaesthetised by intramuscular administration of ketamine HCl 40 mg/kg plus xylazine hydrochloride 5 mg/kg to the left forefoot muscle after eight hours of fasting. During the procedure, the rats were kept breathing spontaneously. The rats were placed on the table in the supine position under a heat lamp.

A midline median laparotomy was performed in the skin of all rats from just below the xiphoid to 0.5 cm above the pubis. After laparotomy, the intestines were deviated to the right with the help of a damp cloth. The infrarenal abdominal aorta and bilateral kidneys were explored by blunt dissection. Low-dose (100 µU/kg) heparin was administered to all rats for anticoagulant purposes. An atraumatic microvascular clamp was placed on the infrarenal abdominal aorta.

After clamping, approximately 5 ml of warm saline was sprayed into the peritoneal cavity. To prevent fluid loss, the abdomen was closed with three silk sutures. After one hour of ischaemia, the atraumatic microvascular clamp in the infrarenal abdominal aorta was removed and a two-hour reperfusion period was applied.

In the groups to which the drugs were to be administered, 15 minutes before the start of reperfusion they were administered intraperitoneally, at doses determined based on similar studies. Aortic ischaemia was followed by loss of pulsation in the aorta after clamping, and reperfusion was followed by the presence of pulsation in the aorta after removal of the clamp. At the end of the experiment, blood samples and the bilateral kidneys of the rats in all groups were taken. After the procedure, the rats were sacrificed.

For histopathological examination, kidney tissue was individually fixed in 10% neutral buffered formaldehyde solution. Paraffin blocks were prepared from the samples. Sections of 3–4 µm were taken from these paraffin blocks with the help of a microtome and stained with haematoxylin–eosin (H+E). Histopathological examination was performed with a light microscope.

In the preparations we evaluated glomerular sclerosis, focal glomerular necrosis, Bowman's capsule dilatation, degeneration of tubular epithelium, necrosis of tubular epithelium, tubular dilatation, interstitial inflammatory infiltration, vessel congestion, vessel wall thickening and interstitial fibrosis. We scored the samples as follows: 0: no change; +1: focal, light; +2: multifocal, medium; +3: prominent, widespread.

Blood samples taken from the rats were transferred to the biochemistry laboratory in yellow-capped biochemistry tubes without anticoagulant. The coagulation process of the samples was delayed. Then, they were separated into serum by centrifugation at 3 000 *g* for 10 minutes. The obtained samples were portioned and stored at –80°C until assaying.

Serum total antioxidant status (TAS) and total oxidant status (TOS) were measured spectrophotometrically in the samples at room temperature on the study day, and the oxidative stress index (OSI) was calculated. Serum arylesterase and lactonase activities were measured kinetically in the spectrophotometer using phenylacetate and dihydrocoumarin, respectively. Serum 8-hydroxydeoxyguanosine level was measured by enzyme-linked immunoassay (ELISA) method according to the package insert of the commercial kit.

Statistical analysis

Based on the study performed by Bakar *et al.*,[10] the effect size was determined as 0.85. Considering this effect size, it was predicted that it would be sufficient to include a total of 36 'observations' in the study at 80% power and 5% significance level. However, since a 30% loss was predicted in the study, it was decided to include 48 'observations' in the study.

The conformity of histopathological and biochemical measurements to a normal distribution was examined with the Shapiro–Wilk test. According to the results of the normality test, the variables in the study are primarily expressed as median (minimum–maximum) values and are supported by mean and standard deviation (SD) values.

The Kruskal–Wallis test was used for comparisons between groups. In the case of general significance after the Kruskal–Wallis test, subgroup analyses were performed using the Dunn–Bonferroni test.

The analyses of the study were performed in SPSS (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, version 23.0. Armonk, NY: IBM Corp) program, and a type I error rate was accepted as 5% in statistical comparisons. The level of significance was determined as $p < 0.05$ in all statistical analyses.

Results

Median (min–max) values and mean values ± SD of the histopathological parameters are given in Table 1. Statistical analysis (*p*-values) of the histopathological data between pairs is given in Table 2.

In the histopathological examination, no difference was found between the groups in levels of glomerular sclerosis, focal glomerular necrosis, interstitial inflammatory infiltration and

Table 1. Histopathological parameters

Groups	*Glomerular sclerosis*	*Focal glomerular necrosis*	*Bowman's capsule dilatation*	*Tubule epithelial degeneration*	*Necrosis of tubule epithelium*	*Tubular dilatation*	*Interstitial inflammatory infiltration*	*Vascular congestion*	*Vascular wall thickening*	*Interstitial fibrosis*
Control	0.00 (0.00–0.00) 0.00 ± 0.00	0.00 (0.00–0.00) 0.00 ± 0.00	0.50 (0.00–2.00) 0.87 ± 0.99	1.00 (0.00–2.00) 0.87 ± 0.64	0.00 (0.00–1.00) 0.12 ± 0.35	0.00 (0.00–1.00) 0.37 ± 0.51	1.00 (1.00–2.00) 1.37 ± 0.51	1.00 (0.00–3.00) 1.00 ± 0.92	0.00 (0.00–1.00) 0.37 ± 0.51	0.00 (0.00–0.00) 0.00 ± 0.00
I/R	0.00 (0.00–0.00) 0.00 ± 0.00	0.00 (0.00–0.00) 0.00 ± 0.00	1.00 (0.00–3.00) 1.25 ± 0.88	2.00 (2.00–3.00) 2.25 ± 0.46	2.00 (1.00–3.00) 1.87 ± 0.64	2.00 (1.00–3.00) 1.87 ± 0.83	2.00 (1.00–3.00) 2.00 ± 0.92	2.50 (1.00–3.00) 2.37 ± 0.74	2.00 (1.00–3.00) 1.75 ± 0.70	0.00 (0.00–0.00) 0.00 ± 0.00
I/R + EA	0.00 (0.00–0.00) 0.00 ± 0.00	0.00 (0.00–0.00) 0.00 ± 0.00	1.00 (0.00–3.00) 1.42 ± 0.97	2.00 (2.00–3.00) 2.42 ± 0.53	1.00 (1.00–3.00) 1.57 ± 0.78	3.00 (3.00–3.00) 3.00 ± 0.00	2.00 (2.00–3.00) 2.28 ± 0.48	3.00 (1.00–3.00) 2.14 ± 1.06	1.00 (0.00–2.00) 0.85 ± 0.69	0.00 (0.00–0.00) 0.00 ± 0.00
I/R + VA	0.00 (0.00–0.00) 0.00 ± 0.00	0.00 (0.00–1.00) 0.12 ± 0.35	0.00 (0.00–1.00) 0.37 ± 0.51	3.00 (2.00–3.00) 2.62 ± 0.51	2.00 (1.00–3.00) 2.25 ± 0.70	1.00 (0.00–3.00) 1.00 ± 0.92	2.00 (0.00–3.00) 2.00 ± 1.06	2.00 (1.00–3.00) 2.12 ± 0.83	2.00 (1.00–3.00) 1.87±0.83	0.00 (0.00–0.00) 0.00 ± 0.00
I/R + RA	0.00 (0.00–0.00) 0.00 ± 0.00	0.00 (0.00–1.00) 0.12 ± 0.35	0.00 (0.00–1.00) 0.12 ± 0.35	2.00 (2.00–3.00) 2.37 ± 0.51	2.00 (1.00–3.00) 1.87 ± 0.83	1.50 (1.00–3.00) 1.75 ± 0.88	2.00 (1.00–3.00) 2.00 ± 0.75	1.00 (0.00–2.00) 1.00 ± 0.75	2.00 (1.00–3.00) 2.25 ± 0.70	0.00 (0.00–0.00) 0.00 ± 0.00
I/R + EA + VA + RA	0.00 (0.00–0.00) 0.00 ± 0.00	0.00 (0.00–0.00) 0.00 ± 0.00	2.00 (1.00–3.00) 1.75 ± 0.70	2.00 (1.00–3.00) 2.00 ± 0.75	1.50 (1.00–3.00) 1.62 ± 0.74	3.00 (2.00–3.00) 2.87 ± 0.35	2.00 (1.00–3.00) 2.12 ± 0.83	3.00 (1.00–3.00) 2.75 ± 0.70	1.00 (1.00–2.00) 1.12 ± 0.35	0.00 (0.00–0.00) 0.00 ± 0.00
p-value		0.55	< 0.01*	< 0.01*	< 0.01*	< 0.01*	0.28	< 0.01*	< 0.01*	

I/R: ischaemia/reperfusion, EA: ellagic acid, VA: vanillic acid, RA: rosmarinic acid. The values are of the tissues according to the histopathological scoring system. Values are given as median (min–max) and mean and standard deviation. *Statistically significant ($p < 0.05$).

interstitial fibrosis. Tubular epithelial degeneration, necrosis of the tubule epithelium and vessel wall thickening were significantly higher in the I/R group.

Significant tubular dilation, significant vessel wall thickening (Fig. 1A–C) and significant Bowman's capsule dilatation (Fig. 1D) in the I/R group were seen. Bowman's capsule dilatation and vessel wall thickening in the I/R + EA group, Bowman's capsule dilatation in the I/R + VA group, and vessel congestion in the I/R + RA group were found to be lower than in the I/R group and close to that of the control group, although it was not statistically significant.

A case without Bowman's capsule dilatation in the EA group is shown in Fig. 2A. A case without Bowman's capsule dilatation in the VA group is shown in Fig. 2C.

Median (min–max) values and mean ± SD of biochemical parameters are given in Table 3. Statistical analysis (p-values) of biochemical data between pairs is given in Table 4.

In the biochemical examination, no difference was found between the groups with regard to arylesterase or lactonase activity and 8-hydroxydeoxyguanosine levels. TOS and OSI were significantly higher and TAS was significantly lower in the I/R group. Although not statistically significant in the I/R + EA, I/R + VA and I/R + RA groups, TAS was higher, and TOS and OSI were lower than in the I/R group.

Table 2. Statistical analysis (p-values) of histopathological data between pairs

Groups	*Bowman's capsule dilatation*	*Tubule epithelial degeneration*	*Necrosis of tubule epithelium*	*Tubular dilatation*	*Vascular congestion*	*Vascular wall thickening*
Control – I/R	> 0.99	0.03*	< 0.01*	0.16	0.15	0.02*
Control – I/R + EA	> 0.99	< 0.01*	0.1	< 0.01*	0.57	> 0.99
Control – I/R + VA	> 0.99	< 0.01*	< 0.01*	> 0.99	0.57	0.01*
Control – I/R + RA	> 0.99	0.01*	< 0.01*	0.28	> 0.99	< 0.01*
Control – I/R + EA + VA + RA	0.79	0.23	0.05	< 0.01*	0.01*	> 0.99
I/R – I/R + EA	> 0.99	> 0.99	> 0.99	0.71	> 0.99	0.67
IR – I/R + VA	0.79	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
I/R – I/R + RA	0.14	> 0.99	> 0.99	> 0.99	0.14	> 0.99
I/R– I/R + EA + VA + RA	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
I/R + EA – IR + VA	0.42	> 0.99	> 0.99	0.01*	> 0.99	0.43
I/R + EA – I/R + RA	0.07	> 0.99	> 0.99	0.44	0.53	0.04*
I/R + EA – I/R + EA + VA + RA	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99
I/R + VA – I/R + RA	> 0.99	> 0.99	> 0.99	> 0.99	0.53	> 0.99
I/R + VA – I/R + EA + VA + RA	0.03*	> 0.99	> 0.99	0.01*	> 0.99	> 0.99
I/R + RA – I/R + EA + VA + RA	< 0.01*	> 0.99	> 0.99	0.66	0.01*	0.17

I/R: ischaemia/reperfusion, EA: ellagic acid, VA: vanillic acid, RA: rosmarinic acid. *Statistically significant ($p < 0.05$).

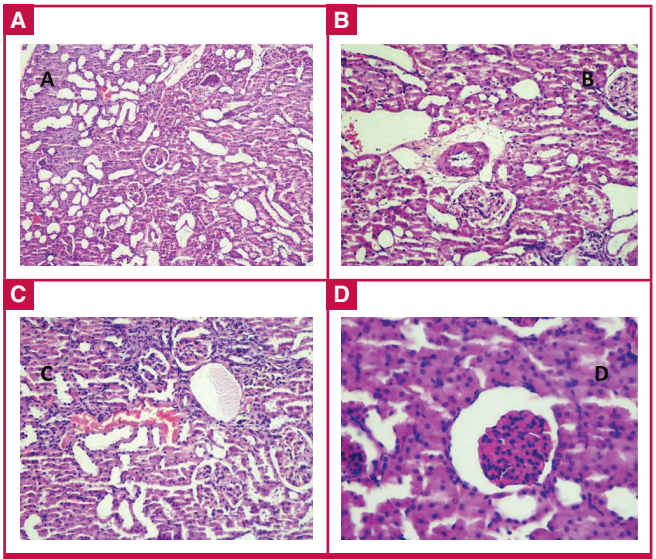

Fig. 1. A. Case without dilatation of Bowman's capsule in the EA group (H&E × 400). B. Case without vascular congestion in the RA group (H&E × 400). C. Case without dilatation of Bowman's capsule in the VA group (H&E × 400). D. Mild tubular dilatation in VA group (H&E × 400)

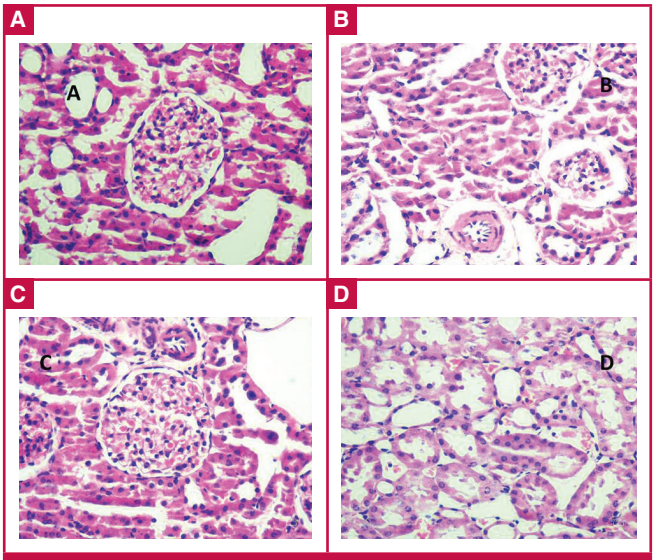

Fig. 2. A. Case without dilatation of Bowman's capsule in the EA group (H&E × 400). B. Case without vascular congestion in the RA group (H&E × 400). C. Case without dilatation of Bowman's capsule in the VA group (H&E × 400). D. Mild tubular dilatation in VA group (H&E × 400).

Table 4. Statistical analysis (p-values) of biochemical data between pairs

Groups	*TOS (µmol H_2O_2 eq/l)*	*TAS (mmol eq trolox/l)*	*OSI*
Control – I/R	0.04*	< 0.01*	< 0.01*
Control – I/R + EA	> 0.99	0.7	> 0.99
Control – I/R + VA	> 0.99	0.56	> 0.99
Control – I/R + RA	< 0.01*	0.3	< 0.01*
Control – I/R + EA + VA + RA	0.01*	> 0.99	0.34
I/R – I/R + EA	> 0.99	0.57	0.24
IR – I/R + VA	> 0.99	0.5	0.28
I/R – I/R + RA	> 0.99	0.9	> 0.99
I/R – I/R + EA + VA + RA	> 0.99	< 0.01*	0.94
I/R + EA – IR + VA	> 0.99	> 0.99	> 0.99
I/R + EA – I/R + RA	0.13	> 0.99	0.3
I/R + EA– I/R + EA + VA + RA	> 0.99	> 0.99	> 0.99
I/R + VA – I/R + RA	0.08	> 0.99	0.36
I/R + VA – I/R + EA + VA + RA	> 0.99	> 0.99	> 0.99
I/R + RA – I/R + EA + VA +RA	> 0.99	> 0.99	> 0.99

I/R: ischaemia/reperfusion, EA: ellagic acid, VA: vanillic acid, RA: rosmarinic acid, TOS: total oxidant status, TAS: total antioxidant status, OSI: oxidative stress index. *Statistically significant ($p < 0.05$).

Discussion

Considering the results of our study, we found a statistically and clinically significant difference between the control and the I/R group. In the I/R + EA, I/R + VA and I/R + RA groups, we achieved a clinically significant decrease in oxidative values/increase in antioxidant values, although not statistically significant, compared to the I/R group.

The reason why no statistically significant results could be obtained in the I/R + drug groups compared to the I/R group may have been due to the intraperitoneal route of administration of the drugs and the low dose. In addition, the duration of administration of the drugs may have affected drug efficacy.

A certain period of ischaemia is required for experimental I/R damage to occur in the kidneys. It has been shown that reperfusion injury occurs in the rat kidney after a 60-minute ischaemic period.[11] In our study, 60 minutes of ischaemia followed by 120 minutes of reperfusion was applied.

Since it has been shown that free radicals form rapidly, within 15–20 seconds after reperfusion, it is known that if an agent is to be used to reduce reperfusion damage, it is effective when given 15 minutes before reperfusion, and its preventative property is not effective if given after reperfusion.[2,12] We tried to ensure that our study was effective by applying EA, VA and RA 15 minutes before the aortic clamp was removed.

It has been shown in experimental studies that the negative effects of I/R of the lower extremity on distant organ damage are reduced by some substances such as melatonin, aprotinin, ascorbic acid and *n*-acetylcysteine.[4,13,14,15] In addition, experimental animal studies on the antioxidant effects of therapeutic agents such as EA, VA and RA, which we used in our study, are available in the literature.[16-19] However, this is the first study in the literature on the effects of EA, VA and RA on kidney damage caused by I/R in the lower extremities.

Hwang *et al.*[20] showed in their study that the antioxidant and cytoprotective properties of EA prevented liver damage caused by various types of oxidative stress. Kapan *et al.*[21] also investigated the possible protective effect of EA on the liver and distant organs against I/R injury. Although they observed

Table 3. Biochemical parameters

Groups	*TOS (µmol H_2O_2 eq/l)*	*TAS (mmol eq trolox/l)*	*OSI*	*Arilesterase activity (U/l)*	*Lactonase activity (U/l)*	*8-hydroxy deoxy-guanosine (ng/ml)*
Control	9.01 (7.29–17.73) (10.06 ± 3.35)	1.58 (1.15–1.78) 1.54 ± 0.18	0.59 (0.41–1.55) 0.69 ± 0.36	50.22 (44.73–58.59) 50.52 ± 4.81	7.16(5.69–8.46) 7.02 ± 0.89	5.94 (4.83–7.58) 6.17 ± 1.03
I/R	16.45(14.58–25.12) 18.26 ± 3.70	0.95 (0.72–1.30) 1.01 ± 0.20	1.88 (1.25–2.87) 1.86 ± 0.53	46.53 (38.93–52.62) 46.64 ± 5.05	6.18 (4.17–8.42) 6.22 ± 1.42	7.22 (4.91–8.66) 7.05 ± 1.19
I/R + EA	14.19 (12.02–19.51) 14.78 ± 2.70	1.32 (1.17–1.50) 1.32 ± 0.10	1.10 (0.80–1.44) 1.12 ± 0.23	45.23 (39.38–51.45) 45.63 ± 4.49	7.30 (5.25–8.38) 7.14 ± 1.04	6.45 (5.08–7.72) 6.42 ± 0.83
I/R + VA	12.85 (8.57–21.18) 14.39 ± 4.93	1.36 (0.98–1.56) 1.27±0.22	1.05 (0.59–2.08) 1.17±0.50	42.86 (33.04–53.50) 43.39 ± 7.31	8.02 (6.55–9.76) 8.15 ± 0.96	5.92 (4.76–9.00) 6.32 ± 1.45
I/R + RA	21.97 (18.03–27.49) 22.40 ± 3.82	1.30 (0.98–1.46) 1.27 ± 0.16	1.96 (1.35–2.08) 1.78 ± 0.33	45.67 (41.08–55.25) 46.32 ± 4.65	6.58 (4.91–9.76) 6.66 ± 1.50	6.77 (5.62–8.81) 7.02 ± 1.19
I/R + EA + VA + RA	19.95 (12.02–26.70) 19.49 ± 5.35	1.49 (1.10–2.06) 1.53 ± 0.30	1.23 (0.75–1.95) 1.29 ± 0.37	43.82 (38.14–49.52) 44.29 ± 4.09	7.58 (5.60–9.21) 7.44 ± 1.29	5.60 (4.83–9.40) 6.47 ± 1.76
p-value	< 0.01*	0.001*	< 0.01*	0.259	0.064	0.527

I/R: ischaemia/reperfusion, EA: ellagic acid, VA: vanillic acid, RA: rosmarinic acid, TOS: total oxidant status, TAS: total antioxidant status, OSI: oxidative stress index. Values are given as median (min–max) and mean and standard deviation. *Statistically significant ($p < 0.05$).

improved histopathological changes with EA application, they could not obtain statistically significant results. In our study, we obtained improved histopathological changes in vessel congestion and vessel wall thickening in the EA group compared to the I/R group, although it was not statistically significant.

Yao *et al*.[22] showed in their study that VA can alleviate acute myocardial hypoxia/reoxygenation injury by preventing oxidative stress. Stanely Mainzen Prince *et al.*[23] observed in their study that there was a decrease in lipid peroxidation with VA treatment in cardiotoxic rats induced by isoproterenol, and that VA administration at a dose of 10 mg/kg was more effective than 5 mg/kg.

Luo *et al*.[24] gave daily RA (50, 75 or 100 mg/kg) via gavage seven days before pulmonary I/R injury in their study published in 2022. They found an increase in hypoxaemia, pulmonary oedema and serum inflammatory cytokines when they caused pulmonary I/R damage. They showed that RA pretreatment (75 and 100 mg/kg) effectively restored injury parameters, while 50 mg/kg RA pretreatment had less of an effect.

Considering the histopathological results of our study, we found a statistically and clinically significant difference between the control and I/R groups. In the I/R + EA, I/R + VA, and I/R + RA groups, we achieved improvement according to the histopathological scoring system, although it was not statistically significant, compared to the I/R group.

In all groups, we found clinically significant scores compared to the control group, which means better results [Bowman's capsule dilatation (control: 0.87, I/R + EA: 1.42, $p > 0.99$) and vessel wall thickening (control: 0.37, I/R + EA: 0.85 $p > 0.99$)]. [Bowman's capsule dilatation (control: 0.87, I/R + VA: 0.37, $p > 0.99$)]. [Vascular congestion was similar in both groups (control: 1.00, I/R + RA: 1.00, $p > 0.99$)].

Uzar *et al*.[25] found that catalase, paraoxonase (PON-1) and TAS values returned to normal levels in rats treated with EA in their study. Oxidative stress was measured in the nerve endings of diabetic rats. They found that malondialdehyde (MDA), TOS, nitric oxide and OSI values were decreased compared to untreated rats. In our study, we found lower TOS and OSI values and higher TAS values in the I/R + EA group compared to the I/R group.

Radmanesh *et al*.[26] showed a decrease in infarct size, MDA and myocardial dysfunction in the VA group in their study by creating I/R in rat cardiac tissue. In our study, in the I/R + VA group, we also found a decrease in TOS and OSI values, which indicates oxidative status, and an increase in TAS values, which indicates antioxidant status.

Murillo-González *et al*.[27] found that PON-1 lactonase activity was lower in patients with cardiovascular disease compared to the control group. In our study, lactonase activity decreased in the I/R group compared to the control group, and a decrease in antioxidant activity was observed (control: 7.02 U/l, I/R: 6.22 U/l). Lactonase activity in the I/R + EA group (7.14 U/l) and in the I/R + VA group (8.15 U/l) was higher than in the I/R and control groups and an increase in antioxidant activity was observed. A *p*-value close to a statistically significant value was found in lactonase activity ($p = 0.064$).

The major limitation of this study is the animal model. The limitation relates to the creation of isolated I/R without thrombotic tendency. Thrombosis metabolism may lead to different results in different study groups with thrombotic tendencies.

Conclusion

The mechanism of I/R injury is complex and multifactorial. Studies on drugs and methods of therapeutic or prophylactic treatment of I/R injury are ongoing. In our study, it was observed that lower-extremity I/R injury caused histopathological and biochemical damage in renal tissue. EA, VA and RA may possess antioxidant properties that could potentially alleviate post-reperfusion renal injury in acute lower-extremity ischaemia. This research hopes to provide valuable insights for future clinical trials.

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