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ORIGINAL PAPER

Hasan Erdogan · Ersin Fadillioglu · Murat Yagmurca
Muharrem Uçar · M. Kemal Irmak

Protein oxidation and lipid peroxidation after renal ischemia-reperfusion injury: protective effects of erdosteine and *N*-acetylcysteine

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Abstract Oxygen radicals have roles in the renal ischemia-reperfusion (IR) injury usually encountered in several conditions such as renal transplantation. The aim of this study was to investigate the effects of erdosteine and *N*-acetylcysteine (NAC) on the oxidant/antioxidant status and microscopy of renal tissues after IR injury. Male Sprague–Dawley rats were randomly assigned to four groups: control untreated rats, IR (30 min ischemia and 120 min reperfusion), IR + NAC (i.p.; 180 mg/kg) and IR + erdosteine (oral; 50 mg/kg/day for 2 days before experiments) groups. After unilateral renal IR, the right kidney was rapidly excised and sectioned vertically into two pieces for microscopic examination and biochemical analysis. Erdosteine and NAC treatment did not cause any significant change in the activity of superoxide dismutase (SOD) in comparison with the IR group, even if the SOD activity increased in IR groups than in the control group. Catalase (CAT) activity was decreased in the IR group in comparison with control and IR + erdosteine groups ($P < 0.05$), whereas it was higher in the IR + erdosteine group than in the IR + NAC group ($P < 0.05$). Xanthine oxidase (XO) activity was higher in all the IR-performed groups than in the control group ($P < 0.05$). Thiobarbituric acid-reactive substances (TBARS) level and protein carbonyl (PC) content were increased after IR injury ($P < 0.05$). Erdosteine or NAC treatments ameliorated these increased TBARS and PC contents in comparison with the IR group ($P < 0.05$). Light microscopy of the IR group showed tubular dilatation, tubular necrosis and vacuole formation in epithelial cells. Erdosteine but not NAC apparently reduced the renal tissue damage. The pathological damage score after IR was significantly reduced after erdosteine treatment ($P < 0.05$), but not after NAC treatment. In conclusion, renal IR resulted in oxidative damage as seen in biochemical lipid peroxidation and protein oxidation results with aggravated tubular necrosis. Erdosteine and NAC treatments improved the biochemical results of IR injury. However, on microscopic evaulations, animals receiving erdosteine showed a great reduction in renal damage when compared with the NAC group.

Keywords Renal · Ischemia-reperfusion · Erdosteine · *N*-*A*cetylcysteine · Light microscopy · Protein oxidation

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H. Erdogan (✉)
Department of Physiology, Medical Faculty of Gaziosmanpasa University, Tip Fakultesi, 60100 Tokat, Turkey
E-mail: haserdogan@yahoo.com
Tel.: +90-356-2133294
Fax: +90-356-2133179

E. Fadillioglu
Department of Physiology, Medical Faculty of Inonu University, Malatya, Turkey

M. Yagmurca
Department of Histology and Embryology, Medical Faculty of Afyon Kocatepe University, Afyon, Turkey

M. Uçar
Department of Histology and Embryology, Medical Faculty of Inonu University, Malatya, Turkey

M. Kemal Irmak
Department of Histology and Embryology, Gulhane Military Medical Academy, Ankara, Turkey

Introduction

Renal ischemia-reperfusion (IR) injury is common in several clinical situations, including renal transplantation. IR-induced acute renal failure is associated with decreased allograft survival in patients with transplanted kidneys and high mortality and morbidity in patients with native kidneys [1]. It was demonstrated that reactive oxygen species (ROS) increase in the areas of ischemia and reperfusion, which is responsible for renal damage. Renal ischemia causes tissue hypoxia and leads to a complex cascade of events resulting in renal injury. ROS-mediated cellular damage can be

expected to occur when oxygen is supplied to the tissue by reperfusion and ROS formation exceeds the high cellular detoxification capacity of the tissue [2, 3]. During loss of blood supply, ATP production is diminished relative to the limited oxygen availability. Then, the alterations in membrane ion gradients cause an elevation of the cytosolic Ca^{2+} concentrations, which activates proteases capable of transforming xanthine dehydrogenase to xanthine oxidase (XO). During reperfusion, the molecular oxygen provided is consumed as an electron acceptor and used for the formation of two superoxide anions ($O_2^{\bullet -}$) by XO [3, 4]. The reduction of dioxygen to $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2) and the hydroxyl radical (•OH) is believed to cause the actual injury induced by IR. The major intracellular antioxidant enzymes, copper/zinc-superoxide dismutase (Cu/Zn-SOD) in the cytoplasm and manganese-superoxide dismutase (Mn-SOD) in the mitochondria, rapidly and specifically reduce $O_2^{\bullet -}$ to H_2O_2. The other endogenous antioxidant enzyme, GSH-Px, acts to detoxify H_2O_2 to water [5]. Oxidant injury affects cellular molecules including DNA, proteins, membrane lipids, etc. Demonstration of both protein oxidation and lipid peroxidation will help to understand better the exact mechanism of IR on renal tissue. Lipid peroxidation and protein oxidation are important indices of oxidant injury. Serteser et al. [6] demonstrated a protein oxidation and lipid peroxidation in liver tissues after renal IR injury. Accordingly, the measurement of lipid peroxidation was evaluated by thiobarbituric acid (TBA)-reactive substances (TBARS) and protein oxidation was evaluated by measurement of the protein carbonyl (PC) content which is brought out during the oxidative injury [7].

Several agents are proposed to be useful in the clinical setting of renal IR damage, including free radical scavengers. *N*-acetylcysteine (NAC) is one of the scavengers used for the prevention of renal IR [2, 8]. Erdosteine is a molecule containing two blocked sulphydril groups in its structure, and its active metabolites show antioxidant activity with these blocked sulphydril groups [9, 10]. It was demonstrated that erdosteine prevented lipid peroxidation in renal tissue induced by IR [11]. However, the effects of erdosteine on morphology of renal tissue and protein oxidation have not been indicated after IR injury till now and there has been no comparative study on erdosteine and NAC on kidney IR injury. Therefore, the aim of this study was to investigate the effects of erdosteine and NAC on the oxidant/antioxidant status and light microscopic changes of renal tissue after IR injury.

Materials and methods

Male Sprague–Dawley rats were used in the experiments. The animals were housed in quiet rooms with 12:12 h light–dark cycle (from 7:00 a.m. to 7:00 p.m.) and the experiments were performed in accordance with "Guide for the Care and Use of Laboratory Animals, DHEW publication no. (NIH) 85-23, 1985" and approved by the local ethical committee at the Medical School of Inonu University.

Rats were randomly assigned to one of four groups: control untreated rats ($n = 8$); IR ($n = 9$); IR + NAC ($n = 9$) and IR + erdosteine ($n = 9$). NAC was injected as a bolus i.p. dose of 100 mg/kg 15 min before ischemia and then eight equal doses (just before reperfusion and during reperfusion with 15 min interval total dose of 80 mg/kg). Erdosteine 50 mg/kg/day was applied for 2 days before experiments [12]. Rats were anesthetized with urethane (i.p.; 1.2 g/kg), and right renal vascular pedicle was exposed via a midline laparatomy. After unilateral renal ischemia (30 min) by placing an occlusive clamp across the right renal pedicle and reperfusion (120 min), the right kidney was rapidly excised and sectioned vertically into two pieces for microscopic examination and biochemical analyses. The renal tissue was stored at −70°C until biochemical analyses.

Homogenization and determination of protein

After weighing the kidney tissue, the homogenate, the supernatant and the extracted samples were prepared as described elsewhere [3], and the following determinations were made on the samples using commercial chemicals supplied by Sigma (St. Louis, MO, USA). After the kidney was cut into small pieces with a pair of scissors, tissues were homogenized for 3 min at 16,000 rpm in four volumes of ice-cold Tris–HCl buffer (pH 7.4) using a homogenizer (IKA Ultra-Turrax T25 basic homogenizer, Germany). Tissue PC contents and TBARS levels were determined from the homogenate. The tissue homogenate was then centrifuged at 5,000*g* for 60 min to remove debris in order to get the supernatant for catalase (CAT) and XO analyses. Then, the clear upper supernatant fluid was taken and CAT and XO activities and protein determination were carried out at this stage. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (5:3 v/v). After centrifugation at 5,000g for 60 min, the upper ethanol phase was taken and used in the SOD and protein assays. Protein measurements were analyzed in the homogenate, supernatant and extracted samples according to the method of Lowry et al. [13].

Determination of catalase activity

Catalase (CAT, EC 1.11.1.6) activity was determined according to Aebi's method [14]. The principle of the assay is based on the determination of the rate constant (s^{-1}, k) or the H_2O_2 decomposition rate at 240 nm.

Determination of thiobarbituric acid reactive substances level

The tissue TBARS level was determined by a method [15] based on the reaction with TBA at 90–100°C. In the TBA test reaction, MDA or MDA-like substances and TBA react, producing a pink pigment having an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 90°C for 15 min. The sample was mixed with two volumes of cold 10% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifugation, and an aliquot of the supernatant was reacted with an equal volume of 0.67% (w/v) TBA in a boiling water bath for 10 min. After cooling, the absorbance was read at 532 nm. The results were expressed according to the standard graphic which was prepared by the measurements done with a standard solution (1,1,3,3-tetramethoxypropane).

Determination of superoxide dismutase activity

Total (Cu–Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al. [16]. The principle of the method is based on the inhibition of nitroblue-tetrazolium (NBT) reduction by the xanthine–XO system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate.

Determination of tissue protein carbonyl content

The tissue PC content was determined spectrophotometrically by a method based on the reaction of the carbonyl group with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone [7]. 2,4-Dinitrophenylhydrazine was the reagent originally used for proteins subjected to metal-catalyzed oxidation. The samples were pipetted into eppendorf tubes and precipitated with trichloroacetic acid. Then, 2,4-dinitrophenylhydrazine in 2 M HCl was added and allowed to stand at room temperature for 1 h. After the addition of 20% trichloroacetic acid, the tubes were centrifuged at 11,000*g* for 3 min. Then, the pellet was washed with ethanol–ethyl acetate (1:1) solution to remove free reagent. At the last stage, the precipitated protein was redissolved in 100 mM NaOH solution. After centrifugation to remove any insoluble material, spectrophotometric analysis was performed at 360 nm. Carbonyl content was calculated using a molar absorption coefficient of 22,000. The results were given as nanomoles carbonyl per milligram protein.

Determination of xanthine oxidase activity

Tissue XO (EC 1.2.3.2) activity was measured spectrophotometrically by the formation of uric acid from xanthine through the increase in absorbance at 293 nm, according to Prajda and Weber's method [17]. One unit of activity was defined as 1 μmol of uric acid formed per minute at 37°C, pH 7.5.

Light microscope

For microscopic evaluation, formalin-fixed coronal kidney samples were embedded in paraffin, and 4 μm sections were prepared. The sections were then stained with hematoxylin and eosin and scored with a previously described semiquantitative scale designed to evaluate the degree of renal damage (tubular cell necrosis, cytoplasmic vacuole formation, hemorrhage and tubular dilatation) [3, 18]. Specifically, one whole deep coronal section was examined under the microscope and graded according to the extent of damage, based on the percentage of involvement of the kidney. Higher scores represent more severe damage, with maximum score being 4: 0, normal kidney; 1, minimal damage (0–5% involvement); 2, mild damage (5–25% involvement); 3, moderate damage (25–75% involvement); and 4, severe damage (75–100% involvement).

Statistical analysis

Data were analyzed using a commercially available statistics software package (SPSS® for Windows v. 9.0, Chicago, IL, USA). Distributions of the groups were analyzed with one sample Kolmogrov–Smirnov test. All groups showed a normal distribution, so that parametric statistical methods were used to analyze the data. One-way ANOVA test was performed and post hoc multiple comparisons were done with LSD. Results were presented as mean ± SEM. P values < 0.05 were regarded as statistically significant.

Results

Biochemical results

The results of the biochemical analysis of renal tissue are illustrated in Table 1. The renal TBARS level and PC content were higher in the IR group than in the other groups ($P < 0.05$). The erdosteine and NAC treatments caused significant reverse effect of protein oxidation and lipid peroxidation induced by IR. Also, the TBARS levels and the PC contents of the IR + NAC and IR + erdosteine groups were not significantly different compared to the control group ($P > 0.05$). IR + NAC and IR + erdosteine groups' TBARS levels and PC contents were not significantly different ($P > 0.05$) either.

There was a non-significant increase in the SOD activity of the IR group in comparison with the control group ($P = 0.055$). However, the activity of SOD in renal

Table 1 The activities of superoxide dismutase (SOD), catalase (CAT) and xanthine oxidase (XO) and levels of thiobarbituric acid-reactive substances (TBARS) and protein carbonyl (PC) content in renal tissue in control, ischemia-reperfusion (IR), IR + *N*-acetylcysteine (IR + NAC) and IR + erdosteine groups (mean ± SEM)

	SOD (U/mg prot)	CAT (k/g prot)	XO (U/g prot)	TBARS (nmol/g wet tissue)	PC (nmol/mg prot)
Control ($n=8$)	0.178 ± 0.022	1.775 ± 0.039	1.772 ± 0.137	39.57 ± 2.28	1.046 ± 0.041
IR ($n=9$)	0.231 ± 0.014	1.629 ± 0.038	2.476 ± 0.188	55.01 ± 4.27	1.390 ± 0.111
IR + NAC ($n=9$)	0.246 ± 0.017	1.646 ± 0.056	2.191 ± 0.065	46.95 ± 1.67	1.074 ± 0.063
IR + erdosteine ($n=9$)	0.261 ± 0.019	1.809 ± 0.053	2.342 ± 0.101	41.66 ± 2.17	0.864 ± 0.075
P values					
Control–IR	NS	0.041	0.001	0.001	0.005
Control–IR + NAC	0.015	NS	0.033	NS	NS
Control–IR + erdosteine	0.003	NS	0.005	NS	NS
IR–IR + NAC	NS	NS	NS	0.048	0.007
IR–IR + erdosteine	NS	0.011	NS	0.002	0.0001
IR + NAC–IR + erdosteine	NS	0.020	NS	NS	NS

tissue was increased in the IR + NAC and IR + erdosteine groups in comparison with the control group ($P<0.05$). The CAT activity of IR group was decreased in comparison with the control and IR + erdosteine ($P<0.05$) groups, whereas it was higher in the IR + erdosteine group than in the IR + NAC group ($P<0.05$). However, CAT activity in the IR + NAC group was insignificantly increased in comparison with the control ($P=0.068$). The XO activity was increased in all IR-performed groups in comparison with the control group ($P<0.05$). There was no significant difference in XO activity between IR-performed groups.

Light microscopic examination results

Histological damage ranged from normal (control group) to mild (erdosteine group) and severe (IR and NAC groups), with cortical rather than medullary tubules demonstrating the most marked changes. Histological changes, including tubular dilatation, tubular necrosis and vacuole formation, were clearly observed in the kidneys of IR (Fig. 1). The most sensitive indices were the overall cortical damage and the degree of cortical tubular dilatation. Vacuole formation was observed in all groups to some extent except in control group (Fig. 2). Erdosteine but not NAC apparently reduced the renal tissue damage, although there is some improvement with NAC. The pathological damage score (3.4 ± 0.1) after IR injury was significantly reduced in the erdosteine group (2.6 ± 0.2) but not in the NAC group (3.1 ± 0.4). Kidneys in the erdosteine group showed mild histological changes with slight tubular dilatation and vacuole formation (Fig. 3), while the tubular injury in the NAC group was more severe and associated with generalized hemorrhage (Fig. 4).

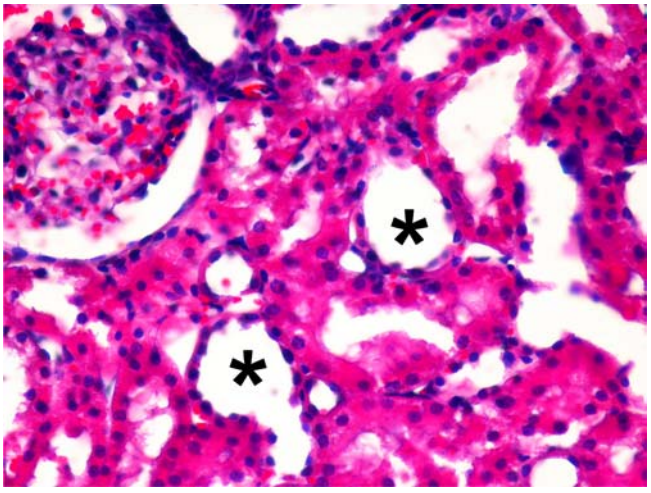

Fig. 1 Photomicrograph of renal cortical tubules after ischemia-reperfusion period. The tubules appear dilated (*asterisks*) and lined by flattened epithelial cells with vacuoles (hematoxylin–eosin ×500)

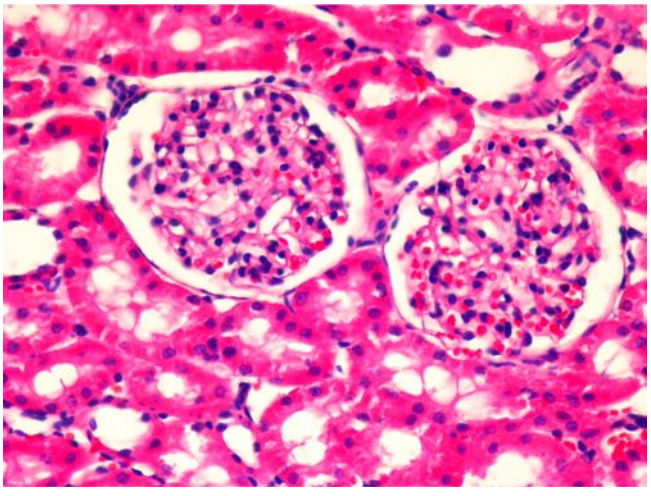

Fig. 2 Photomicrograph of renal cortex in the control group. Tubules and glomeruli appear normal (hematoxylin–eosin ×500)

Discussion

The IR injury is of paramount importance to organ transplantation and is clearly a major determinant of early graft dysfunction. Loss of blood supply results in

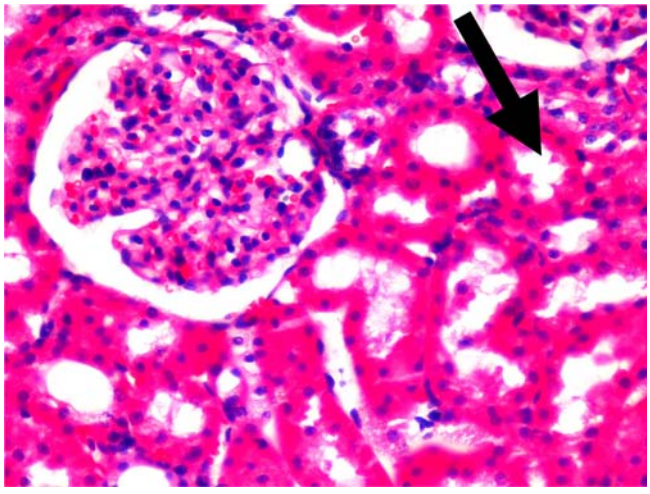

Fig. 3 Photomicrograph of renal cortical tubules after IR following treatment with erdosteine. The tubules and the interstitium are almost normal, but the cytoplasm of some of the tubular epithelial cells are filled with fine vacuoles (*arrow*) (hematoxylin–eosin ×500)

ischemia, which rapidly damages metabolically active tissues such as renal tissue [19]. Following reperfusion, significant production of ROS occurs due to hypoxanthine oxidation. Xanthine dehydrogenase–oxidase conversion and hypoxanthine formation have been implicated in the oxidative damage of reperfusion injury following reoxygenation of the organ [20, 21]. Our results showed that all IR groups had high XO activity. However, Irmak et al. [3] demonstrated that XO activity was decreased in IR-performed groups. They made 30 min ischemia and 30 min reperfusion on the renal tissue. So our results substantiated that the duration of reoxygenation might be an important factor for the xanthine dehydrogenase–oxidase conversion. Joannidis et al. [22] measured arteriovenous lipid peroxidation difference and XO activity in rats after IR. They found elevated

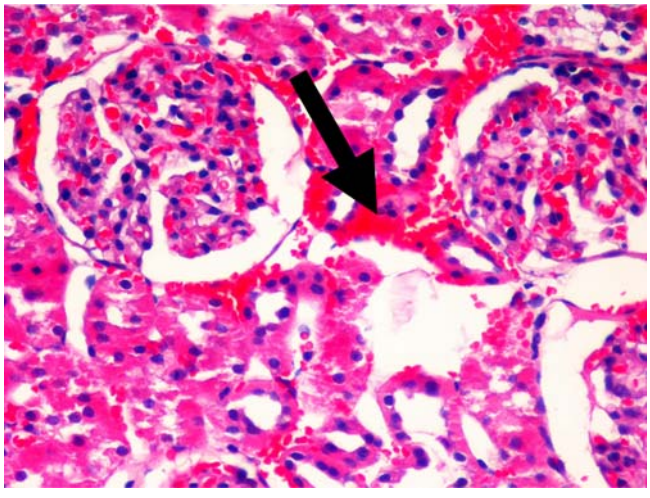

Fig. 4 Photomicrograph of renal cortical tubules after IR following treatment with NAC. Notice the dilated tubules and intense hemorrhage (*arrow*) (hematoxylin–eosin ×500)

lipid peroxidation and no significant conversion of xanthine dehydrogenase to XO during renal IR when comparing arteriovenous differences. However, the role of XO in renal IR injury cannot be ruled out because it is an intracellular enzyme and causes an increase in renal injury as our study demonstrated.

The present study demonstrated that renal IR resulted in raised TBARS and PC content in renal tissue. These data are in good agreement with the work of Irmak et al. [3] who found high lipid peroxidation after renal IR injury in rats. The IR injury caused ROS production which could not be abolished by endogenous antioxidant systems. In normal conditions, the body systems can easily detoxify the produced ROS with endogenous antioxidants such as SOD and CAT. However, if there is a pathological condition, like IR injury, ROS is produced more than usual. Then, these high productions of ROS cannot be detoxified to water by antioxidant enzymes. Increased production of $O_2^{\bullet -}$ leads to H_2O_2, and non-detoxified H_2O_2 reacts with Fe^{2+} (known as Fenton reaction) to produce $\bullet OH$, which is the most toxic ROS to cellular organisms in the oxidative system. However, antioxidant therapy usually prevents the toxic effect of ROS not detoxified by endogenous antioxidants. Our biochemical results demonstrated that antioxidant therapy with erdosteine or NAC prevents lipid peroxidation and protein oxidation, which are two important indices of oxidative injury to the tissue. Erdosteine and NAC treatment caused increased activity of SOD in renal tissue after IR injury in rats. However, erdosteine treatment showed higher CAT activity than did the IR and IR with NAC treated groups. Renal IR resulted in low CAT activity in comparison with the control group in the present study.

Histological findings were almost parallel with the degree of lipid peroxidation. In the IR and NAC groups, apparent tubular dilatation may stem from precipitated proteins that obstructed tubules raising intratubular pressure. This suggestion is consistent with the result of studies in animal models that showed increased intratubular pressure in ischemic injury [23]. Fine vacuoles observed in the cytoplasm of some of the proximal tubular epithelial cells may result from osmotic changes in tubular fluid. Histological examination also confirmed the protective action of erdosteine. In the erdosteine group, the severity of damage was less when compared to the IR and NAC groups. Erdosteine may protect the tubular epithelium effectively from reperfusion injury. The renal damage score tended to be less in the NAC group compared with that in the IR group, but this did not reach statistical significance. However, the erdosteine group had a marked and statistically significant reduction in renal damage score.

In conclusion, the present study demonstrated that renal IR injury resulted in oxidative damage to cellular injury as seen in biochemical lipid peroxidation and protein oxidation results under light microscopic examinations. Erdosteine and NAC treatments improved the biochemical results; however, only erdosteine effectively

treats pathological renal damage in IR injury. So, our results indicate that renal reperfusion aggravated tubular necrosis and animals receiving erdosteine showed a marked reduction in renal damage. Although the exact mechanisms remain to be clarified, erdosteine could be an effective regimen to enhance therapeutic efficacy and reduce IR injury of kidney in clinics.

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