Original Article

Protective Effect of the Nitric Oxide Pathway in L-Citrulline Renal Ichaemia-Reperfusion Injury in Rats

(L-citrulline / ischaemia-reperfusion / nitric oxide synthase / L-NAME)

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Abstract. To observe the protective effects of L-citrulline on the renal I/R injury and elucidate the mechanisms involved, 48 rats were randomized into eight groups: Group 1: sham operated; Group 2: I/R (45 min renal ischaemia and 24 h reperfusion); Group 3: I/R + L-citrulline (300 mg/kg, i.g.); Group 4: I/R + L-citrulline (600 mg/kg, i.g.); Group 5: I/R + L-citrulline (900 mg/kg, i.g.); Group 6: I/R + normal saline (NS, i.g.); Group 7: I/R + N sup ω nitro-L-arginine ester (L-NAME, 20 mg/kg, i.p.); Group 8: I/R + L-citrulline (900 mg/kg, i.g.) + L-NAME (20 mg/kg, i.p.). At the end of the reperfusion period, serum was collected and the kidneys underwent histological and biochemical examinations. Our results showed that pre-treatment with L-citrulline (300, 600, and 900 mg/kg) significantly ameliorated the renal injury caused by I/R. Moreover, L-citrulline prevented induction of lipid peroxidation and increased the activity of superoxide dismutase and the levels of glutathione and nitric oxide. The I/R-induced decreases in total nitric oxide synthase activity, inducible nitric oxide activity, constitutive nitric oxide activity and endothelial nitric oxide protein expression in the renal cortex were significantly prevented. However, the L-citrulline-mediated protection was significantly antagonized by co-administration of L-NAME. These results suggested that L-citrulline administration exhibited significant protection against renal I/R injury. This protective effect, at least in part, via up-regulation of the endothelial nitric oxide protein expression and constitutive nitric oxide synthase activity, maintained production of nitric oxide at the basal level.

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Abbreviations: ARF – acute renal failure, BUN – blood urea nitrogen, cNOS – constitutive NOS, eNOS – endothelial NOS, GFR – glomerular filtration rate, GSH – glutathione, iNOS – inducible NOS, I/R – ischaemia-reperfusion, L-Arg – L-arginine, L-NAME – N sup ω nitro-L-arginine ester, MDA – malondialdehyde, NADPH – nicotinamide adenine dinucleotide 3-phosphate, NO – nitric oxide, NOS – nitric oxide synthase, NTB – nitro-blue tetrazolium, ROS – reactive oxygen species, Scr – serum creatinine, SOD – superoxide dismutase, TNOS – total nitric oxide synthase.

Introduction

Ischaemia-reperfusion (I/R) injury is one of the causes of acute renal failure (ARF) in humans, especially following renal transplant surgery, renal artery stenosis and cardiovascular surgery, leading to grave morbidity and mortality (Yamashita et al., 2001), in which there has not been any dramatic decrease over the past 50 years (Chander and Chopra, 2006). The mechanisms underlying renal I/R injury are likely multifactorial and interdependent. Several studies have shown excessive production of reactive oxygen species (ROS) during reperfusion (Bonventre, 1993; Rodríguez-Reynoso et al., 2004), which are responsible for renal injury (Zimmerman and Granger, 1992; Rasoulian et al., 2008). During reperfusion, the provided molecular oxygen is converted to superoxide radicals, which are converted to hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and peroxyl-(ROO^-) radicals, causing lipid peroxidation in the membranes. These radicals damage the structural integrity of the lipid bilayer and enhance the membrane permeability, with consequent alterations in both the structure and functions of mitochondria, lysosomes and plasma membranes.

Nitric oxide (NO), a biologically active substance, is a potential endogenous vasodilator involved in the regulation of angiotasis in normal physiological states. NO is produced from L-arginine (L-Arg) in three different isoforms of nitric oxide synthase (NOS): endothelial (e), neuronal (n), and inducible (i) (Moncada et al., 1991; Moody and Calvert, 2011). Several studies have shown that there is a decrease in NO levels in the renal cortex during the renal ischaemia reperfusion (Lefer, 2006), and the function of NO on renal injury remains biphasic. There are some reports that eNOS-derived NO has a protective effect against tissue injury (Yamashita et al., 2003; Yamasowa et al., 2005), whereas other researchers reported that the excessive iNOS-derived NO had a deleterious effect (Lien et al., 2003; Mark et al., 2005; Liu et al., 2013). Furthermore, previous studies (Weight et al., 1999) confirmed that cNOS inhibition could impair the glomerular filtration rate (GFR), and upregulation of the cNOS activity attenuated the injury.

Several studies have shown that acute L-Arg infusion was beneficial in renal ischaemia *in vivo* (Schneider et al., 2003). However, oral supplementation of L-Arg can be subject to extensive pre-systemic and systemic elimination by bacteria and by arginases (Morris, 2004). L-citrulline is the natural precursor of L-Arg, substrate for NOS in the biosynthesis of NO in NO-producing tissues. In the kidneys, vascular endothelium and other tissues, L-citrulline can be readily converted to L-Arg, thus raising plasma and tissue levels of L-Arg and increasing NO production (Mori and Gotoh, 2000; Schneider et al., 2003). Indeed, previous studies have indicated that supplementation with L-citrulline is effective in improving gastric ischaemia-reperfusion injury and glycerol-induced ARF, with the related mechanisms of maintenance of the constitutive NOS activity (Gou et al., 2011; Liu. et al., 2013). However, there is no literature available as to the effect of L-citrulline on renal I/R injury.

In the present study, we attempted to determine the protective effect of L-citrulline on renal I/R injury and investigated the possible underlying mechanism of the effects in rats. Renal function was assessed by determination of serum creatinine (Scr) and blood urea nitrogen (BUN) concentrations, while histological damage was assessed with haematoxylin-eosin (H-E) staining of renal tissues under a light microscope. Superoxide dismutase (SOD) activities and malondialdehyde (MDA), glutathione (GSH), NO contents were examined in the renal cortex tissues. In addition, the enzymatic activities of total NO synthase (TNOS), constitutive NOS (cNOS), and inducible NOS (iNOS) were examined. The protein levels of endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) in the renal cortex tissues were determined by Western blot.

Material and Methods

Animals

Adult male Sprague-Dawley rats, weighing 200–250 g, were provided by the Experimental Animal Centre of Xuzhou Medical College. The animals were housed under standard conditions of 12:12 h light and dark cycle (7 a.m. to 7 p.m.) with *ad libitum* access to food and water. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental grouping

Drugs (L-citrulline, NS, L-NAME) were administered for seven consecutive days before the animals were starved for 12 h for the experiment, in which animals received sodium pentobarbital (50 mg/kg) 10 min in advance. In the sham-operated rats, only the renal pedicles were dissected without induction of ischaemia. L-citrulline was administered via gavage prior to intraperitoneal injection of L-NAME. Renal ischaemia was maintained for 45 min, followed by reperfusion for 24 h. At the end of the surgical procedures, the midline incision was sutured, and the animals were allowed recovery from anaesthesia.

A total of 64 rats were divided into eight groups (N = 8 each). Group 1: sham operated; Group 2: I/R (45 min renal ischaemia and 24 h reperfusion); Group 3: I/R + L-citrulline (300 mg/kg); Group 4: I/R + L-citrulline (600 mg/kg, i.g.); Group 5: I/R + L-citrulline (900 mg/kg, i.g.); Group 6: I/R + normal saline (NS); Group 7: I/R + N sup ω nitro-L-Arg ester (L-NAME, 20 mg/kg, i.p.); Group 8: I/R + L-citrulline (900 mg/kg, i.g.) + L-NAME (20 mg/kg, i.p.). At the end of 24 h of reperfusion, the animals were sacrificed with a high dose of anaesthesia and blood was collected through abdominal aorta. Serum was isolated and used for the assessment of renal function tests. A midline abdominal incision was performed and the left kidneys were isolated. Two kidneys from each group were randomly used as samples for histological studies, and the remaining kidneys in each group were deep frozen until further enzymatic analyses.

Assessment of renal functions

The levels of serum creatinine (Scr) and blood urea nitrogen (BUN) were measured as indicators of renal functions by microplate spectrophotometry.

Histological procedure and assessment

For pathological examinations, all samples were fixed in 4% formaldehyde buffer. The tissues were embedded in paraffin, and pathological sections were sliced along the longitudinal axis. From each sample, 5-μm-thick sections were obtained and stained with haematoxylin-eosin to evaluate renal morphology.

Biochemical assay

Renal cortices were thawed and homogenized in a proportion of 1 : 9 (w/v) ice-cold normal saline. The homogenates were centrifuged at 4000 rpm at 4 °C for 10 min in high-speed centrifuge Eppendorf 5415R Microcentrifuge (Eppendorf /Shanghai/ International Trade Co., Ltd., Shanghai, China), and then they served for determination of the levels of MDA, GSH and NO, as well as the activities of SOD, TNOS, iNOS and cNOS.

Determination of MDA level

MDA was measured by the method of Ohkawa et al. (1979). The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium lauryl sulphate (8.1%) were added to 0.1 ml of each processed tissue sample, and the samples were heated at 95 °C for 60 min. The mixture was cooled with tap water and centrifuged at 4000 rpm for 10 min, the organic layer was separated and absorbance was measured at 532 nm using microplate spectrophotometry. Protein estimation was conducted (Lowry et al., 1951) and the data were expressed as nmol/mg protein.

Determination of SOD activity

The activity of SOD was assessed by measurement according to Sun et al., 1988. The estimation was based on the generation of superoxide radicals (produced by xanthine and xanthine oxidase), which thereafter reacted with nitro-blue tetrazolium (NTB) to form formazan dye. The SOD activity was then measured at 550 nm by the degree of inhibition of this reaction. One unit of enzyme was defined as the amount of enzyme required at the inhibition rate of 50 %. The activity of superoxide dismutase was expressed as units/mg protein.

Determination of GSH level

GSH concentrations were determined by the procedures of Elmann (1959). Briefly, 0.5 ml homogenate was mixed with 1.5 ml 0.15 M KCl and 3 ml deproteinization solution. Each sample was centrifuged at 3000 rpm for 10 min to obtain supernatant, and this was followed by the addition of 2 ml phosphate solution and 0.5 ml DTNB into the 0.5 ml supernatant, with the absorbance read at 412 nm and compared with GSH standards.

Determination of NO level

Nitrite and nitrate are the primary oxidation products of NO subsequent to the reaction with oxygen and, therefore, the nitrite/nitrate concentration in renal homogenate was used as an indicator of NO synthesis. Quantitative analysis of nitrate and nitrite was based on the Griess reaction, in which a chromophore with a strong absorbance at 550 nm was formed by the reaction of nitrite with a mixture of naphthyl-ethylenediamine and sulphanilamide. The nitrate was reduced to nitrite by 10 min of incubation with nitrate reductase in the presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Total nitrite/nitrate concentration was calculated using sodium nitrate as a standard and the results were expressed as μmol/l.

Determination of NOS activity

NOS activity was measured on the basis of formation of L-citrulline from L-Arg (Bredt and Snyder, 1989). Frozen tissues from the fundic stomach were homogenized at 0 °C in five volumes of buffer containing 320 mmol/l sucrose, 1 mmol/l DL-dithiothreitol, phenylmethylsulphonyl fluoride (100 μg/ml), leupeptin (10 μg/ml), soybean trypsin inhibitor (100 μg/ml) and aprotinin (2 μg/ml) in 50 mmol/l HEPES (pH 7.0). Following centrifugation of the homogenate at 100 000× *g* for 1 h, the supernatants were added to the reaction mixture containing 50 mmol/l Tris (pH 7.4), L-[U-14C] arginine (specific activity 11.8 GBq/mmol), 10 μg/ml calmodulin, 1 mmol/l $CaCl_2$, and 50 mmol/l L-valine. The samples were incubated for 20 min at 37 °C prior to termination of the reaction by the addition of 0.1 volume of 20% (v/v) $HClO_4$. L-[U-14C]-Citrulline was isolated from L-[U-14C] arginine with passage through Dowex 50W (Na^+ form, Sigma-Aldrich., St. Louis, MO) and quantified by liquid-scintillation counting. The level of iNOS activity was measured by the addition of 1 mmol/l EGTA and the total activity was determined by the addition of 2 mmol/l L-NMMA. The cNOS activity was determined by subtraction of iNOS activity from the total NOS activity. Protein concentration of the supernatant was determined by spectrophotometry using a commercial assay kit (Bio-Rad Laboratories, Richmond, CA).

Western blot analysis

Tissue lysates were prepared with cold 1% Nonidet P-40 in 50 mmol/l Tris-HCl (pH 7.4) containing 120 mmol/l NaCl, 1 mmol/l EDTA, 50 mmol/l NaF, 0.1 mmol/l Na_3VO_4, and protease inhibitor cocktail. Protein content was quantified by the bicinchoninic assay. The lysates (200 μg total protein) were then subjected to SDS-PAGE and transferred onto a nitrocellulose membrane. Blotting filters were blocked with 3% bovine serum albumin in Tris-buffered saline/Tween-20 buffer (137 mmol NaCl, 20 mmol Tris-HCl (pH 7.4), 0.1% Tween-20). After rinsing in Tris-buffered saline/Tween-20 buffer, the membranes were incubated with a 1 : 300 dilution of anti-iNOS, anti-nNOS, and anti-eNOS at room temperature overnight with constant agitation. The filters were then washed and subsequently probed with horseradish peroxidase-conjugated anti-rabbit for iNOS, nNOS and eNOS at a dilution of 1 : 500. Immunocomplexes were detected by the Supersignal West Pico Chemiluminescent Kit (Pierce, Rockford, Ill). Comparison between different treatment groups was performed by determination of the examined protein/actin protein ratio of the immunoreactive area by densitometry.

Statistical analysis

The statistical analysis was performed using SPSS 13.0 software. The results were expressed as mean ± SEM. All data were analysed statistically by one-way analysis of

variance (ANOVA), followed by Dunnett's *t*-test. A significant difference was reached when $P < 0.05$.

Results

Histological results

The sham-operated group did not show any morphological changes. By contrast, the kidneys of untreated ischemic rats showed glomerular lesions, necrosis or collapse of massive tubular epithelial cells, and tubular dilatation. Treatment with L-citrulline (300, 600, and 900 mg/kg) preserved the normal morphology of the kidneys (Fig. 1) and showed normal glomeruli and slight oedema of the tubular cells, particularly at the dose of 900 mg/kg. However, this protective effect was significantly reversed by prior administration of L-NAME.

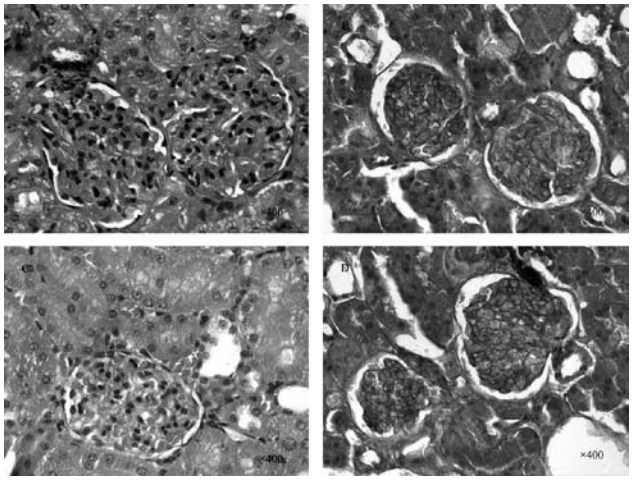

Fig. 1. Effect of L-citrulline on renal morphology in I/R-induced renal injury in rats. Renal injury was evidenced by glomerular lesions, massive tubular epithelial cell necrosis or collapse, and tubular dilatation. (A) Sham: sham-operated control; (B) I/R: 45 min ischaemia prior to 24 h reperfusion; (C) L-Cit_{900}: L-citrulline pre-treatment (900 mg/kg-1). (D) L-Cit_{900} + L-NAME: L-citrulline (900 mg/kg-1, i.g.) + L-NAME (20 mg/kg, i.p.). H-E staining was used to assess the degree of acute renal tubule damage in I/R-induced renal injury. Sections were observed under a light microscope (×400).

Effects of L-citrulline on the levels of Scr and BUN

The results demonstrated that renal I/R caused a marked increase in Scr as well as BUN levels ($P < 0.001$). Pre-treatment with L-citrulline at different doses (300, 600 and 900 mg/kg) significantly reduced the levels of Scr and BUN ($P < 0.05$), as shown in Fig. 2. However, this protective effect was significantly reversed ($P < 0.001$) by prior administration of L-NAME (20 mg/kg, i.p.). There was no significant statistical difference between the I/R group and the I/R + NS group ($P < 0.05$).

Effects of L-citrulline on the levels of MDA and GSH and on the activity of SOD in rat renal tissue

The results showed that I/R induced an increase in the MDA level. However, this increase was significantly reversed by L-citrulline administration for pre-treatment, resulting in the inhibition of lipid peroxidation. GSH was dramatically decreased by renal I/R and significantly increased by L-citrulline at the doses of 300, 600 and 900 mg/kg ($P < 0.05$). As to the antioxidase activity, in the I/R group, the activity of SOD was markedly decreased as compared to the sham-operated group, while a significant elevation was observed in the L-citrulline pre-treatment group when compared with the I/R group. However, this protective effect was significantly reversed ($P < 0.05$) by prior administration of L-NAME (Table 1).

Effects of L-citrulline on the level of NO and on the activities of TNOS, iNOS and cNOS

Table 2 shows the NO levels and NOS activities in all groups. The NO level was significantly lower in the I/R

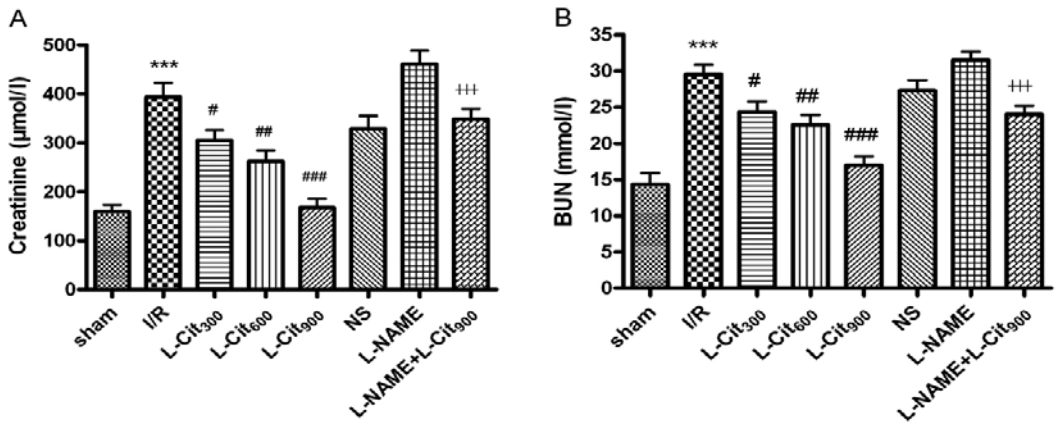

Fig. 2. Effects of L-citrulline on the levels of Scr and BUN. All values are expressed as mean ± SEM (N = 6 each). (A) Effect of L-citrulline on the level of Scr. (B) Effect of L-citrulline on the level of BUN. $^{***}P < 0.001$, when compared with the sham-operated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the I/R group, $^{+++}P < 0.001$ when compared with the L-Cit_{900} group.

Table 1. Effects of L-citrulline on I/R-induced injury in the renal cortex. All values are expressed as mean ± SEM (N = 6 each), $^{***}P < 0.001$, when compared with the sham-operated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the I/R group; $^{+}P < 0.05$, $^{++}P < 0.01$ when compared with the L-Cit$_{900}$ group.

Group/dose (mg/kg)	MDA (nmol/mg)	GSH (mg/g)	SOD (U/mg)
Sham-operated	1.630 ± 0.113	10.313 ± 0.631	231.228 ± 13.087
I/R	2.940 ± 0.162***	3.094 ± 0.484***	134.371 ± 10.870***
L-Cit$_{300}$	2.313 ± 0.170$^{\#}$	5.693 ± 0.693$^{\#}$	171.128 ± 10.055$^{\#}$
L-Cit$_{600}$	2.032 ± 0.189$^{\#\#}$	7.438 ± 0.858$^{\#\#}$	195.269 ± 13.276$^{\#\#}$
L-Cit$_{900}$	1.417 ± 0.173$^{\#\#\#}$	8.748 ± 0.788$^{\#\#\#}$	199.298 ± 11.998$^{\#\#}$
NS	2.928 ± 0.313	2.911 ± 0.252	139.684 ± 10.479
L-NAME + L-Cit$_{900}$	2.555 ± 0.218^{++}	4.584 ± 0.796^{++}	152.372 ± 10.423^{+}
L-NAME	3.030 ± 0.246	2.424 ± 0.401	107.253 ± 8.397

Table 2. Effect of L-citrulline on the NO level and NOS activities in I/R-induced renal injury. All values are expressed as mean ± SEM (N = 6 each), $^{**}P < 0.01$, when compared with the sham-operated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, when compared with the I/R group; $^{+}P < 0.05$ when compared with the L-Cit$_{900}$ group.

Group/dose (mg/kg)	NO (μmol/L)	TNOS (U/mg prot)	cNOS (U/mg prot)	iNOS (U/mg prot)
Sham-operated	1.249 ± 0.107	0.429 ± 0.027	0.333 ± 0.026	0.095 ± 0.006
I/R	0.724±0.089**	0.266 ± 0.031**	0.206 ± 0.025**	0.061 ± 0.008**
L-Cit$_{300}$	1.044 ± 0.064$^{\#}$	0.372 ± 0.021$^{\#}$	0.287 ± 0.016$^{\#}$	0.085 ± 0.006$^{\#}$
L-Cit$_{600}$	1.070 ± 0.069$^{\#}$	0.392 ± 0.030$^{\#}$	0.300 ± 0.024$^{\#}$	0.093 ± 0.007$^{\#}$
L-Cit$_{900}$	1.214 ± 0.098$^{\#\#}$	0.431 ± 0.037$^{\#\#}$	0.333 ± 0.031$^{\#\#}$	0.098 ± 0.007$^{\#\#}$
NS	0.859 ± 0.120	0.292 ± 0.037	0.222 ± 0.031	0.070 ± 0.008
L-NAME + L-Cit$_{900}$	0.852 ± 0.077^{+}	0.285 ± 0.031^{+}	0.211 ± 0.035^{+}	0.073 ± 0.006^{+}
L-NAME	0.662 ± 0.060	0.175 ± 0.019	0.141 ± 0.015	0.034 ± 0.007

group than in the sham-operated group ($P < 0.01$), while it was markedly elevated ($P < 0.05$) in the L-citrulline pre-treatment groups compared to the I/R group. However, the L-citrulline-mediated protection was significantly antagonized by co-administration of L-NAME ($P < 0.05$). As compared to the sham-operated group, the activities of TNOS, iNOS and cNOS in the I/R group were markedly decreased, while there was a significant elevation in NOS activities in the L-citrulline pre-treatment group when compared with the I/R group. However, this protective effect was significantly reversed ($P < 0.05$) by prior administration of L-NAME.

Effect of L-citrulline on the protein expression of eNOS, nNOS and iNOS in renal cortex

As shown in Fig. 3, a significant decrease in eNOS expression was observed in the I/R group with respect to the control ($P < 0.001$); L-citrulline (300, 600, 900 mg/kg) pre-administration significantly attenuated this decrease ($P < 0.05$). No significant statistical difference in nNOS or iNOS expression was observed between the IR group and the control. In the L-citrulline-treated I/R group, there was a modest increase in nNOS expression and a slight decrease in iNOS expression compared with the I/R group (Figs. 4 and 5).

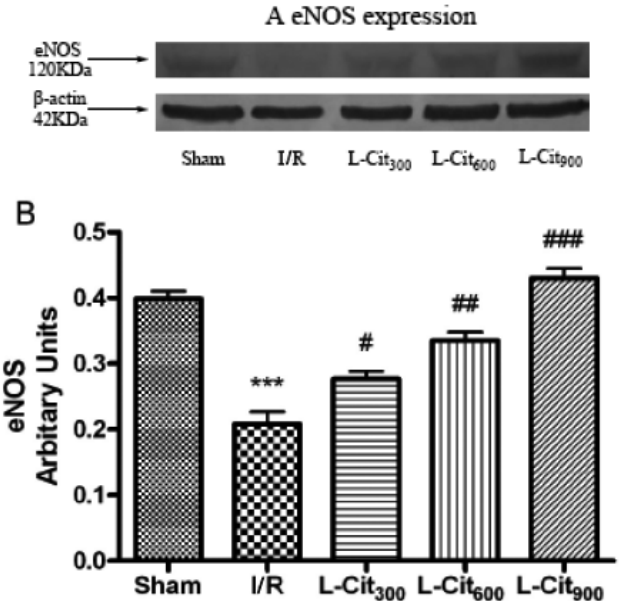

Fig. 3. (A) Endothelial nitric oxide synthase (eNOS) protein levels in renal cortex homogenate in all groups were visualized by immunoblotting with antibodies against eNOS. (B) Densitometric analysis was performed following normalization with actin. Data represent mean ± SEM, *** $P < 0.001$ compared with the sham group; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the I/R group.

Discussion

In the present study, L-citrulline exhibited significant protection against renal I/R injury in rats. These protective effects were observed when L-citrulline was administered by gavage at doses of 300, 600 and 900 mg/kg. Our results have shown that renal I/R caused kidney damage, with an increase in the levels of Scr and BUN, and significant alterations in the histopathological fea-

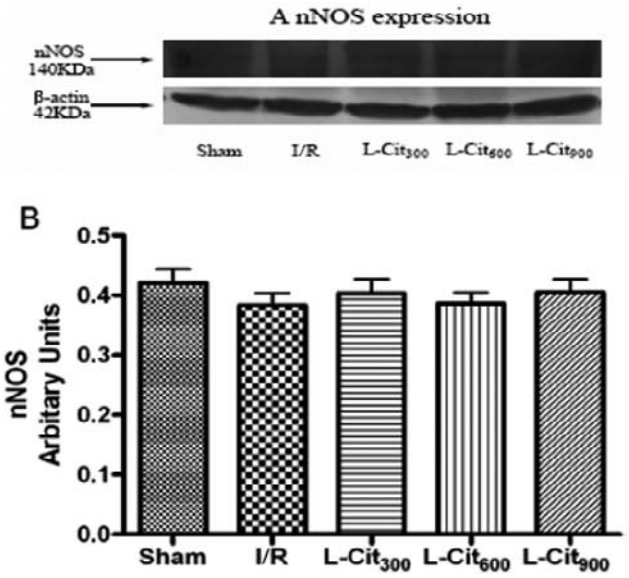

Fig. 4. (A) Neuronal nitric oxide synthase (nNOS) protein levels in the renal cortex homogenate in all groups were visualized by immunoblotting with antibodies against nNOS. (B) Densitometric analysis was performed following normalization with actin. Data represent mean ± SEM.

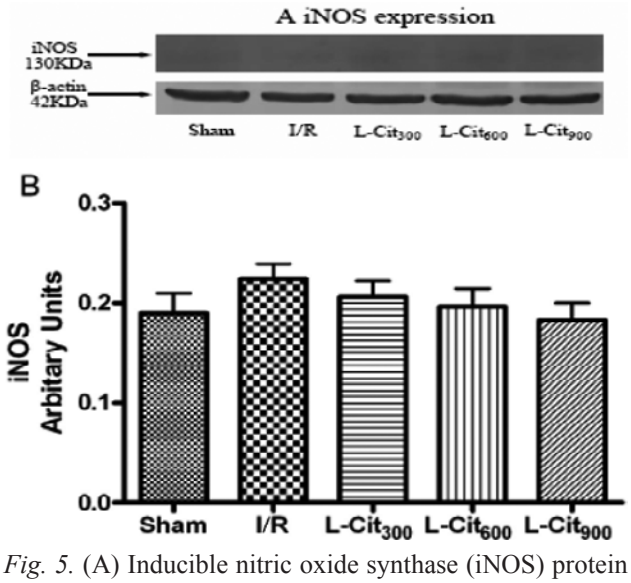

Fig. 5. (A) Inducible nitric oxide synthase (iNOS) protein levels in the renal cortex homogenate in all groups were visualized by immunoblotting with antibodies against iNOS. (B) Densitometric analysis was performed following normalization with actin. Data represent mean ± SEM.

tures when compared to the sham-operated rats. Pre-treatment with L-NAME aggravated the renal injury. Furthermore, pre-treatment with L-citrulline improved renal functions, reduced the levels of Scr and BUN, and preserved the normal morphology of kidneys. However, the L-citrulline-mediated protective effect was significantly antagonized by co-administration of L-NAME.

ARF due to ischaemia is characterized by renal vasoconstriction, severe reduction in glomerular filtration rate (GFR), extensive tubular damage, tubular cell necrosis and glomerular injury (Bird et al., 1988; Weinberg, 1991). Despite the significant advances in critical care medicine, it remains a major clinical problem, producing grave morbidity and mortality that has not decreased significantly over the last 50 years (Chander and Chopra, 2006). During renal ischaemia-reperfusion, a large number of ROS such as superoxide radical (O_2^-), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) were produced, leading to oxidative damage (Rodriguez-Reynoso et al., 2004). Singh and Chopra (2004) have shown that in the kidneys with I/R, there is a significant increase in renal oxidative stress that leads to both structural and functional damage to rat kidneys. In our previous study we also found the level of oxidative stress increased in glycerol-induced ARF in rats (Liu et al., 2013). Treatment with L-NAME, a non-selective NOS inhibitor, aggravated the renal damage. Our results were consistent with these findings. In the present study, I/R caused a marked renal oxidative stress, increased the level of MDA, and reduced the activity of SOD and the level of GSH compared to the sham-operated rats. Pre-treatment with L-NAME worsened all these parameters. Pre-treatment with L-citrulline for seven consecutive days led to alleviation of the renal oxidative stress, reduction of the level of MDA, elevation in the activity of SOD and the level of GSH, and inhibition of lipid peroxidation, which is in agreement with our previous study (Liu et al., 2013). However, the protective effect mediated by L-citrulline was significantly antagonized by co-administration of L-NAME.

NO, a potential endogenous vasodilator, is another important factor in renal ischaemia-reperfusion. Basal production of NO plays an important role in maintaining the normal glomerular functions, as the inhibition of the NO synthesis may increase both efferent glomerular arteriolar resistance and glomerular capillary pressure, and induce considerable changes in renal histology (Waz et al., 1998). These results are strengthened by other reports showing that administration of L-Arg, a NO precursor, has protective effects on tissue I/R injury (Burra et al., 1997; Nilsson et al., 1997), while administration of L-NAME, a non-selective NOS inhibitor, exacerbates the renal damage. Previous studies have reported that L-citrulline could increase plasmatic L-Arg concentration via the L-citrulline-NO cycle (Solomonson et al., 2003). In the present study, I/R caused a marked reduction in the NO level. Pre-treatment with L-citrulline at doses of 300, 600 and 900 mg/kg increased the NO content. However, the effect mediated by L-citrulline was significantly antagonized by co-administration of L-NAME.

NO is produced from L-Arg in three different isoforms of NOS: endothelial (e), neuronal (n) and inducible (i) (Moncada et al., 1991). Several studies have shown that eNOS-derived NO has a protective effect against tissue injury (Yamashita et al., 2003; Yamasowa et al., 2005). Furthermore, previous studies (Weight et al., 1999) have confirmed that the inhibition of cNOS

could impair the GFR, and upregulation of the cNOS activity ameliorated the injury. L-citrulline is a kind of ubiquitous amino acid in mammals (Curis et al., 2005). In the kidneys, vascular endothelia and other tissues, L-citrulline can be readily converted to L-Arg, thereby providing a recycling pathway for the conversion of L-citrulline to NO via L-Arg (Mori and Gotoh, 2000; Schneider et al., 2003). Recent studies have shown that oral administration of L-citrulline can improve the myocardial damage caused by I/R (Ikeda et al., 2000). Gou et al (2011) have also demonstrated that L-citrulline has a protective effect on the gastric ischemic reperfusion injury, with the related mechanisms of maintaining the constitutive NOS activity, at least in part. In the present study, I/R caused a marked reduction in cNOS activity and eNOS protein expression. The iNOS activity was also decreased in the I/R group. However, the NO content and iNOS activity in this study is contrary to our previous study on glycerol-induced ARF in rats (Liu et al., 2013). In that study, we observed that the content of NO and iNOS activity was increased in the glycerol-induced ARF in rats, and the adverse condition was reversed by administration of L-citrulline. Many studies have suggested that iNOS plays a deleterious role during renal I/R injury, and iNOS can increase the NO level anomalously, which is associated with generation of oxygen-based and nitrogen-based radicals, including $ONOO^-$ (Lien et al., 2003; Mark et al., 2005; Sezgin et al., 2013). In our present study, we found that pre-treatment with L-citrulline at doses of 300, 600 and 900 mg/kg significantly elevated iNOS activity and eNOS protein expression, and maintained the cNOS activity in normal physiological conditions. However, there were no significant statistical differences in the nNOS and iNOS protein expression. The preventive and therapeutic effect of L-citrulline on glycerol-induced ARF and I/R-induced renal injury are still being investigated, and we hope to be able to achieve more protective effects of L-citrulline on renal disease.

Overall, the results of our study show that NO is an important factor in renal ischaemia-reperfusion injury. Pre-treatment with L-citrulline for seven consecutive days significantly prevented reduction of the NO level and attenuated the I/R-induced renal damage. The decrease in total NOS activity, inducible NOS activity, constitutive NOS activity and endothelial NOS protein expression in the renal cortex were inhibited by L-citrulline administration at different concentrations. Moreover, L-citrulline prevented lipid peroxidation and increased the SOD activities and the GSH levels. However, the L-citrulline-mediated protection was significantly antagonized by co-administration of L-NAME.

Conclusion

L-citrulline was confirmed to have protective effects on I/R-induced renal injury in rats. The protective effect of L-citrulline occurs, at least in part, via the up-regulation of the eNOS protein expression and cNOS activity, thus maintaining the basal production of NO. Moreover, the antioxidant action might also be involved in the protective effect of L-citrulline against I/R-induced renal injury, indicating that L-citrulline could serve as a potential candidate in the treatment of I/R-induced renal injury.

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