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Original Article

Protective effects of icariin on lipopolysaccharide-induced acute renal injury in mice

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Abstract: Objective: Sepsis can cause serious acute kidney injury inbacterium infected patients, especially for intensive care patients. Icariin is a bioactive flavonoid, which has renal protection and anti-inflammation effects. This study investigated the mechanism underlying the attenuation of Lipopolysaccharide (LPS) induced renal injury by icariin. Methods: ICRmice were treated with LPS (25 mg/kg) with or without treatment with icariin (20 or 40 mg/kg for three days). Renal function, histological changes, degree of oxidative stress and tubular apoptosis were examined. The effects of icariinon LPS-induced expression of renal TNF-α, NF-κB, MCP-1, ICAM-1, cleaved caspase-3 and Bcl-2 family proteins were evaluated. Results: Treatment of mice with LPS resulted in renal damage, showing an increase in blood urea nitrogen and creatinine levels, tubular damage, oxidative stress and apoptosis. These renal changes could be significantly improved by icariin treatment, both at two dosages. Examination of cytokines, chemokines and molecular mediators involving in inflammation and apoptosis of the kidney revealed that treatment of LPS increased levels of TNF-α, NF-κB, IL-1β, cleaved caspase-3, MCP-1, ICAM-1and decreased the level of BCL-2. All these disturbed expressions were reversed by treatment with icariin. Conclusions: These results indicate that icariin ameliorates the LPS-mediated nephrotoxicity via improving renal oxidant status, consequent NF-κB activation and inflammation cascade and apoptosis, and the following disturbed expression of apoptosis related proteins.

Keywords: Acute kidney injury, LPS, icariin, inflammation, apoptosis

Introduction

The kidneys are the preferential targets of xenobiotics such as drugs or environmental contaminants. Acute renal damage, characterized by acute tubular cell injury and kidney dysfunction, mainly develops following toxic or ischemic insults [1]. Indeed, severe sepsis and septic shock are the leading causes of acute kidney injury (AKI) in intensive care patients and may be responsible for more than 50% of cases of AKI in such patients. Septic patients who developed AKI have overall increased morbidity, and required prolonged hospital stays; they occupied more healthcare resources, and have increased mortality. The pathophysiology of septic AKI is complex, multi-factorial and distinct from non-septic AKI [2-6].

Although the mechanism of LPS-induced toxicity is not well known, it has been proposed to be multi-factorial in nature. These include reactive oxygen species (ROS), apoptosis and inflammation [7]. The enhanced production of reactive oxygen species (ROS) and the decrease in the antioxidant enzymes are involved in the early stage of LPS-induced nephrotoxicity [8, 9], result in oxidative damage in different tissues, and reaction with thiols in protein and glutathione which could cause cell dysfunction [10]. The activation of TLR4 signaling pathway stimulates the expression of pro-inflammatory chemokines and cytokines such as interleukins (IL-1β, IL-18, IL-6) and tumor necrosis factor-α (TNF-α) in the kidney, which play an important role in the pathogenesis of LPS-induced renal injury and damage [11]. Glomerular fibrin deposition occurred partially due to enhanced induction of nuclear factor-κB and TNFα in the kidney [12].

Therefore, antioxidants, modulators of nitric oxide, diuretics, and anti-apoptotic agents are current strategies for ameliorating or preventing LPS nephrotoxicity [13]. Herb epimedii is frequently used to invigorate the kidney and

strengthen yang [14]. Icariin is the main active components of *Epimedium brevicornum* Maxim [15]. It exhibits many bioactivities including regulating cardiovascular, genital, liver, bone marrow systems [16], and immunoregulatory effect. These effects are mainly related to attenuation of oxidative stress and apoptosis [17, 18], protecting DNA against radical-induced damage [19], and regulation of insulin/IGF1 pathway [20] and Th17/Treg function [21]. Recently studies show renal protective effect of icariin via regulating hypothalamus-pituitary-adrenal axis, and alterating cell cycle distribution and expression of apoptotic genes [22].

Based on previous studies, it was thought possible that icariin may also be useful in ameliorating the LPS-induced acute kidney injury. Testing such a hypothesis and explaining the possible mechanisms were the objective of this study.

Material and methods

Experimental animals

Healthy male ICR mice (20-22 g) were procured from SPF Laboratory Inc. Beijing, China), and were caged under specific pathogen-free conditions in a temperature- and humidity controlled environment. The experiments were carried out in accordance with the guidelines of Animal Ethics Committee of 254th Hospital of PLA. Mice were given food and water ad libitum, and divided into four equal groups (n = 10 per group) after one weeks of acclimatization. Control group: saline was given orally for six consecutive days and on the 3rd day a single intraperitoneal (i.p.) injection of saline was given; Model group: saline was given orally for six consecutive days and on the 3rd day also a single i.p. injection of LPS (25 mg/kg) was given; Icariin group: icariin (20 or 40 mg/kg/day bw) was given orally once daily for three consecutive days and on the 3rd day a single i.p. injection of LPS was given. The body weight of mice was recorded each day. At the end of the experiment, blood samples were obtained by eye enucleation. Mice were then sacrificed by decapitation, and kidney were immediately harvested and processed. One half of the samples was fixed in 4% buffered paraformaldehyde (pH 7.4) at 4°C overnight and embedded in paraffin for histopathological and immunohistochemically examination. The remaining half was frozen in liquid nitrogen immediately for subsequent evaluation.

Serum biochemical indices

Heparin was used as an anticoagulant and plasma samples were obtained by centrifugation at 900 g for 20 min and stored at -80°C. Serum creatinine (Scr) and BUN activities were measured with enzymatic kinetic method using commercial kits (Nanjing Jiancheng Pharmaceuticals, Nanjing, China) following the manufacturer's instructions.

Histopathology examination

Renal samples were fixed immediately in 4% PFA, dehydrated in alcohol series, cleared with xylene and embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E) and examined by light microscopy. All renal sections were examined by experienced histologists in a blinded fashion. Five high-power fields of each section were assessed, and scores representing the approximate extent of necrotic area in the cortical proximal tubules were then averaged as mean ± SD. The degree of damage was graded as 0, no degeneration; 1, minimal degeneration (10% involvement); 2, mild degeneration (10-35% involvement); 3, moderate degeneration (36-75% involvement); and 4, severe degeneration (75% involvement).

TUNEL assay

The terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay was used for *in situ* apoptosis with TUNEL reagent (Promega, Madison, WI). In brief, 10 μm paraffin sections were treated with 20 μg/mL proteinase K and then incubated in a nucleotide mixture containing fluorescein-12-dUTP and TdT. Positive controls were pretreated with 1 U/mL Dnase, and negative controls were incubated without TdT. Dark yellow of apoptotic cells was counted under light microscopy (CX31, Olympus, Japan).

Estimation of oxidative and anti-oxidative parameters in kidney

Kidney tissues were washed with ice-cold saline after immediate removal and weigh. Tissues were homogenized in ice-cold normal saline (1:10, w/v). The homogenate was centri-

Table 1. Blood urea nitrogen, Scr, NGAL values at the post-treatment with the ICARIIN in LPS-induced AKI model

Groups	Normal control	LPS model	ICARIIN (20 mg/kg)	ICARIIN (40 mg/kg)
N	10	10	10	10
BUN (mg/dl)	12.23±1.38	39.14±4.17#	16.82±2.15*	12.76±2.67**
Scr (mg/dl)	1.23±0.11	1.85±0.37#	1.41±0.24*	1.01±0.12**

#P<0.05, compared with the normal control rats; *P<0.05, **P<0.01, compared with the LPS-induced AKI rats.

Table 2. ICARIIN improve the oxidative stress condition

Groups	Normal control	LPS	ICARIIN (20 mg/kg)	ICARIIN (40 mg/kg)
N	10	10	10	10
GSH (mmol/g tissue)	6.45±0.17	2.89±0.09#	3.67±0.18*	5.35±0.14**
MDA (nmol/g tissue)	37.6±2.23	84.9±4.62#	64.4±11.8*	56.5±12.9**
SOD (U/mg protein)	12.3±0.86	5.86±0.54#	7.64±0.44*	10.3±0.64**
Catalase (U/mg protein)	7.6±0.53	3.9±0.42#	5.4±0.48*	6.8±0.39**

#P<0.05, compared with the normal control rats; *P<0.05, **P<0.01, compared with the LPS-induced AKI rats.

fuged at 12,000 g for 10 min at 4°C, and the resultant supernatant was used to determine the activities of superoxide dismutase (SOD), malondialdehyde (MDA), reduced glutathione (GSH), and catalase using commercial kits (Nanjing Jiancheng Pharmaceuticals, Nanjing, China) according to the instructions.

Western blot analysis

The frozen kidney cortex were homogenized and lysed in a lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1 mM EGTA, and 10% Triton X-100) containing a protease inhibitor cocktail. After centrifugation at 4°C, 12,000 rpm for 10 min, the supernatants were collected and their protein concentration was measured by the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of total protein (approximately 50 ug) were boiled for 5 min and loaded onto a 10% SDS-polyacrylamide gel electrophoresis gel. Subsequently, proteins were transferred to polyvinylidene difluoride membranes at 300 mA for 45 min (Millipore Co., Billerica, MA, USA). The membranes were washed in Tris-buffered saline with Tween 20 (TBST) and incubated in 5% skim milk (Sigma) at room temperature for 1 h. Then the membranes were incubated with the primary antibodies for IL1β (1:1000 dilution), TNF-α (1:2000 dilution), NF-κB (1:2000 dilution), MCP-1, ICAM-1 or β-actin (1:1000 dilution) overnight at 4°C. The membranes were washed with TBST and further incubated with the appropriate HRP-conjugated secondary anti-bodies (1:10000 dilution) for 1 h at room temperature. HRP activity was visualized by an enhanced chemiluminescence system (Kodak Medical X-Ray Processor, Rochester, NY, USA). Densitometric analysis was performed using an image analysis program (Fluor Chem 8900, Alpha Innotech Corp, San Leandro, CA, USA).

Immunohistochemistry

After deparaffinization and rehydration of kidney sections embedded in paraffin, the sections were exposed to 3% H_2O_2 for 10 min to bleach endogenous peroxidases, followed by rinsing 3 times in PBS for 10 min. The sections were then irradiated in 0.1 mol/L sodium citratebuffer (pH 6.0) in a microwave oven (medium low temperature) for 20 min. Nonspecific binding sites were blocked with normal goat serum diluted 1:10 in PBS for 30 min, and theslides were then incubated overnight at 4°C with anti-mouse monoclonal antibodies against cleaved caspase 3 and BCL2 in a humid environment. The sections were rinsed in PBS and then incubated in HRP-conjugated secondary anti-bodies for 30 min at 37°C. The sections were rinsed in PBS and then incubated with labelled streptavidin-biotin for 30 min. After washing in PBS, the sections were visualized with DAB and counterstained with hematoxyline. Then the sections were rinsed in tap water for 10 min, dehydrated with an alcohol series and cleared with xylene, mounted with DPX and cover slipped. Slides prepared for each case were examined by light microscopy.

Statistical analysis

Data were shown as mean ± SD and compared by one-way ANOVA with post hoc tests to LPS treated group. A p value less than 0.05 were considered as significant. Statistical analysis

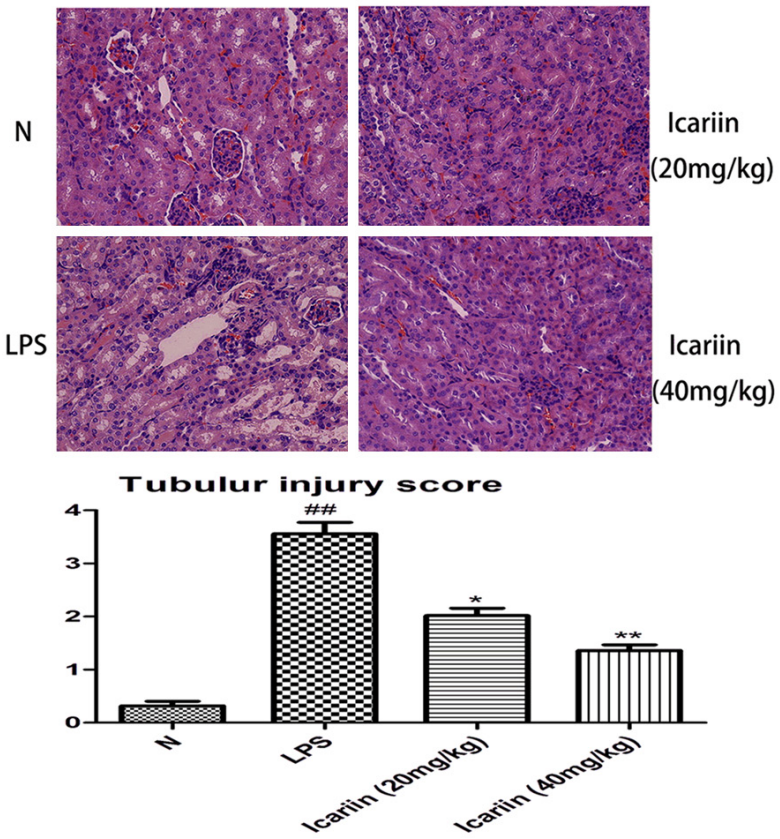

Figure 1. Effect of icariin on LPS-induced renal tubular damage. Histologic sections of kidney at 24 h after treatment with control buffer, LPS and LPS plus icariin (200×). Histopathological scoring of tubular injury was concomitant with histologic analysis (n = 10 for each experimental group). Data are expressed as mean ± SEM. #P<0.05 vs. Control; *P<0.05, **P<0.01 vs. LPS.

was carried out using Graphpad Prism 5.0 (Graphpad Software, San Diego, CA, USA).

Results

Icariin improvesbiochemical indices of renal function

Serum creatinine (Scr) and BUN (blood urea nitrogen) levels in LPS treated mice were significantly increased by 3.2 and 1.5 folds compared to those in the normal mice (39.14±4.17 *vs.* 12.23±1.38 mg/dl, 1.85±0.37 vs. 1.23±0.11 mg/dl, P<0.01) (**Table 1**), indicating the induction of severe septic nephropathy. Compared to model group, icariin at two doses markedly decreased SCr levels by 23.8% and 45.2%, and BUN levels by 20.3% and 46.5%, respectively.

Icariin ameliorates oxidative stress in LPS-challenged kidney tissues

The effects of icariin on LPS-induced renal oxidant status were shown in **Table 2**. LPS induced a significant increase in MDA level compared to the normal group (84.9± 4.62 vs. 37.6±2.23 nmol/g tissue, P<0.01). Meanwhile the antioxidant indices, i.e. GSH concentration, and catalase and SOD activities, showed significant reductions compared to the normal group (P<0.05). The mice received icariin showed significantly alleviated MDA and GSH level compared to the model group, together with the increased activities of catalase and SOD enzyme (P<0.05).

Icariin improves the histology of LPS treated mice

In control group, normal glomerular and tubular histology was seen both in cortical and medullary regions of kidney (**Figure 1**). In LPS treated group, severe diffuse acute tubular necrosis and desquamation and parenchyma degeneration in the cortex could be seen. In detail, tubular congestion and swelling, loss of brush border, tubular cells necrosis, tubular nuclear pyknosis, tubular cells flattening, and severe invasion of inflammatory cells within the interstitium and the perivascular and subvascular areas could be seen (**Figure 1**). The group treated with icariin at 20 mg/kg showed partial improvement of the histological features of renal injury compared to the LPS treated group. Mice treated with icariin at 40 mg/kg demonstrated more significant reduction in injury almost similar to the control. The histological scores also showed increased tubular injury score after LPS treatment, which could be significantly reversed by icariin treatment (**Figure 1**).

Icariin attenuates LPS-induced apoptosis in the kidney

TUNEL staining apoptotic cells were shown in **Figure 2**. There were almost no TUNEL-positive cells in normal mice. The number of TUNEL-

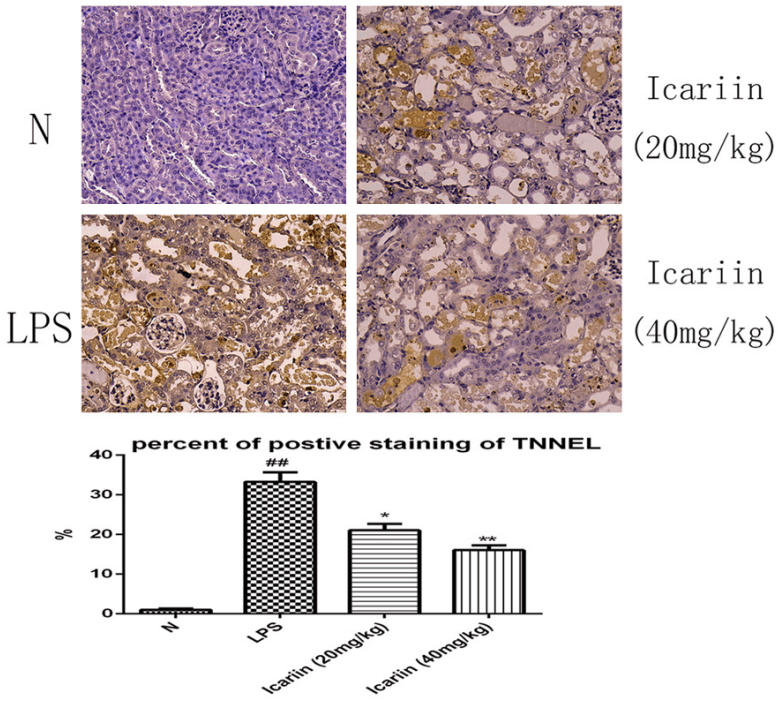

Figure 2. DNA fragmentation was visualized in situ by the TUNEL procedure by light microscopic.

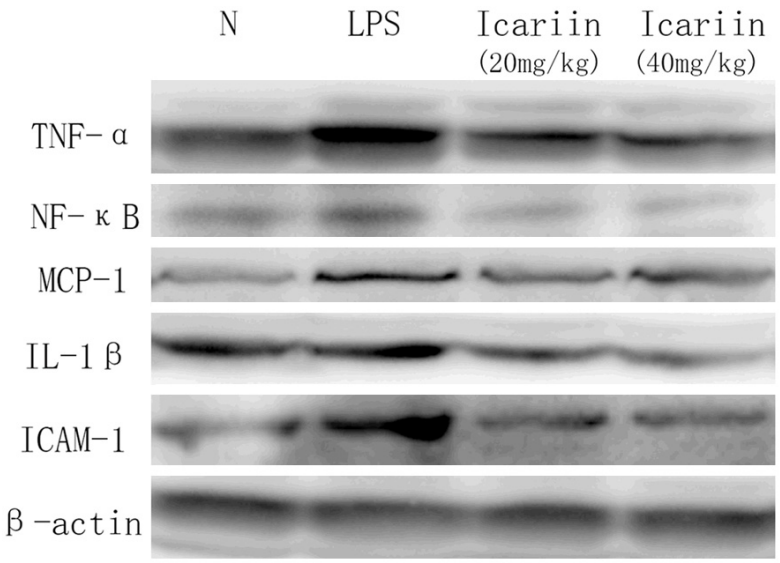

Figure 3. Effects of icariin on LPS-induced TNF-α, NF-κB, IL1β, MCP-1 and ICAM-1 protein expressions. Kidneys from mice treated with control buffer, LPS and LPS plus icariin were evaluated for protein expressions by western blot analysis.

positive cells was significantly increased after LPS treatment compared to the number of the control group. Icariin administration at low dose significantly decreased the number of LPS-induced TUNEL-positive cells, and more significant reduction almost similar to the control at high dose.

Icariin regulates expression of TNF-α, NF-κB, ICAM-1, MCP-1 and IL-1β

As shown in **Figure 3**, compared to the normal group, the protein expression of inflammatory factors such as TNF-α, NF-κB, ICAM-1, MCP-1 and IL-1β were significantly increased in LPS treated group. However, these changes of protein expression could be significantly alleviated by icariin treatment, especially in high dose of icariin treated group.

Icariin suppresses cleaved Caspase-3 and BCL-2 induction in LPS-treated mice

The distribution of cleaved caspase-3 and BCL-2 in kidney sections was assessed by immunohistochemistry (**Figure 4**). Slight staining of cleaved caspase 3 was observed in cortical and medullar structures of kidney in control group. In LPS group, diffuse and strong cleaved caspase-3 staining was seen in all the proximal and distal tubules in renal cortex. The positively stained cells exhibited morphologic changes that were associated with apoptosis (pyknotic, shrunken cell with condensed nucleus). The caspase-3 activation was dramatically reduced by icariin treatment, which was more obvious in higher dose of icariin treatment group. Similar results were obtained for BCL-2, which icariin treatment significantly increased the BCL-2 expression.

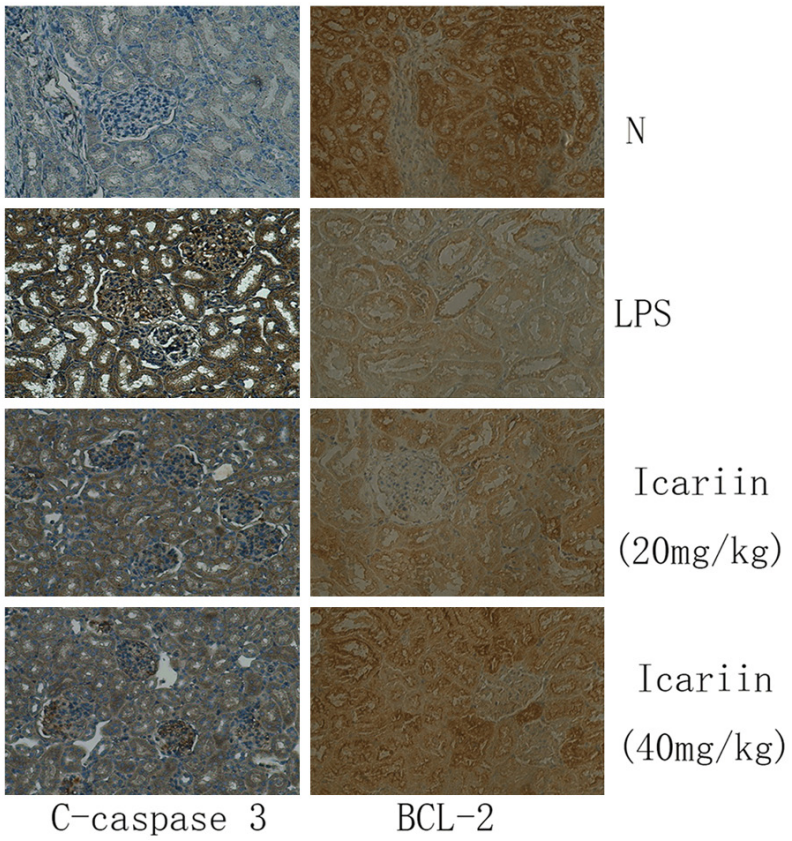

Figure 4. Effect of Icariin on immunohistochemical stain of cleaved caspase 3 and BCL-2 in LPS-induced mice. The brown area was the positive expression area (200×).

Discussions

In this study, we assessed the protective effects of icariin using a mouse model of LPS-induced nephropathy. Results showed that icariin administration reduces LPS-induced oxidative stress, local inflammation, tubular apoptosis, which were incriminated in the pathogenesis of renal dysfunction. The molecular basis was a reduction of LPS-induced increase in expression of inflammatory signals including TNF-α, NF-κB, MCP-1, IL1β, and expression of apoptotic signals including caspase-3 and Bcl-2 family proteins.

The systemic inflammation has been known to mediate organ dysfunction during sepsis. Excessive inflammation plays a major role in the initiation of kidney damage and deterioration of kidney function [23]. Pro-inflammatory cytokines are major mediators of sepsis induced AKI [24]. Proinflammatory mediators are known to regulate acute inflammation by activating the JAK-stat3 signaling [25] and Nuclear factor NF-κB pathways [26]. Controlling the production and downstream signaling of pro-inflammatory mediators could be an effective approach to treat inflammatory kidney injury [27]. However, the regulation of inflammatory signaling in sepsis-induced AKI remains largely unknown. Nowadays attentions have focused on discovering compounds of natural origin preventing, protecting as well as accelerating of tubular cells regeneration against renal injury. Icariin, main bioactive components of *E. brevicornum* have attracted much attention due to effects on invigorating the kidney. Icariin has protective effect on the early stage of experimental diabetic nephropathy via modulating TGF-β1 and type IV collagen expression in rats [16]. Besides, there is also report about its protective effect against LPS-induced acute inflammatory responses via PI3K/Akt and NF-κB signaling pathway [28]. Therefore, we explored the potential pharmacological actions exerted by icariin for LPS-induced mice and possible mechanism.

In this study, a single injection of LPS in mouse model caused renal morphological changes including tubular necrosis desquamation and degeneration in the proximal and distal tubules, as well as increased number of TUNEL-positive cells. This usually caused consequent disturbed renal function, which were also confirmed inour examination of biochemical parameters (elevated BUN and SCr levels, and renal cortical MDA level, as well as reduced kidney GSH level, catalase and SOD activities). Given that induction of nephrotoxicity by LPS is a rapid process that occurs within 4 h following administration, icariin administration started 2 days before LPS administration in the present study. Results showed icariin significantly moved all

the measured biochemical parameters measured towards normalcy, and attenuated necrotic damage, which suggests protective effects on kidney function and histology.

Previous mechanism studies in LPS-induced nephropathy have demonstrated variously implicated key upstream events. Research advances demonstrated that increased oxidative stress was one of the earliest features which lead to lipid peroxidation and GSH depletion [8, 10]. Treatment of LPS-treated mice with icariin here alleviated the disturbed renal oxidant status could partially attributed to the protective action of icariin at an early stage of LPS-induced nephrotoxicity.

NF-κB activation associated with increased ROS generation is pivotal in the consequent expression of proinflammatory cytokines like TNF-α, adhesion molecules (such as ICAM-1) [29], and the pro-apoptotic proteins (BCL-2 family) [30]. LPS treatment activated NF-κB translocation into the nucleus and increased TNF-α mRNA via p38 MAPK [31]. These chemokines may then facilitate migration and infiltration of inflammatory cell and a secondary wave of ROS generation, and further amplify the inflammatory cascade and injury [32]. In this study, icariin suppressed the release of TNF-α of the activated immune cells through attenuating NF-κB activation. It is likely that the protective effect of icariin is mediated in part by its anti-inflammatory effect.

NF-κB activation has been known to regulate various cellular responses, including apoptosis. It was reported that apoptosis could aggravate the pathogenesis of nephrotoxicityvia caspase 3 expression [27]. Mitochondrial oncogene products, Bcl-2 and Bax, are known to function upstream of caspase-3 to regulate apoptosis. Bcl-2 gene expression prevented caspase-3 activation during a variety of proapoptotic conditions [33]. Results demonstrated that expression of the BCL-2 was decreased in LPS-induced mice, and increased significantly by icariin. Besides, LPS-mediated activation of caspase-3 was attenuated by icariin. All these observations indicate a protective effect of icariin on LPS-induced renal tubular apoptosis via modulating expression of Bcl-2 family proteins and consequent caspase 3.

Conclusions

In conclusion, according to the protein expression analysis, proteins related to inflammation response and apoptosis are regulated during LPS-induced acute kidney injury. During this process, icariin treatment improves renal function, tubular damage, oxidative stress and apoptosis. Further study shows icariin ameliorates NF-κB activation, which was probably the key link between the oxidant status in early stage and consequent inflammation cascade and caspase 3 mediated apoptosis.

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Disclosure of conflict of interest

None.

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