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*Original Article*

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

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**Abstract:** Cisplatin chemotherapy often causes acute kidney injury in cancer patients. Icariin is a bioactive flavonoid, which has renal protection and anti-inflammation effects. This study investigated the mechanism underlying the attenuation of cisplatin-induced renal injury by icariin. BALB/c mice were treated with cisplatin (15 mg/kg) with or without treatment with icariin (30 or 60 mg/kg for 5 days). Renal function, histological changes, degree of oxidative stress and tubular apoptosis were examined. The effects of icariin on cisplatin-induced expression of renal TNF-α, NF-κB, cleaved caspase-3 and Bcl-2 family proteins were evaluated. Treatment of mice with cisplatin 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, especially in high dose of icariin group. Examination of molecules involving inflammation and apoptosis of the kidney revealed that treatment of icariin reduced expression of TNF-α, NF-κB, cleaved caspase-3, and Bax, increased the expression of BCL-2. These results indicate that icariin ameliorates the cisplatin-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, cisplatin, 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]. Nowadays cancer therapies using chemotherapeutic agents are the main strategies to improve the quality of life. Cisplatin is an effective and commonly used chemotherapeutic agents in the treatment of a variety of solid tumors. Cisplatin, which could be metabolized to a potent nephrotoxin-reactive thiol [2], selectively accumulates in proximal tubular cells to a 5 times higher degree of the serum concentration [3], damages proximal tubular epithelial cells [4], and finally contributes to nephrotoxicity [5]. Therefore it is a debating issue how to balance its dose-dependent efficiency and side effects, especially the acute kidney injury.

Although the mechanism of cisplatin-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 [6]. The enhanced production of reactive oxygen species (ROS) and the decrease in the antioxidant enzymes are involved in the early stage of cisplatin-induced nephrotoxicity [7], result in oxidative damage in different tissues, and reaction with thiols in protein and glutathione which could cause cell dysfunction [8]. Cisplatin impairs DNA replication and cell division [9] and thus, induced renal tubular cell death and acute deterioration of renal function. 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 are stimulated, initiate and extend inflammation, and play an important role in the pathogenesis of cisplatin-induced renal injury and damage [10]. Glomerular fibrin deposition occurred partially due to enhanced induction of nuclear factor-κB and TNF in the kidney [11].

Therefore, antioxidants, modulators of nitric oxide, diuretics, and anti-apoptotic agents [6] are current strategies for ameliorating or preventing cisplatin nephrotoxicity. Herb epimedii is frequently used to invigorate the kidney and strengthen yang [12]. Icariin is the main active components of *Epimedium brevicornum* Maxim [13]. It exhibits many effects including regulating cardiovascular, genital, liver, bone marrow systems [14], and immunoregulatory effect [15]. These effects are mainly related to attenuation of oxidative stress and apoptosis [16, 17], protecting DNA against radical-induced damage [18], and regulation of insulin/IGF1 pathway [19] and Th17/Treg function [20]. Recently studies show renal protective effect of icariin via regulating hypothalamus-pituitary-adrenal axis [21], 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 cisplatin-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 BALB/c mice (20-22 g) were procured from Vital River Laboratories (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, Peking Union Medical College Hospital. 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 cisplatin (15 mg/kg bw) was given; Icariin group: icariin (30 or 60 mg/kg/day bw) was given orally once daily for six consecutive days and on the 3rd day a single i.p. injection of cisplatin 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 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 immunohistochemical 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. SCr and BUN activities were measured with enzymatic kinetic method using commercially 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 endlabeling (TUNEL) assay was used for *in situ* apoptosis with TUNEL reagent (Promega, Madison, WI). In brief, 10 μm frozen 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. Green fluorescence of apoptotic cells (fluorescein-12-dUTP) was counted on a red background (propidium iodide) under fluorescence microscopy.

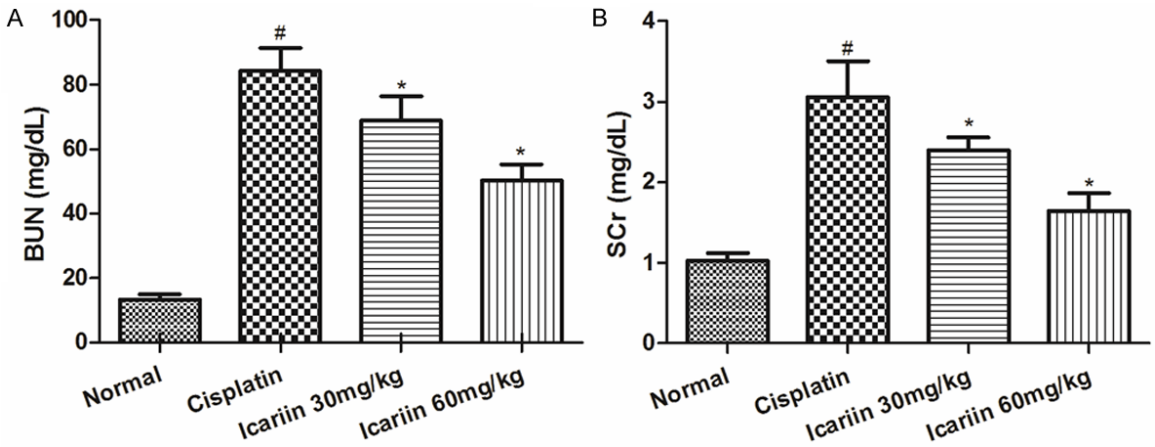


**Figure 1.** Effect of icariin on renal function after cisplatin treatment. Acute kidney injury was induced by cisplatin administration. Mice were treated with icariin or control buffer once a day for six consecutive days and on the 3rd day followed by intraperitoneal cisplatin injection. Blood samples were collected 72 h after cisplatin treatment and BUN (A) and creatinine (B) levels were measured. Data are expressed as mean ± SD (n=10 mice per group). #p<0.01 vs. Control; *p<0.01 vs. Cisplatin.

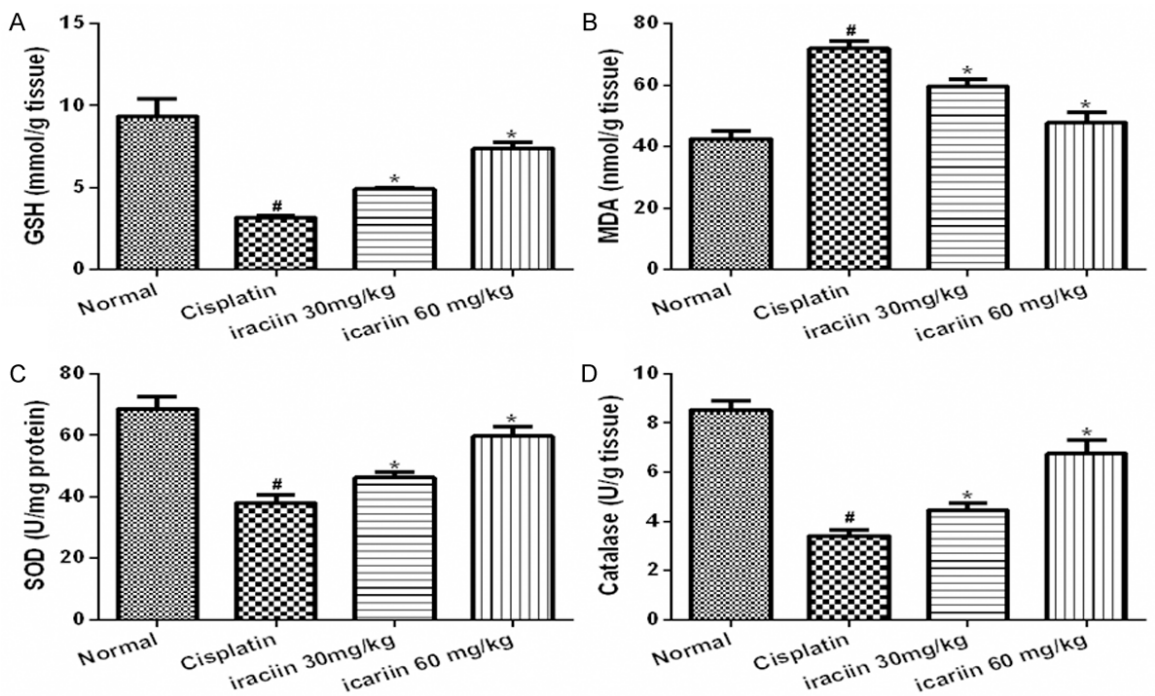


**Figure 2.** Effect of icariin on MDA level GSH content SOD and catalase activities in cisplatin-induced acute kidney injury. Tissue GSH content (A) MDA level (B) catalase (C) and SOD (D) activities were measured in the whole kidney lysate. Data are expressed as mean ± SD. #p<0.01 vs. Control; *p<0.01 vs. Cisplatin.

*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 centrifuged 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.

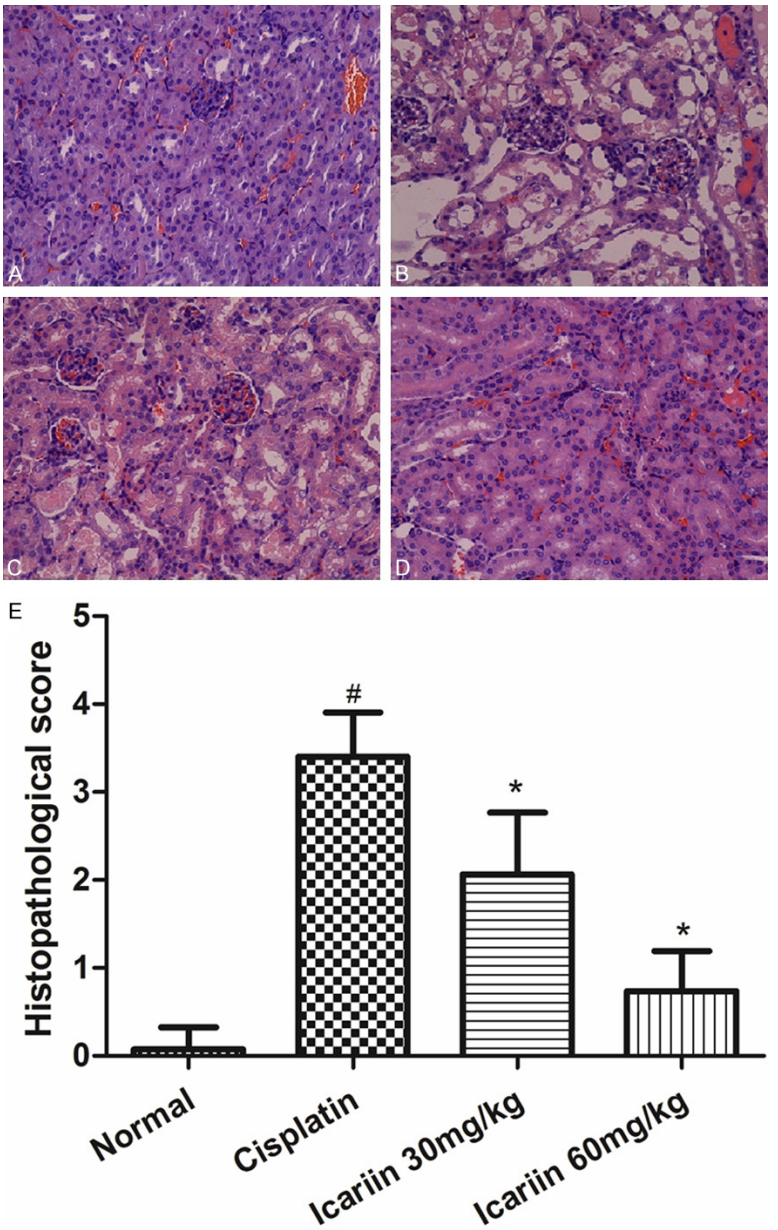


**Figure 3.** Effect of icariin on cisplatin-induced renal tubular damage. Histologic sections of kidney at 72 h after treatment with control buffer (A) cisplatin (B) and cisplatin plus icariin (C and D) (400×). Histopathological scoring of tubular injury was concomitant with histologic analysis (n=10 for each experimental group). Data are expressed as mean ± SD. #p<0.05 vs. Control; *p<0.05 vs. Cisplatin.

*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 cleaved caspase 3 (1:1000 dilution), TNF-α (1:2000 dilution), NF-κB (1:2000 dilution), BCL-2 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 (FluorChem-8900, Alpha InnotechCorp, 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 citrate buffer (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 the slides

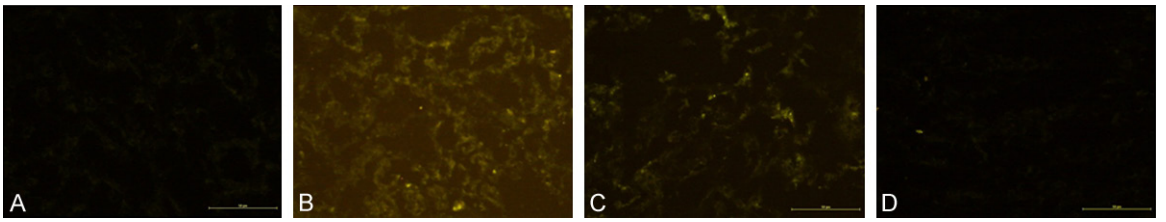


**Figure 4.** DNA fragmentation was visualized in situ by the TUNEL procedure and by electrophoresis. Fluorescence microscopy of TUNEL staining in control (A) cisplatin (B) and cisplatin plus icariin (C and D).

were then incubated overnight at 4°C with anti-mouse monoclonal antibodies against cleaved caspase 3 and Bax 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 cisplatin treated group. A $p$ value less than 0.05 were considered as significant. Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

## Results

*Icariin regulates biochemical indices of renal function*

BUN (blood urea nitrogen) and Serum creatinine (SCr) levels in cisplatin treated mice were significantly increased by 6.4 and 3.0 folds compared to those in the normal mice (84.31±7.14 *vs.* 13.27±1.68 mg/dl, 3.05±0.44 *vs.* 1.02±0.09 mg/dl, $p$<0.01) (**Figure 1**), indicating the induction of severe nephrotoxicity. Compared to model group, icariin at two doses markedly decreased SCr levels by 21.6% and 46.3%, and BUN levels by 18.3% and 40.2%, respectively.

The effects of icariin on cisplatin-induced renal oxidant status were shown in **Figure 2**. Cisplatin induced a significant increase in MDA level compared to the normal group (71.9±2.62 *vs.* 42.6±2.42 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 (3.18±0.12 *vs.* 9.36±1.04 mmol/g tissue, 3.42±0.23 *vs.* 8.54±0.37 U/g tissue, 37.95±2.67 *vs.* 68.67±4.03 U/mg protein, $p$<0.01). Compared to the model group, mice received icariin at two doses alleviated MDA level by 17.1% and 33.5%, increased GSH level by 55% and 132.7%, increased activities of SOD enzyme by 21.9% and 57.4%, and increased catalase activities by 30.7% and 97.4%, respectively ($p$<0.01).

*Icariin improves the histology of cisplatin treated mice*

In control group, normal glomerular and tubular histology was seen both in cortical and medullary regions of kidney (**Figure 3A**). In cisplatin 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 3B**). The group treated with icariin at 30 mg/kg b.wt. showed partial improvement of the histological features of renal injury compared to the cisplatin treated group (**Figure 3C**). Mice treated with icariin at 60 mg/kg b.wt. showed more significant reduction in injury almost similar to the control (**Figure 3D**). The histological scores also showed increased tubular injury score after cisplatin treatment, which could be significantly reversed by icariin treatment (**Figure 3E**).

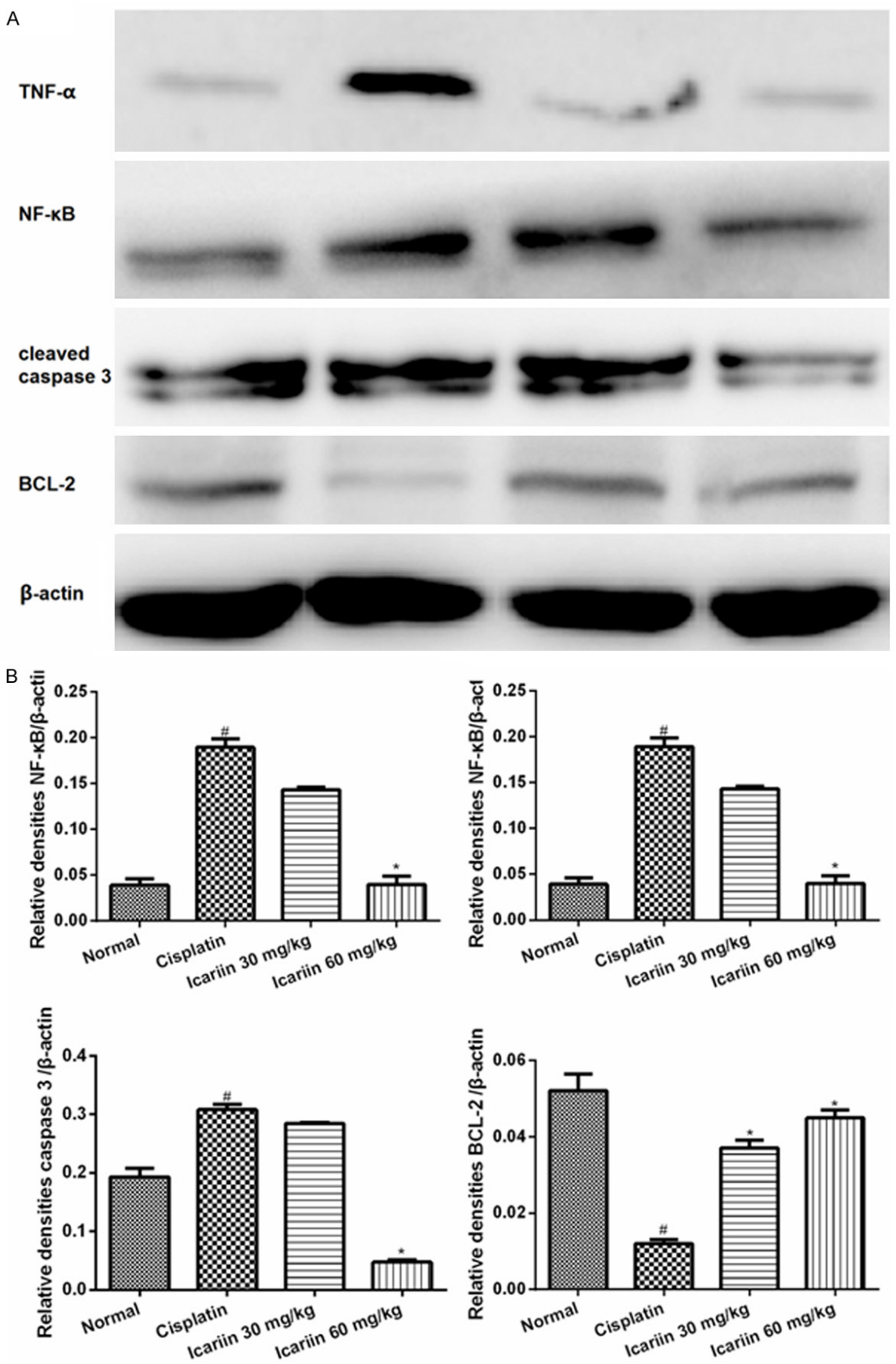
A
TNF-α
NF-κB
cleaved caspase 3
BCL-2
β-actin
B
Relative densities NF-κB/β-actin
Relative densities caspase 3 /β-actin
Relative densities BCL-2 /β-actin
Normal
Cisplatin
Icariin 30 mg/kg
Icariin 60 mg/kg

**Figure 5.** Effect of icariin on cisplatin-induced TNF-α NF-κB cleaved caspase-3 and BCL-2 protein expressions. Kidneys from mice treated with control buffer cisplatin and cisplatin plus icariin were evaluated for protein expressions by western blot analysis (A). Densitometric analyses are presented as the relative ratio of each protein to β-actin (B). Data are expressed as mean ± SD of three independent experiments. #p<0.01 vs. control; *p<0.01 vs. Cisplatin.

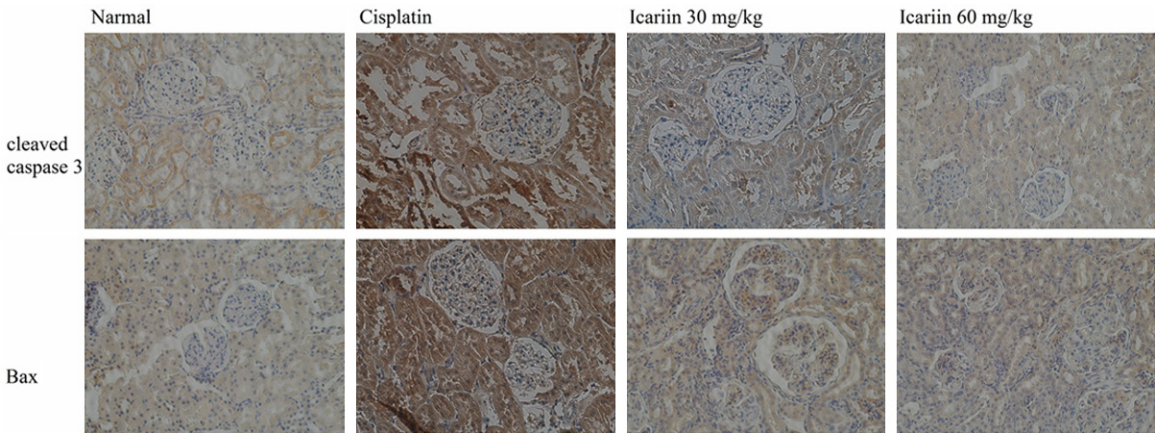


**Figure 6.** Effect of Icariin on immunohistochemical stain of cleaved caspase 3 and Bax in cisplatin-induced mice. The brown area was the positive expression area (400×).

*Icariin attenuates cisplatin-induced apoptosis in the kidney*

TUNEL staining apoptotic cells were shown in **Figure 4**. There were almost no TUNEL-positive cells in normal mice. The number of TUNEL-positive cells was significantly increased after cisplatin treatment compared to the number of the control group. Icariin administration at low dose significantly decreased the number of cisplatin-induced TUNEL-positive cells, and more significant reduction almost similar to the control at high dose.

*Icariin regulates expression of TNF-α, NF-κB, cleaved caspase-3, and BCL-2*

Compared to the normal group, the protein expression of inflammatory factors TNF-α and NF-κB were significantly increased by 18.1 and 2.7 fold in cisplatin treated group. Meanwhile, the protein expression of caspase-3 and BCL-2, which mediated cell apoptosis, were regulated in cisplatin treated group as well (1.5 and 0.3 fold of control group, respectively). Results showed that caspase-3 was significantly up-regulated, while BCL-2 was significantly reduced (0.3 fold of the normal group) ($p$<0.01) (**Figure 5**). However, these changes of protein expression could be significantly alleviated by icariin treatment at two doses.

*Icariin suppresses active Caspase-3 and Bax induction in cisplatin-treated mice*

The distribution of cleaved caspase-3 and Bax in kidney sections was assessed by immunohistochemistry (**Figure 6**). Slight staining was observed in cortical and medullar structures of kidney in control group. In cisplatin group, diffuse and strong cleaved caspase-3 and Bax 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 and Bax activation was dramatically reduced by icariin treatment, which was more obvious in higher dose of icariin treatment group.

## Discussion

In this study, we assessed the protective effects of icariin using a mouse model of cisplatin-induced nephropathy. Results showed that icariin administration reduces cisplatin-induced oxidative stress, local inflammation, tubular apoptosis, which were incriminated in the pathogenesis of renal dysfunction [23]. The molecular basis was a reduction of cisplatin-induced increase in expression of inflammatory signals including TNF-α and NF-κB, and expression of apoptotic signals including caspase-3 and Bcl-2 family proteins.

Cisplatin chemotherapy, an effective strategy for a wide range of malignant tumors, has dose-depedent nephrotoxicity. More than 25% of patients develop acute renal failure after receiving an initial dose of cisplatin due to its preferential accumulation within the proximal tubular epithelial cells [24]. However, the pathogenesis of acute renal injury involved complex pathway crosstalk contributing to increased oxidative stress and inflammation, as well as renal tubular apoptosis, during the disease course. Nowadays attentions have focused on discovering compounds of natural origin preventing, protecting as well as accelerating of tubular cells regeneration against renal injury [25]. 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 [14]. Besides there is also report about its protective effect against LPS-induced acute inflammatory responses via PI3K/Akt and NF-κB signaling pathway [26]. Therefore, we explored the potential pharmacological actions exerted by icariin for cisplatin-induced mice and possible mechanism.

In this study, a single injection of cisplatin 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 [27], which was also confirmed in our 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 cisplatin is a rapid process that occurs within 1 h following administration [28], icariin administration started 2 days before cisplatin 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 mechanistic studies about cisplatin-induced nephropathy demonstrated variously implicated key upstream events. Research advances demonstrated that increased oxidative stress was one of the earliest features, which leads to lipid peroxidation and GSH depletion [1]. Treatment of cisplatin-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 cisplatin-induced nephrotoxicity.

NF-κB activation associated with increased ROS generation [29] is pivotal in the consequent expression of proinflammatory cytokines like TNF-α, adhesion molecules (such as ICAM-1) [23], and the pro-apoptotic proteins (BCL-2 family) [30]. Cisplatin 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 [32], and further amplify the inflammatory cascade and injury [33]. 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 nephrotoxicity via caspase-3 expression [34]. 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 pro-apoptotic conditions [35]. Results demonstrated that expression of the pro-apoptotic Bax was increased in cisplatin-induced mice, and decreased significantly by icariin, accompanied by significantly expression of anti-apoptotic Bcl-2. Besides, cisplatin-mediated activation of caspase-3 was attenuated by icariin. All these observations indicate a protective effect of icariin on cisplatin-induced renal tubular apoptosis via modulating expression of Bcl-2 family proteins and consequent caspase 3.

In conclusion, according to the protein expression analysis, proteins related to inflammation response and apoptosis are regulated during cisplatin-induced acute kidney injury. During this process, icariin treatment improves renal function, tubular damage, oxidative stress and apoptosis. Further study shows icariin amelio-

rates 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.

## Disclosure of conflict of interest

None.

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