

THE PROTECTIVE EFFECT AND THE UNDERLYING MECHANISM OF *EPHEDRA SINICA* STAPF ON RENAL INJURY IN A MURINE MODEL

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Manuscript received: November 2019

Abstract

This study aimed to investigate the protective effect and the underlying mechanism of *Ephedra sinica* Stapf on renal injury in a murine model. Sixty rats were randomly divided into 5 groups with 12 animals per group as follows: control group, model group, high dose group of *Ephedra* (20 g/kg bw/day), middle dose group of *Ephedra* (10 g/kg bw/day), and low dose group of *Ephedra* (5 g/kg bw/day). Renal injury was induced in rats using a renal ischemia-reperfusion model. Blood urea nitrogen (BUN), serum creatinine (SCr) and malondialdehyde (MDA) in the model group increase significantly while superoxide dismutase (SOD), nitric oxide (NO), and endothelial nitric oxide synthase (eNOS) decrease significantly compared with the control group ($p < 0.05$). In *Ephedra* groups BUN, SCr, and MDA decrease and SOD, NO, and eNOS increase compared to the model group in a dose-dependent manner. The histo-pathological evaluation showed in the model group appears a renal tubular protein cast with inflammatory cell infiltration, renal interstitial congestion, and visible turbid swelling of renal tubular epithelial cells. The histo-pathological changes are significantly reducing or even disappearing after the treatment with *Ephedra*. The model group showed severe tissue damage and a large number of apoptotic cells, up-regulation of Bax protein and down-regulation of Bcl-2 protein expression compared to control group ($p < 0.05$). Tissue damage in the *Ephedra* treatment groups is minor, and only scattered apoptotic cells were seen. Bax protein expression in the *Ephedra* groups was significantly down-regulated and Bcl-2 protein expression was significantly up-regulated in a dose-dependent manner compared to the model group ($p < 0.05$). *Ephedra* can reduce renal injury and improve renal function by reducing oxidative damage, free radical generation, and inhibiting apoptosis.

Rezumat

Acest studiu a urmărit investigarea efectului protector și mecanismul de bază al *Ephedra sinica* Stapf asupra leziunilor renale la șobolani. Șaizeci de șobolani au fost împărțiți aleatoriu în 5 loturi a 12 animale: lotul control, lotul de animale bolnave, lotul tratat cu doză mare de *Ephedra* (20 g/kgc/zi), lotul tratat cu doză medie de *Ephedra* (10 g/kgc/zi) și lotul tratat cu doză mică de *Ephedra* (5 g/kgc/zi). Leziunea renală a fost indusă la șobolani folosind un model de ischemie-reperfuție renală. Ureea din sânge (BUN), creatinina serică (SCr) și malondialdehidă (MDA) din lotul de animale bolnave cresc semnificativ, în timp ce superoxid-dismutaza (SOD), oxidul nitric (NO) și nitric oxid sintetaza endotelială (eNOS) scad semnificativ în comparație cu lotul control ($p < 0,05$). În loturile tratate cu *Ephedra*, BUN, SCr și MDA scad și SOD, NO și eNOS cresc comparativ cu lotul de animale bolnave, doză dependent. Evaluarea histo-patologică a arătat că în lotul de animale bolnave apare o proteină care produce inflamație, congestie interstițială renală și gonflare vizibilă a celulelor renale epiteliale tubulare. Modificările histo-patologice se reduc semnificativ sau chiar dispar după tratamentul cu *Ephedra*. Lotul de animale bolnave a prezentat leziuni tisulare severe și un număr mare de celule apoptotice, up-reglarea proteinei Bax și down-reglarea expresiei proteice Bcl-2 în comparație cu lotul control ($p < 0,05$). Deteriorarea țesuturilor în loturile tratate cu *Ephedra* este mică și s-au observat doar celule apoptotice împrăștiate. Expresia proteinei Bax din grupurile *Ephedra* a fost semnificativ down-reglată, iar expresia proteinei Bcl-2 a fost semnificativ up-reglată, doză dependent, comparativ cu lotul de animale bolnave ($p < 0,05$). *Ephedra* poate reduce leziunile renale și poate îmbunătăți funcția renală.

Keywords: *Ephedra*, renal injury, ischemia-reperfusion, oxygen free radical, apoptotic protein

Introduction

Ischemia-reperfusion injury (IRI) refers to ischemia caused by various causes that determine tissue or organ damage caused by blood perfusion recovery [1, 2].

The kidney is one of the organs affected by ischemia-reperfusion injury in the clinic [3]. Renal ischemia-reperfusion injury (RIRI) is clinically common in acute kidney injury (AKI) and after kidney transplantation

[4, 5]. It is one of the main factors that affect the prognosis in the treatment of AKI and the early functional recovery and long-term survival of grafts after renal transplantation [6]. Oxidative stress (OS) is the main pathological factor and mechanism of RIRI [7, 8]. The increased number of free radicals that appear during IRI, induce inflammatory response and apoptosis, and affect kidney function [9, 10]. *Ephedra* is a dry herbaceous stem of *Ephedra sinica* Stapf with acrid and slightly bitter taste. It has the effects of inducing sweat, freeing lung, relieving asthma and inducing diuresis to alleviate oedema, being used in the traditional medicine for cold, chest tightness, cough, oedema and bronchial asthma [11, 12]. *Ephedra* also has the effect of scavenging oxygen free radicals [13]. Studies that investigated the antioxidant and scavenging properties against oxygen free radicals of *Ephedra* polysaccharide used hot water extraction method in order to obtain a pure *Ephedra* polysaccharide sample [14, 15]. They showed that *Ephedra* polysaccharides could scavenge oxygen free radicals and had antioxidant effects. Its use in renal ischemia-reperfusion injury has not been previously reported. Therefore, this study aimed to evaluate the protective effect and the underlying mechanism of *Ephedra* in renal ischemia-reperfusion injury on a murine model.

Materials and Methods

Animals

Male CD-SD rats with the body weight of 240 ~ 260 g were obtained from the animal house of Heilongjiang University of Traditional Chinese Medicine. The rats were acclimatized to the new laboratory conditions for one week before the experiment and routinely raised. They were fasted, but not banned from the water overnight before surgery. The temperature of the animal room was controlled at $25 \pm 2^\circ\text{C}$, the relative humidity was controlled at 50% ~ 60% and the circadian rhythm was mimic by 12 hours light/dark cycle. The ethics committee of Heilongjiang University of Traditional Chinese Medicine approved the animal protocol and the experiment also followed the regulations of the State Science and Technology Commission on the management of laboratory animals.

Ephedra extract solutions preparation

The *Ephedra* pieces (Chinese Herbal Pieces Factory of Guangdong Medicine Company, China) were soaked in water for 30 minutes, followed by decoction for 50 minutes (defoaming during decoction). The residues were removed from the decoct by filtration in gauze, and then the solution was concentrated with a rotary evaporator (Heidolph Laborota 4000, Merck KGaA, Germany) to the final concentration of 1.0 g raw *Ephedra*/mL. Then, the stock solution was diluted with distilled water to obtain the treatment solutions of 0.5 g/mL and 0.25 g/mL. The treatment was administered to the animals by oral gavage in the

volume of 0.2 mL/10 g. The groups that receive 1.0, 0.5, 0.25 g/mL of *Ephedra* water decoction were equivalent to the doses of 20, 10, 5 g *Ephedra*/kg.

Murine model of renal ischemia-reperfusion injury and animal groups

Sixty rats were randomly divided into 5 groups: control group, model group, high dose group of *Ephedra* (20 g/kg), middle dose group of *Ephedra* (10 g/kg), and low dose group of *Ephedra* (5 g/kg), 12 rats per group.

The rat model of renal ischemia-reperfusion injury:

The rats from the model group and *Ephedra* groups were inhalator anaesthetized with sevoflurane (Chongqing Shenghuaxi Pharmaceutical Co., Ltd., China) and fixed on the operation table. The abdominal skin was cut with surgical scissors, and the abdominal cavity was opened. The right renal hilus was separated and after ligating with a surgical line, the right kidney and renal capsule were separated, and the right kidney was removed. After the left renal hilus were separated, the arteries and veins of the renal hilus were ligated lively with a surgical line to block the renal arteries and veins. After blocking for 45 minutes, the slipknot was released for the kidney blood reperfusion. When the blood reperfusion started the kidney colour changed from dark to red for successful of the model. The abdominal wall and skin were sutured, and the surgical wound was disinfected with iodine solution (Dezhou Tongde Disinfection Products Technology Co., Ltd., China). The penicillin (100,000 IU/kg) (Suzhou Erye Pharmaceutical Co., Ltd., China) was injected intraperitoneally to prevent infection. After the modelling was completed, each rat was sent to the cage for observation and feeding.

The control group was a sham operation group in which the kidney was not removed and the renal arteries and veins were not ligatured. The rest of the procedures were the same as in the model group of rats.

The control group and the model group received orally 2 mL/100 g physiological saline solution per day for two weeks while the *Ephedra* groups received *Ephedra* decoct solution in specified concentrations for 2 consecutive weeks.

During the 2 weeks of the experiment the hair, diet, urination volume, mental activity status, and death of the rats were observed. After 2 weeks of treatment the rats were sacrificed by exsanguination after anesthetized with 10% chloral hydrate (Guangzhou Peiyu Biological Products Co., Ltd., China). The blood was collected from the abdominal aorta for biochemical determination. Rat kidneys were collected, and the left kidney was placed in 10% neutral formalin solution (Nanjing Haeckel Biotechnology Co., Ltd., China) for haematoxylin and eosin (HE) staining. The right kidney was deep freeze at -80°C for biochemical index detection.

Biochemical indexed determination

2 mL of blood collected from abdominal aorta was centrifuged at 1170 g for 15 minutes to determine the blood urea nitrogen (BUN) and serum creatinine (SCr)

(BUN and SCr test kits, Renoir Technology Industry Co., Ltd., Shenzhen, China) by the picric acid method and diacetyl method according to the kit instructions. 1 g of the renal cortex was homogenated for the determination of superoxide dismutase (SOD), nitric oxide (NO), malondialdehyde (MDA) and endothelial nitric oxide synthase (eNOS). SOD was measured by the xanthine oxidase method (xanthine solution, Beijing Zhongsheng Ruitai Technology Co., Ltd., China). MDA was measured by the thiobarbital method (sorbic acid, Hunan Huari Pharmaceutical Co., Ltd., China; thiobarbituric acid, Shanghai Xinyu Biotechnology Co., Ltd., China), and NO was measured by the nitrate reductase method (NO test kit, Wuhan Chundu Biotechnology Co., Ltd., China) according to the instructions of the kits. The content of endothelial nitric oxide synthase (eNOS) in renal tissue was tested by enzyme-linked immunoassay (ELISA) (ELISA kit, Shanghai Sifeng Biotechnology Co., Ltd., China).

HE staining

The kidney tissue was fixed in a 10% neutral formalin solution. The fixed tissue block was dehydrated in ascending series of ethanol (Guangzhou Dongye Chemical Industry, China), cleared in xylene (Jinan Aohui Chemical Co., Ltd., China) and embedded in paraffin. The embedded tissue block was cut in pieces of 3 μ m. The tissue was stained with haematoxylin and eosin (Shanghai Tianhe Biotechnology Co., Ltd., China), cleared in xylene, sealed with resin (Shanghai Tianhe Biotechnology Co., Ltd., China). Then, the morphological changes of the tissue were observed under a light microscope (Weifang Mingyang Experimental Analytical Instrument Co., Ltd., China).

The kidney cells apoptosis determination by TUNEL method

TUNEL kit was purchased from Roche, USA. 3 μ m paraffin sections of kidney tissue were deparaffinised and incubated with proteinase K, a mixed solution of nucleotide and terminal deoxynucleotidyl transferase, horseradish peroxidase-labelled secondary antibody. Then the slides were stained with diaminobenzidine (DAB). Tissue sections were magnified 400 times with a colour image recording input device and a full-automatic image analyser (Sony Corporation, Japan). 5 visual fields from each section were randomly taken for positive cells (tan or brownish-yellow particles) and total cell count. Then, the apoptotic index (AI) was calculated as follows: AI = number of apoptotic cells/the total number of cells \times 100%.

Expression of Bcl-2 and Bax in kidney tissues measured by immunohistochemistry

Bcl-2 and Bax were determined in kidney paraffin sections by immunohistochemistry. Normal goat serum working solution sealed the antigen. Rabbit anti-rat Bax polyclonal antibody and rabbit anti-rat Bcl-2 polyclonal antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) were respectively used as primary antibodies. The paraffin sections were incubated at

37°C for 1 hour with the primary antibodies. Normal goat serum was used to seal the antigen. Then the samples were incubated with the secondary antibody-biotin-labelled goat anti-rabbit IgG (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China), followed by incubation with horseradish peroxidase-labelled streptavidin solution at 37°C for 30 min and finally stained with DAB Brownish-yellow staining was considered positive.

Expression of Bcl-2 and Bax by Western blot

Each group of tissue protein was extracted, and the total protein concentration was measured. After SDS-PAGE (dodecyl sulphate, sodium salt (SDS)-Polyacrylamide gel electrophoresis), the protein was transferred to a 0.2 μ m PVDF (polyvinylidene fluoride) membrane. 5% skimmed milk powder was sealed at room temperature for 2 h, adding rabbit anti-rat Bcl-2 and Bax primary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China). B-actin was used as internal control and the samples were incubated at 4°C overnight with rabbit anti-rat β -actin antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China). Then the membrane were washed with TBST (Tris buffered saline + Tween) (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) for 10 minutes, 3 successive times. HRP (Horseradish Peroxidase) goat anti-rabbit secondary antibody (Beijing Zhongshan Jinqiao Biotechnology Co., Ltd., China) was added and incubated at room temperature for 2 h followed by membrane wash with TBST for 10 min for 3 successive times. Then the samples were coloured with ECL (Electro-Chemiluminescence) solution (Beyotime Biological Technology Co., Ltd.) and then analysed using a Gel Doc 2000 gel imager (Bio-Rad, USA) and the bands were analysed with Image J software (National Institutes of Health, USA). The ratio of the grey value of the target gene band to the grey value of the internal reference β -actin band was used to reflect the expression level of the target gene.

Statistical analysis

SPSS 19.0 (IBM, USA) software was used for the single-factor analysis of variance. The data were expressed as mean \pm standard deviation ($\bar{x} \pm s$), and the statistical significance was considered if $p < 0.05$ [16].

Results and Discussion

General observations

Compared with the control group, the rats in the model group show mental debility, reduced activity, and often bury their heads and curl up. The above phenomenon also appears in each administration group, but it improves significantly compared with the model group, and gradually disappears with the increase of the dosage.

Biochemical parameters

In the model group, it is observed a significant increase of BUN, SCr and MDA levels and a significant decrease of SOD, NO, and eNOS compared to the control group

($p < 0.05$). In *Ephedra* treatment groups, the treatment determine a significant decrease of BUN, SCr, and MDA and a significant increase of SOD, NO, and

eNOS in a dose-dependent manner compared to the model group ($p < 0.05$) (Table I).

Table I

Effects of *Ephedra* on biochemical indexes in rats

Group	Control group	Model group	Low dose <i>Ephedra</i> group	Middle dose <i>Ephedra</i> group	High dose <i>Ephedra</i> group
BUN (mmol/L)	6.68 ± 0.69	25.87 ± 3.97**	20.86 ± 4.67*	15.17 ± 2.87 **	9.46 ± 1.56##&@
SCr (μmol/L)	38.86 ± 8.23	88.57 ± 9.96**	72.52 ± 8.21**	68.24 ± 7.63**	43.35 ± 5.24##&@
SOD (U/mL)	43.45 ± 9.38	15.5 ± 4.35**	19.54 ± 4.32**	30.53 ± 6.95**	38.46 ± 7.64##&@
MDA (nmol/mL)	2.23 ± 0.43	4.56 ± 0.89*	4.12 ± 0.67*	3.73 ± 0.65#	2.88 ± 0.54#&@
NO (μmol/mL)	13.34 ± 1.32	7.88 ± 2.25*	9.39 ± 2.4	15.12 ± 1.3#	22.30 ± 1.02***&@
eNOS (ng/mL)	1.96 ± 0.47	0.98 ± 0.24**	1.47 ± 0.37*#	1.85 ± 0.48#	2.27 ± 0.53***&@

* – $p < 0.05$, ** – $p < 0.01$ compared with the control group; # – $p < 0.05$, ## – $p < 0.01$ compared with the model group;

& – $p < 0.05$ compared with the *Ephedra* low dose group; @ – $p < 0.05$ compared with the *Ephedra* middle dose group.

Histopathological changes

Pathological observation of the kidney tissue under the light microscope showed that the renal tubule lumen of the control group was clear. Epithelial cells were regular in shape and arranged neatly. The glomerular structure was intact. The epithelial cells in the model group showed turbidity with the disorderly arrangement, degeneration and necrosis. The lumen of the renal tubule was narrow with unclear boundaries, and the glomerular atrophy appeared. In the low dose *Ephedra* group, some renal tubule lumens were irregular, and the epithelial cells were turbid and swollen. The nucleus

was located on the luminal side of the epithelial cells, and the glomerular had mild degeneration symptoms. In the middle dose *Ephedra* group, the epithelial cells were slightly turbid and swollen, and the epithelial nucleus was located in the top of the cells. The glomerular structure was slightly damaged. In the high dose *Ephedra* group, the epithelial cells were normal in structure, regular in shape, and arranged neatly. The epithelial cells were slightly turbid and the glomerular structure was slightly damaged, which was almost normal (Figure 1).

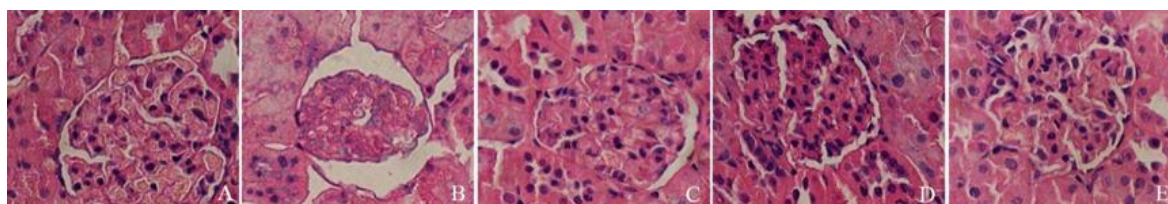


Figure 1.

Pathological changes in kidney tissue (A: control group; B: model group; C: low dose *Ephedra* group; D: middle dose *Ephedra* group; E: high dose *Ephedra* group of *Ephedra*) (HE ×400)

Renal tissue apoptosis

No significant apoptotic cells were seen in the control group. The model group shows severe tissue damage

and a large number of apoptotic cells. Tissue damage in the *Ephedra* groups was slight with scattered apoptotic cells (Table II).

Table II

Renal tissue apoptosis

Group	Control group	Model group	Low dose <i>Ephedra</i> group	Middle dose <i>Ephedra</i> group	High dose <i>Ephedra</i> group
AI	1.67 ± 0.05	35.18 ± 1.27**	18.17 ± 0.82**	10.25 ± 0.62***&	7.26 ± 0.27***&

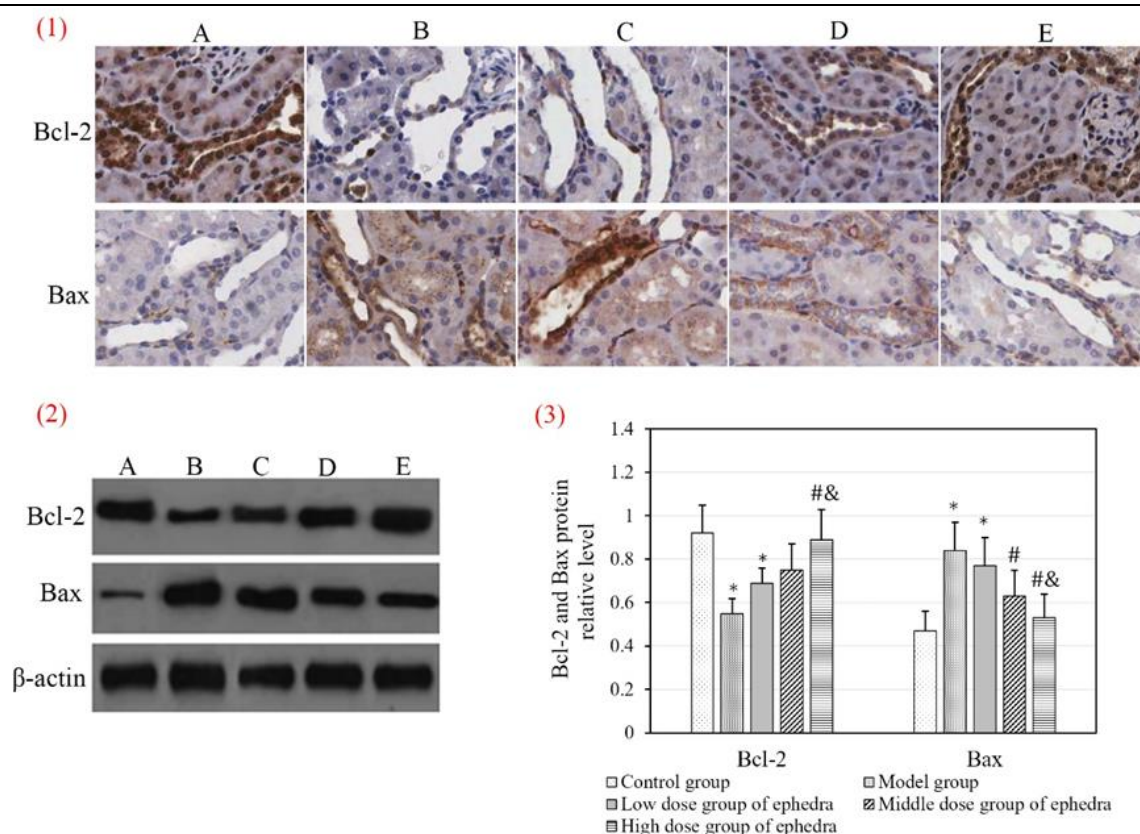
* – $p < 0.05$, ** – $p < 0.01$ compared with the control group; # – $p < 0.05$, ## – $p < 0.01$ compared with the model group;

& – $p < 0.05$ compared with the low dose group of *Ephedra*.

Bcl-2 and Bax protein expression in kidney tissue

Bax protein expression in the model group was significantly up-regulated ($p < 0.05$), and Bcl-2 protein expression was significantly down-regulated ($p < 0.05$) compared with the control group. Bax protein expression

in the *Ephedra* groups was significantly down-regulated ($p < 0.05$), while Bcl-2 protein expression was significantly up-regulated ($p < 0.05$) in a dose-dependent manner compared with the model group (Figure 2).

**Figure 2.**

(1): Various protein expressions tested by immunohistochemistry, $\times 400$; (2): Expressions of Bcl-2 and Bax protein tested by Western blot; (3): Statistics of relative grey values of Bcl-2 and Bax protein

* – $p < 0.05$ compared with the control group; # – $p < 0.05$, compared with the model group; & – $p < 0.05$ compared with the low dose *Ephedra* group.

The kidney is a hyperperfused organ and is quite sensitive to ischemia and ischemia-reperfusion. In this study, the renal injury model is replicated by clipping the renal arteries and veins. The results show that renal function was significantly impaired in the model group. Scr and BUN were significantly increased and the renal tubular epithelial cells were severely damaged. After the administration of *Ephedra* at different doses, Scr and BUN in rats were reduced considerably. The renal tissue damage was alleviated. These results showed that *Ephedra* can improve kidney function, reduce kidney tissue damage, and has a specific protective effect on renal ischemia-reperfusion in rats. Oxidative stress appears when the organism is exposed to various harmful stimuli that determine the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in excess [17]. When oxidative stress appears, the oxidation system and the antioxidant system are imbalanced, resulting in tissue damage [18]. SOD is the main antioxidant enzyme in the body [19]. It is mainly involved in the conversion of superoxide anion free radicals to H_2O_2 , removing excess reactive oxygen species, protecting chromosomes from damage and preventing gene mutations [20]. The change in SOD activity directly affects the content of intracellular oxygen free radicals, lipid peroxides and their

MDA levels [21]. Therefore, MDA concentration and SOD activity can be used as indexes of the degree of lipid peroxidative damage [22]. The results of this study show that compared with the control group, the MDA levels in the model group were significantly increased, and the SOD activity was reduced considerably. Compared with the model group, the MDA levels in *Ephedra* groups decreased significantly with the increase of the dose, and the SOD activity increased. It shows that *Ephedra* can protect against ischemia-reperfusion kidney injury by reducing oxygen free radical damage.

Recent studies have shown that nitric oxide (NO) is involved in the pathophysiology of ischemia-reperfusion injury [23]. Nitric oxide synthase (NOS) is the rate-limiting enzyme produced by NO [24]. Under normal circumstances, eNOS produced by endothelial cells is used to maintain a certain amount of NO production in the body. It plays an essential role in regulating vascular tone and blood pressure, relaxing bronchial smooth muscle, and inhibiting platelet aggregation and adhesion [25, 26]. The results of this study showed that compared with the control group, the renal function of the rats in the model group was significantly impaired, and the level of NO was reduced considerably. Compared with the model group, the level of NO

and eNOS in the *Ephedra* groups increase gradually with the increase of the dose. Also, the levels of NO and eNOS in the high dose *Ephedra* group was significantly higher than that in the control group. It shows that NO plays a decisive regulatory role in the kidney and is beneficial to improve the impaired renal function.

Studies have found that when the ischemia-reperfusion injury occurs, renal tubular epithelial cells are mostly apoptotic cells [27]. Some of the genes that regulate apoptosis are Bcl-2, Bax, p53 and fas [28]. Bcl-2 is an apoptosis-inhibiting gene, and Bax is a pro-apoptotic gene, which together forms a heterodimer [29]. The Bcl-2/Bax ratio reflects the comparison of pro-apoptotic and anti-apoptotic power and is a key factor in the regulation of apoptosis. Both of them act on the endogenous mitochondrial apoptotic pathway [30, 31]. The results of this study showed that compared with the control group, the apoptosis rate in the model group was significantly increased. Bcl-2 protein was decreased, and Bax protein was increased. After *Ephedra* administration, the apoptosis rate begins to decrease. Bcl-2 begins to be up-regulated and Bax begins to be down-regulated. Also, the effect of the high dose group of *Ephedra* was the most significant. It is suggested that *Ephedra* may have a protective effect on the kidney by up-regulating Bcl-2 expression and down-regulating Bax expression.

Conclusions

In conclusions, after renal injury caused by ischemia-reperfusion, ischemia and hypoxia affect the kidney tissues, and a large amount of oxygen-free radicals are generated. These leads to pathological changes in renal tissues and renal function damage with increase in renal tubular apoptosis. *Ephedra* can reduce kidney damage and improve kidney function through anti-oxidant effects, reducing free radical generation, and inhibiting apoptosis by regulating the expression of Bcl-2 and Bax genes implicated in apoptosis. Further studies should be done to evaluate the kidney protective effect of *Ephedra* in acute kidney injuries associated with ischemia-reperfusion injuries.

Acknowledgement

This work was supported by Natural Science Foundation Project of Heilongjiang Province (JJ2019LH1585).

Conflict of interest

The authors declare no conflict of interest.

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