

Original Article

Cordyceps sinensis prevents contrast-induced nephropathy in diabetic rats: its underlying mechanism

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Abstract: Apoptosis is recognized as an important mechanism in contrast-induced nephropathy (CIN). This study investigated the renal protective effect of cordyceps sinensis (CS) in a diabetic rat model of CIN and the mechanism of its effect. Sixty SD rats were randomly divided into 4 groups, the control group, model group, probucol group, and CS group. We used a diabetic rat model of Iodixanol-induced CIN. Serum creatinine (Scr), blood urea nitrogen (BUN), urinary kidney injury molecule-1 (KIM-1), neutrophil gelatinase associated lipocalin (NGAL) levels were measured to evaluate renal function. Total antioxidative ability (T-AOC), superoxide dismutase (SOD), and malonaldehyde (MDA) levels were assessed to discuss the effect of probucol and CS on oxidative stress. The pathologic changes in the kidney were observed by hematoxylin and eosin (HE) staining and periodic acid-Schiff (PAS) staining. Apoptosis was assessed by transmission electron microscopy and TUNEL staining. Caspase-3, Bax, Bcl2 and phospho-p38 mitogen-activated protein kinase (MAPK) protein expressions were assessed by Western blotting. The model group of rats showed significantly elevated levels of BUN, Scr, urinary KIM-1, NGAL, and parameters of oxidative stress ($P<0.05$). Both the probucol and CS groups demonstrated significantly lower Scr, BUN, and urinary KIM-1, NGAL levels compared to the model group ($P<0.05$), with no significant difference between these two groups. The probucol group and the CS group had significantly lower MDA and higher T-AOC, SOD than the model group after modeling ($P<0.05$). Caspase-3, Bax activation were effectively repressed while Bcl-2 expression was increased by probucol and CS pretreatment. Mechanistically, probucol and CS decreased the expression of JNK protein and increased the expression of ERK protein. CS can effectively reduce kidney damage caused by contrast medium. The underlying mechanism may be that CS accelerates the recovery of renal function and renal pathology by reducing local renal oxidative stress and influencing MAPK signal pathways.

Keywords: Cordyceps sinensis, contrast induced nephropathy, Phospho-p38 mitoge-activated protein kinase signals, oxidative stress

Introduction

The prevalence of contrast-induced nephropathy (CIN) is rising refer to the increased use of radio contrast media (CM) in diagnostic coronary angiography or percutaneous coronary intervention (PCI) procedures. CIN is generally defined as an otherwise unexplained acute impairment in renal function, manifested as serum creatinine (Scr) increases of 44.2 $\mu\text{mol/L}$ or $\geq 25\%$ from the baseline value within 3 days after the administration of contrast medium (CM) [1, 2]. To date, chronic kidney disease, dehydration, diabetes mellitus (DM), advanced age, increased volume of CM and recurrent administration are well-known risk factors of

CIN [3]. CIN has become the third most common cause of hospital-acquired acute renal failure [4, 5]. Despite the advent of advanced CM and improvements in preventive strategies, the prevention of CIN remains challenging, and no specific prevention besides adequate periprocedural hydration is available [6]. Thus, it is urgent to uncover the pathogenesis of CIN and to identify novel preventive therapies decrease CIN incidence and to improve clinical prognosis.

Although the exact pathophysiological mechanism of CIN still remains poorly understood, it has been generally demonstrated that CIN appears to be the result of combined effects of

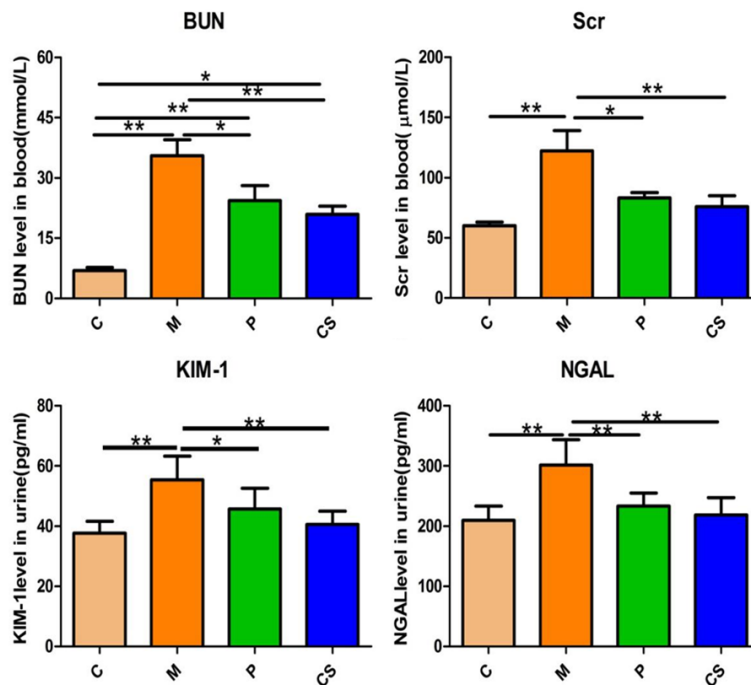


Figure 1. Blood and urine of all rats were harvested for testing BUN, Scr, KIM-1 and NGAL. Diabetic SD rats were injected intravenously with iodixanol to induce CIN. They were randomly divided into 4 groups: control (C) group, model (M) group, probucol (P) group, and cordyceps (CS) group. The rats of C group showed significantly higher levels of BUN, Scr, KIM-1 and NGAL than the M group. Those levels of rats in the two drug treatment groups were decreased. * $P < 0.05$ vs C, M and P; ** $P < 0.01$ vs C, M and P.

direct nephrotoxicity of CM and hypoxic renal injury. Mounting evidence has shown that impaired renal blood flow and ischemia-reperfusion injury plays an important role in the pathogenesis of CIN [7]. In addition, reactive oxygen species (ROS), inflammation, and apoptosis also contribute to the renal tubular cell injury.

Probucol is a universally recognized CIN prevention drug, as it demonstrates strong antioxidant effects and is often used in combination with hydration to prevent the occurrence of CIN [8]. Cordyceps sinensis (CS), a time-honored herbal medicine in China, can improve the microcirculation; increase the tolerance to ischemia in patients with microcirculatory disorders [9]. In addition to renoprotective abilities, CS also has anti-inflammatory, anti-oxidative effects, as well as anti-ischemic effects [10]. Recently, CS has been demonstrated to protect against CIN in type 2 diabetics with renal insufficiency undergoing coronary angiography [11]. This study utilized a rat CIN model to investi-

gate the renal protective effect of CS and the underlying mechanism responsible for this protection.

Materials and methods

Experimental materials

A total of 60 clean-grade male Sprague-Dawley (SD) rats at 8-10 weeks of age and a weight of 200~250 g from the Tianjin Acute Abdominal Diseases Institute Animal Research Center were used. All the rats were adaptively fed in the clean animal room for one week. Rats were housed in an air-conditioned room maintained at 23°C with a 12/12-hour light/dark cycle. Food and water were provided ad libitum except for the day of dehydration. The study protocol was approved by the Medicine Animal Ethics Committee of Tianjin Nankai Hospital (Tianjin, China).

CS were purchased from Hangzhou Zhongmei Huadong Pharmaceutical Co. Ltd. (China); Probucol was purchased from Chengde Jing Fu-kang Pharmaceutical Co. Ltd. (China); Iodixanol was purchased from GE Healthcare Co. Ltd. (Ireland); Streptozotocin (STZ) was purchased from Sigma Co. Ltd. Total antioxidative ability (T-AOC), Superoxide dismutase (SOD), and malondialdehyde (MDA) kit were purchased from Nanjing Jiancheng Company (China). The microscopic image acquisition and analysis system was from the Motic Med 6.0 CMIAS Image Analysis System (MoticChinaGroupCo., Ltd).

Model and grouping

Sixty SD rats were randomly divided into 4 groups of 15 rats each: control (C) group, model (M) group, rats treated with probucol (P) group, and rats treated with CS. Rat diabetes was induced by abdominal single dose injection of STZ (60 mg/kg) intraperitoneally. The blood glucose ≥ 16.7 μmol/L was used as the stan-

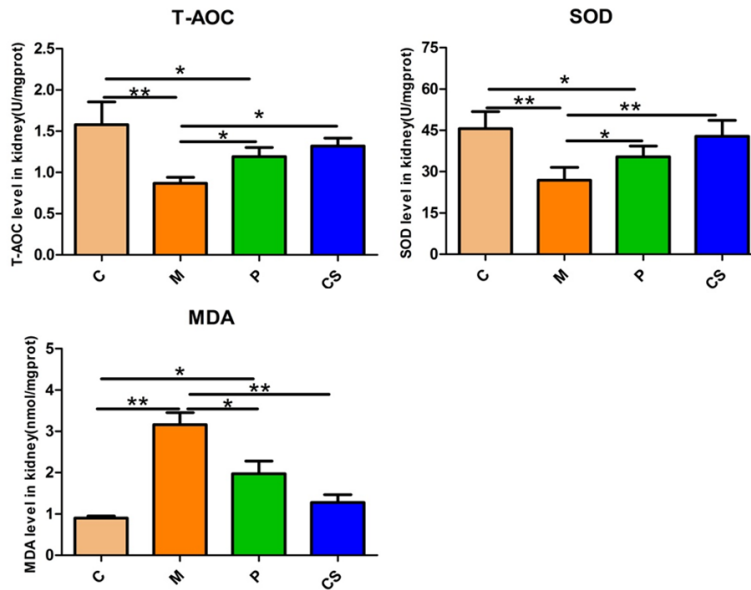


Figure 2. Kidney of all rats were harvested for testing T-AOC, SOD and MDA. Diabetic SD rats were injected intravenously with iodixanol to induce CIN. They were randomly divided into 4 groups: control (C) group, model (M) group, probenecid (P) group, and cordyceps (CS) group. The T-AOC, SOD levels were decreased, whereas MDA level of M group were increased ($P < 0.01$). Compared with M group, SOD levels of the two drug treatment groups were lower ($P < 0.05$ & < 0.01), and MDA levels were higher ($P < 0.05$ & < 0.01). * $P < 0.05$ vs C, M and P, ** $P < 0.01$ vs C, M and P.

dard for the success of diabetic rat model [12]. After ten weeks feeding, we use iodixanol to induce CIN. Rats in the CS treatment group received intragastric administration of CS for 7 days. The dosage of CS was 3 g/kg. Thereafter water deprivation was conducted for 24 hours, and then the diabetic rats were injected intravenously with iodixanol to induce CIN. Drugs administered consisted of iodixanol at a dose of 2 g/kg [13]. This quantity is the dose of CM that is standard for clinical use and for other relevant experiments in rat model. A relative ($\geq 25\%$) or an absolute ($\geq 44.2 \mu\text{mol/L}$) increase in Scr from the baseline is used as the standard for the success of CIN rat model [14]. Rats were anesthetized with 60 mg/kg pentobarbital. Pentobarbital sodium anesthesia was followed by CIN induction, which was performed with drug administration into a tail vein. Baseline blood samples were collected from the tail vein under ether anesthesia for analysis of Scr and blood glucose. Baseline 24-hour urine samples were collected for determination of kidney injury molecule-1 (KIM-1) and neutrophil-gelatinase-associated lipocalin (NGAL).

Observation indicators

General condition

The mental state, activity, body weight, food intake, and mortality of rats were observed after the procedure. After modeling, all rats were killed. After the rats were anesthetized for the modeling procedure, 1 ml of blood was obtained from the inner canthus for blood biochemical examination prior to modeling.

Detection of relevant renal oxidative stress indicators

After the rats were killed, the kidney tissues were quickly removed. In addition, 3 ml of blood was obtained from the abdominal aorta. Tissue blocks of the appropriate size were taken, placed in pre-cooled ice-cold normal saline, and made into tissue homogenate at a ratio of 1:9. The supernatant was removed after 15 minutes of centrifugation at 3000 rpm, and the purchased kits were used to measure the levels of T-AOC, SOD, and MDA in renal tissues.

Renal pathological examination

Tissue blocks from rat kidney were fixed in 10% formalin and prepared for hematoxylin and eosin (HE) staining, transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining, periodic acid-Schiff (PAS) staining. Renal pathological changes were observed using light microscopy. For TUNEL staining, sections were stained using In Situ Cell Death Detection Kit (Roche Applied Science) according to the protocol. TUNEL-positive tubular cell numbers were counted at random in 20 non-overlapping cortical fields under 400 \times magnification. Renal cortex was fixed in phosphate buffer (pH 7.2) containing 3% glutaraldehyde and 0.22 mmol/L sucrose. After fixing in 1% osmium tetroxide, the samples were dehydrated in an ethanol gradient and embedded in epoxy resin. The pathology of the kidney ultra-

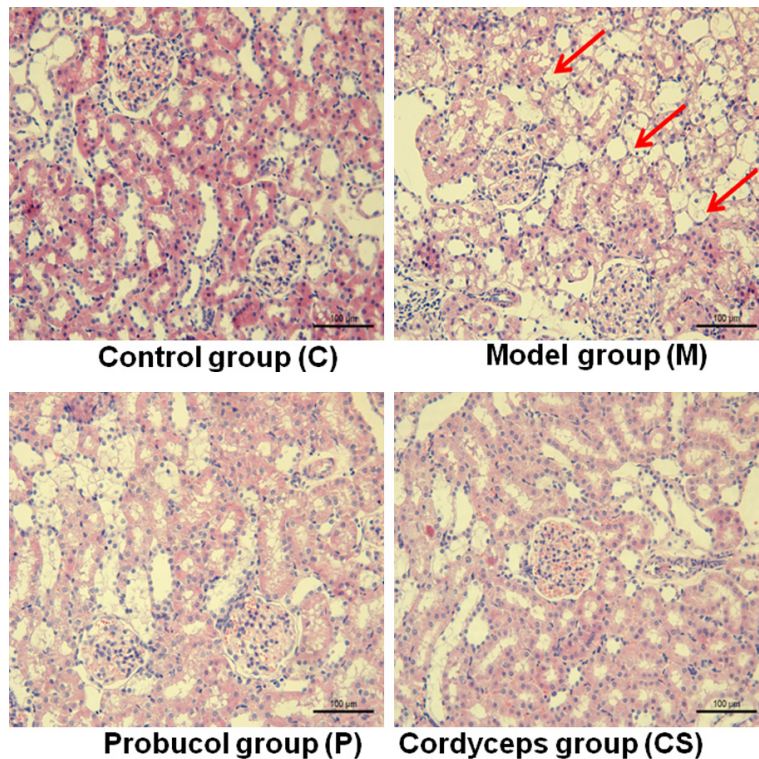


Figure 3. HE staining of kidney tissues (×200). In the C group, the renal tubular presented a normal morphology and structure. The M group showed obvious tubular epithelial vacuolar degeneration and disintegration (the red arrow). The treatment groups showed milder pathological changes than the M group.

structure was examined using a HitachiH-600 transmission electron microscope. All slides were observed by two blinded examiners independently.

Western blotting analysis

Proteins (30 μg) were subjected to electrophoresis on 6~12% polyacrylamide gel and transferred topolyvinylidene fluoride (PVDF) membrane. The membranes were blocked with 5% non-fat milk in TBST for 2 hours at room temperature, and incubated with the primary antibodies overnight at 4°C. The bands were detected by Gel Doc XR (BioRad, USA) after a second incubation with appropriate secondary antibodies. Protein expression was quantified by Image J 1.45 software (Wayne Rasband, NIH, Bethesda, Md., USA) after scanning the film. Primary antibodies included the following: anti-phospho Caspase-3 (Santa Cruz Biotechnology, 1:1,000); anti-phospho ERK1/2 (1:750); anti-phospho JNK (1:1,000); anti-phospho p38 MAPK (1:1,000); anti-Bcl-2 (Santa Cruz

Biotechnology, 1:1000); anti-Bax (Santa Cruz Biotechnology, 1:1,000); anti-α-actin (Santa Cruz Biotechnology, 1:1,000). All experiments were performed at least three times under the same conditions.

Statistical analyses

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Continuous variables and categorical variables are expressed as the mean ± standard deviation (SD) and percentages, respectively. All samples were tested to ascertain if they followed a normal distribution. Categorical variables were compared using the χ^2 test or the Fisher's exact test where appropriate. One-way analysis of variance (ANOVA) was used for the analysis of continuous variables among the 4 groups. Two-tailed *P* values of *P*<0.05 were considered significant.

Results

General condition of the rats

There were no deaths among the rats involved in this study. The rats in the C group did not show significant abnormalities in feeding or activity, although rats in the other groups demonstrated various levels of malaise, less mobility, reduced appetite, and weight loss, with the M group of rats showing the most significant symptoms.

Comparison of renal function among various treatment groups

BUN, Scr, urinary KIM-1, and NGAL levels in the rats of each group were all at the same baseline level before CIN induction. After CIN, the M group showed significantly higher levels of BUN, Scr, and urinary KIM-1, and NGAL levels than the C group (*P*<0.01). Both the CS and P groups demonstrated significantly lower BUN, Scr, urinary KIM-1, and NGAL levels compared to the M group (*P*<0.05), with no significant difference between the two groups (**Figure 1**).

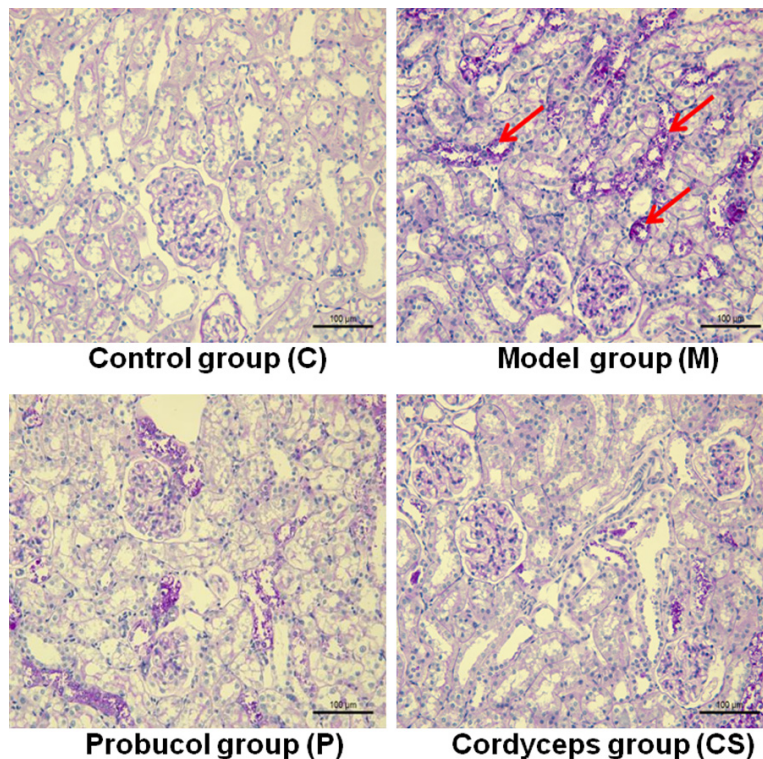


Figure 4. PAS staining of kidney tissues (×200). In the C group, the renal tubular presented a normal morphology and structure. The M group showed purple cell plasma (the red arrow). The treatment groups showed milder changes than the M group.

P groups showed milder pathological changes than the M group, they presented slightly vacuolar degeneration and brush border loss (**Figure 3**).

PAS staining of kidney tissues showed that the renal tubular epithelial cells of the C group presented a normal morphology and structure, without any luminal expansion or urinary casts. However, after CIN, the M group showed obvious tubular epithelial vacuolar degeneration and disintegration and shedding of the brush border, as well as visible cell casts and protein casts in regions of the lumen. In comparison, the CS group showed milder pathologic changes than the M group, but still presented fairly obvious vacuolar degeneration and brush border loss, and the P group showed milder pathologic changes than the CS group (**Figure 4**).

Detection of relevant indicators of oxidative stress in renal tissue

After CIN, the T-AOC, SOD, and MDA levels in the renal tissues of the C group showed no significant changes in comparison to the baseline levels. However, after CIN, the T-AOC and SOD levels of the M group were decreased ($P < 0.05$), whereas the MDA levels were increased ($P < 0.05$). In addition, CS and P groups showed significantly milder changes in the above indicators compared to the M group ($P < 0.05$), with no significant difference between the 2 groups (**Figure 2**).

Renal morphological changes

HE staining of kidney tissues showed that the renal tubular epithelial cells of the C group presented a normal morphology and structure, without any luminal expansion or urinary casts. In contrast, in the M group we found obvious tubular epithelial vacuolar degeneration, disintegration, and shedding of the brush border, as well as visible cell casts and protein casts in regions of the lumen. However, both the CS and

Electron microscopy showed that the mitochondria of renal tubular epithelial cells in the M group were swollen and had ridge fractures. The CS and P groups showed milder mitochondria swelling compared with the M group (**Figure 5**).

CS mitigated renal apoptosis caused by CM

To explore whether CS beneficial effects on CIN were associated with the alternation of apoptosis, we performed TUNEL staining on the kidney sections from rats with CIN. Compared to the C group, contrast injection led to elevated apoptosis in kidney, which was inhibited by CS treatment (**Figure 6**).

Moreover, the expressions of Caspase-3, Bax and Bcl-2 levels were analyzed. Similar to TUNEL assay, western blotting analysis suggested that CIN led to substantial increase in cleaved Caspase-3, Bax expression and decrease in anti-apoptotic proteins Bcl-2 expression as indicated in **Figure 7**. Our data proved that Caspase-3, Bax activation were effectively repressed while Bcl-2 expression was increased

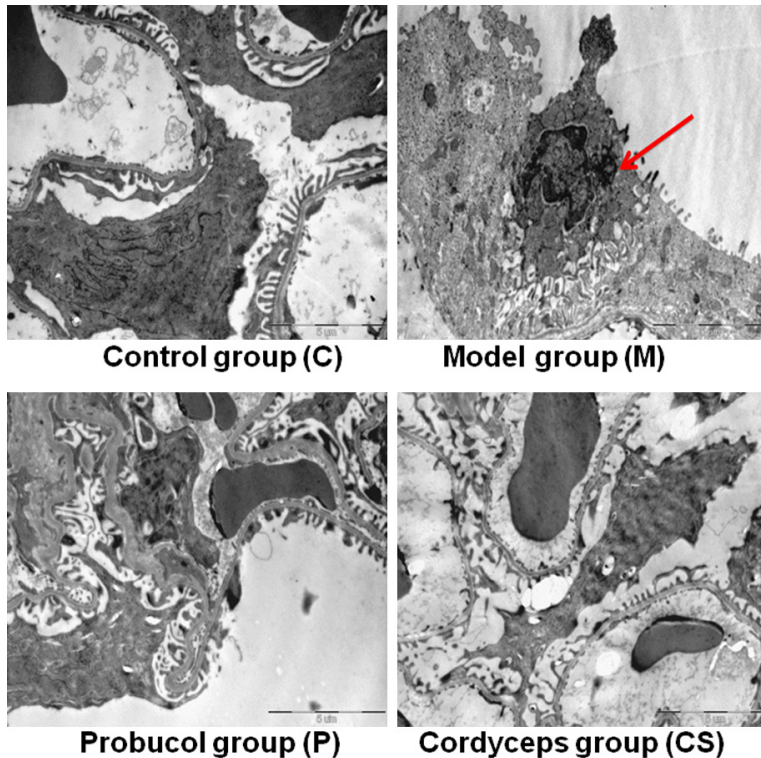


Figure 5. Scanning electron microscopic of kidney ($\times 10,000$). In the C group, there was normal morphology and structure. The M group showed basement membrane thickening, mitochondrial swelling, and apoptotic cells (the red arrow). The P and CS groups showed milder changes than M group.

by CS pretreatment. In summary, CS can mitigate renal tubular cell apoptosis following CIN.

Effects of CS on MAPK signaling pathways

To test the hypothesis that the MAPK pathways are activated in CIN, we examined the expressions of MAPK (including ERK and JNK) signaling. Western blotting analysis suggested that CIN led to decrease in ERK signal expression, and increase in JNK signal expression. Obviously, CS can reverse the above changes caused by CM. Pretreatment with CS can reduce JNK signaling and promote ERK signaling. We also tested the influence of CS on the apoptotic signaling pathway. The activation of p38 MAPK pathway was blocked by CS treatment (**Figure 8**).

Discussion

Contrast-induced nephropathy (CIN) is a serious complication caused by radiological imaging. CIN results in increased health care costs,

prolongs hospital length of stay, and increases both short- and long-term mortality [15]. The diagnosis of CIN often relies on a relative ($\geq 25\%$) or an absolute ($\geq 44.2 \mu\text{mol/L}$) increase in Scr from the baseline value within 3 days after intravascular administration of CM [16]. However, the change of Scr level is not a sensitive indicator of CIN. Recent studies indicate that urinary KIM-1, and NGAL levels had good sensitivity to diagnose kidney injury early [17, 18]. Our data showed that pretreatment with CS remarkably suppressed the increase of these proteins in urine.

The exact pathophysiology of CIN is not yet fully elucidated, but includes renal hypoperfusion, direct tubular injury, increased oxidative stress and inflammatory responses [19]. ROS imbalance causes lipid peroxidation, thereby leading to cytotoxic damage. In agreement with previous studies [20], we observed a remarkable increase in renal MDA and decrease in renal T-AOC, SOD in CIN rats, which indicated the presence of oxidative damage. However, CS preconditioned rats exhibited lower renal MDA and higher renal T-AOC, SOD levels. We believe that the renoprotective effect of CS can be attributed to the direct inhibition of ROS production.

Apoptosis also contributed to the occurrence of CIN. Cumulative evidence has suggested that CM leads to renal tubular cell apoptosis through the ROS pathway and the intrinsic apoptotic pathway [21, 22]. Our study proved that Caspase-3 activation, in other words, the intrinsic apoptotic pathway was involved in the CM induced renal injury, as reported in our recent studies [23, 24] and other reports [25]. CS might exert its inhibitory effect on apoptosis via direct inhibition of caspases. The present study demonstrated that CS pre-administration could protect against CIN. Our data indicated that CS ameliorated deterioration of renal function and histopathological kidney injury in CIN rats,

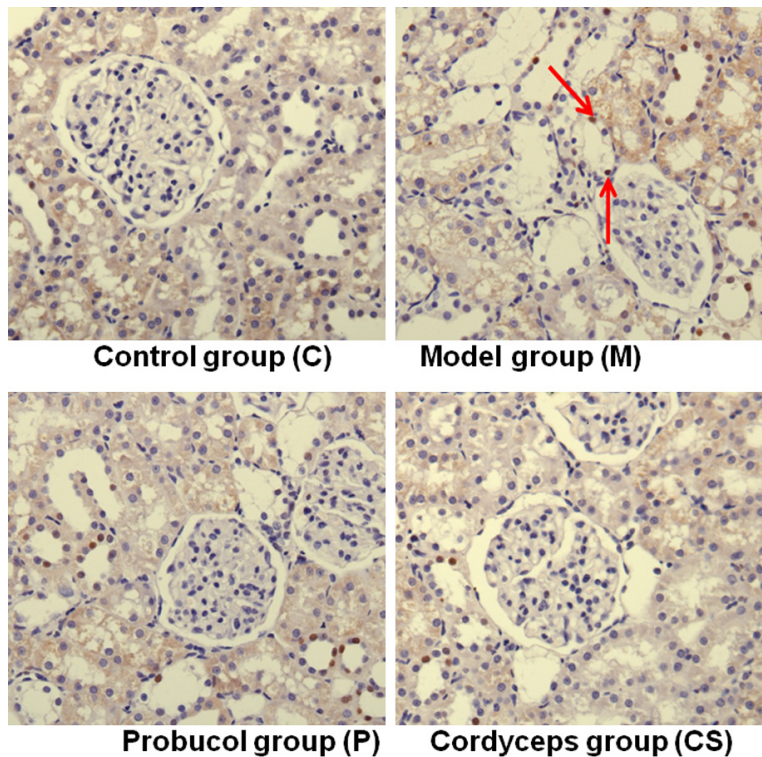


Figure 6. TUNEL staining in kidney (×400). In the C group, the renal tubular cells rarely had apoptosis. The M group showed obvious apoptosis cells, represented by brown points (the red arrow). The CS treatment groups showed fewer apoptotic cells than the M group.

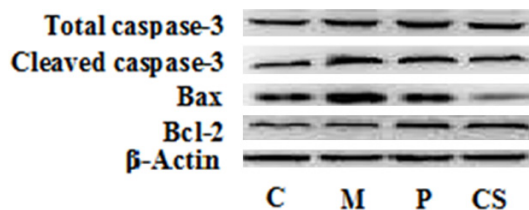


Figure 7. The expression of Caspase-3, Bax, Bcl-2 in kidney. Rats were randomly divided into 4 groups: control (C) group, model (M) group, Probucol (P) group cordyceps (CS) group. WB demonstrated that cleaved caspase-3 and Bax proteins were increased, but Bcl-2 protein was decreased in M group compared with C group. In P and CS groups, the caspase-3 and Bax proteins expression were decreased, and Bcl-2 expression was increased.

which was accompanied by reduced oxidative stress and apoptosis. Additionally, CS pretreatment inhibited MAPK signaling pathways in vivo. These data suggest that CS may mediate its reno-protective effects through anti-oxidation, anti-inflammation, and anti-apoptosis mechanisms.

CS, a time-honored traditional Chinese herb, consisting of many amino acids and inorganic

elements, can elevate the tolerance of the body to anoxia and ameliorate poor microcirculation. Some basic and clinical studies have proved its efficacy for treating microcirculatory disorders. CS can improve non-alcoholic fatty liver disease in mice by improving microcirculation disturbance, as reported by Li J et al [26]. CS can reduce the renal vascular resistance, ameliorate renal ischemia, increase the renal blood flow, and attenuate the damage of renal microvessels and tissue structure [27]. CS may ameliorate nephrotoxicity-induced renal dysfunction in the rats by antioxidant, anti-apoptosis, and anti-autophagy mechanisms [28]. In addition, by activating SOD in renal tissues, CS can scavenge ROS and produce antioxidant effects [29]. Another study suggests that supplementation with CS improves exercise performance and might contribute

to wellness in healthy older subjects [30]. In recent years, CS has been used to prevent contrast-induced renal impairment. In our previous study, prophylactic treatment with CS in patients with acute coronary syndrome and type 2 diabetes undergoing coronary intervention could prevent CIN [31, 32]. In accordance with previous studies, our results demonstrate that CS pretreatment was effective in attenuating the biochemical and histologic changes of CIN.

Probucol is a tested antioxidant, considering also its double properties as a free-radical scavenger, as well as being a drug able to increase the vasodilating effect of nitric oxide (NO) [20]. In particular, probucol can effectively reduce CM induced oxidative stress and protect the kidneys via multiple mechanisms, such as removing ROS, inducing the synthesis of glutathione, and stabilizing NO [33]. Therefore, the current study used probucol as a positive control drug for comparisons with CS.

Oxidative stress exerts both agonistic and antagonistic effects on apoptotic signaling through regulation of apoptosis, mediating cell

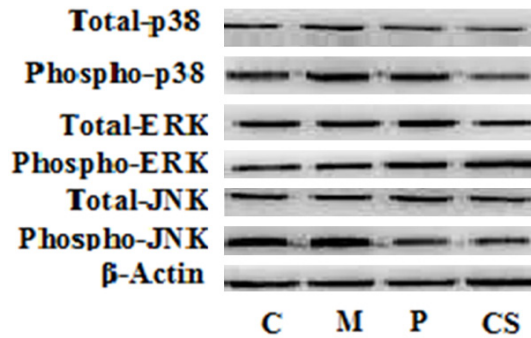


Figure 8. The expression of p-38 MAPK (JNK, ERK) in kidney. Rats were randomly divided into 4 groups: control (C) group, model (M) group, Probucol (P) group cordyceps (CS) group. WB demonstrated that phospho-p38 MAPK and phospho-JNK proteins were increased, but phospho-ERK protein was decreased in M group compared with C group. In P and CS groups, the phospho-p38 MAPK and phospho-JNK proteins expression were decreased, and phospho-ERK expression was increased.

proliferation and differentiation [34]. CIN fractionated the cellular signal traffic to mitochondria, activated of Bax in a caspase-dependent manner and initiated apoptosis. Bcl-2 regulates apoptosis and acts along the intrinsic mitochondrial apoptosis pathway that is activated in response to oxidative stress [35, 36]. In this present study, tubular epithelial cell apoptosis in CIN was confirmed. The MAPK signals are involved in the apoptotic response to a variety of environmental stresses and are critical for renal tubular cell apoptosis triggered by CM. MAPK (including JNK, ERK) signals were regulated by CS in the upstream signaling pathway, and then influenced the downstream caspase apoptotic pathway. After injecting Iodixanol into diabetic rats, the expressions of a series of signaling pathways in the kidney were changed. On one hand, the upstream ERK signals dephosphorylate, resulting in the signal inactivation and decline of antiapoptotic ability. On the other hand, apoptotic signal phosphorylates, resulting in the enhancement of JNK expression. These facts could influence the balance of Bcl-2/Bax, and then apoptosis was induced by the caspase pathway. Obviously, CS can reverse the above changes caused by CM.

A major strength of the present study is the potential protective role of CS in a whole animal model. However, further studies should address whether CS is protective against toxicity induced by CM of different osmolality and vis-

cosity as well as the potential use in humans to prevent CIN.

Conclusions

Our data suggested that CS attenuated renal injury in diabetic CIN rats and these beneficial effects are mainly mediated via anti-oxidation and anti-apoptotic effects. Thus, CS may represent a potential drug for prevention of CIN. Nevertheless, further studies are required to determine its reno-protective effects in the future.

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Disclosure of conflict of interest

None.

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