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Protective effect of a polysaccharide from stem of *Codonopsis pilosula* against renal ischemia/reperfusion injury in rats

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ABSTRACT

In this study, we purified a homogeneous polysaccharide (S-CPPA1) with a molecular weight (Mw) of 133.2 kDa from the stem of *Codonopsis pilosula* for the first time. Gas chromatography (GC) analysis identified that S-CPPA1 contained glucose, galactose, and arabinose with a molar ratio of 10.5:3.4:1.7, along with a trace of mannose. Methylation analysis suggested S-CPPA1 was a branched polysaccharide, with five glucosidic linkage forms, namely (1→4)-linked Glc*p* (residue A), (1→6)-linked Gal*p* (residue B), (1→2,6)-linked Glc*p* (residue C), (1→5)-linked Ara*f* (residue D), and non-reducing terminal (1→)-linked Glc*p* (residue E). The protective effect of S-CPPA1 on kidney ischemia/reperfusion (I/R) injury was also evaluated. Blood urea nitrogen (BUN), creatinine and TNF-α levels, as well as lactate dehydrogenase (LDH) and alanine transaminase (AST) activities were elevated in the I/R group as compared to the sham group. On the other hand, S-CPPA1 treatment reversed all these biochemical indices, as well as histopathological alterations, which were induced by I/R. The findings imply that S-CPPA1 plays a causal role in the protection against I/R-induced renal injury and its renoprotective effect is probably mediated by inhibiting the proinflammatory cytokine TNF-α release.

1. Introduction

Codonopsis pilosula is an herb that has been used in traditional Chinese medicine (TCM) for thousands of years, and distributed in the Northeast Provinces of China. It exhibits similar therapeutic effect to *Panax ginseng* and is sometimes used as a substitute of the much more costly *P. ginseng* (Sun & Liu, 2008). Modern pharmacological studies have revealed the effects of *C. pilosula* in protecting against peptic ulceration and promoting its healing, regulation of bowel movement, enhancing learning and memory behavior, enhancing immunity, and increasing red blood cells and hemoglobin (Singh et al., 2004). Furthermore, it has generally been used in Chinese folk medicine to treat heart conditions, strokes, and other ischemic conditions. Results from both experimental and clinical studies support that *C. pilosula* have beneficial effects in cerebral and intestinal reperfusion injury, cerebral blood flow improvement in the ischemic hemisphere and platelet aggregation inhibition in animal models (Han et al., 2002; Tang, Ren, Zhang, & Du, 2002). In several studies, *C. pilosula* has been reported to have vasodilating and anti-bacterial activities and protective effects in rats with chronic renal failure (Chung, Yokozawa, & Oura, 1986). *C. pilosula* also seems to reduce IR injury and thus improves graft function after experimental kidney transplantation, which may be explained by decreased lipid peroxidation and inhibition of apoptosis (He et al., 2011; Guan et al., 2009). Recently, several investigators reported that the polysaccharides extracted from the roots of *C. pilosula* exhibited several bioactivities, such as immunopotentiation, improving the compensatory hematopoiesis of spleen (Yang, Li, Liu, & Xian, 2005; Zhang, Zhu, Hu, Lai, & Mo, 2003) and oxidation resistance (Li & Yang, 2001). Meanwhile, documents about the reflux extraction, ultrasonic extraction, purification and structural elucidation of polysaccharides from the roots of *C. pilosula* appeared too (Han, Cheng, & Chen, 2005; Sun & Liu, 2008; Zhang, Zhang, Yang, & Liang, 2010; Zou, Chen, Yang, & Liu, 2011). Taken together, although many investigations about renal injury protection have been performed on the extract or saponins from the roosts of *C. pilosula*, the isolation and structural elucidation of the polysaccharides from the stem of *C. pilosula* and their influence on I/R-induced renal injury in mice have not been investigated. To address this issue, we therefore aimed to investigate the kidney-protective activity of the polysaccharide from the stem of *C. pilosula* against I/R-induced kidney injury in rats to ascertain its medicinal potentials.

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2. Materials and methods

2.1. Materials and chemicals

The stem of *C. pilosula* was collected from Medicine Mountain, Liaoning, China in July 2011. DEAE cellulose and Sepharose CL-6B were purchased from Pharmacia (GE, USA). Trifluoroacetic acid (TFA) was purchased from Sigma–Aldrich (St. Louis, USA). All other chemicals and solvents were analytical grade and used without further purification.

2.2. Isolation and purification of polysaccharide

The stem of *C. pilosula* (200 g) was extracted successively with 600 mL of petroleum ether and ethanol, respectively. The resulting residue was dried and then extracted twice with 2000 mL of warm water (85 °C) each time for 2 h. All water extracts were combined, filtered, and precipitated with three volumes of 95% EtOH at 4 °C overnight. The resulting precipitate was collected by centrifugation (4000 × *g*, 15 min, 4 °C) and deproteinated by the Sevag method (Staub, 1965). Finally, the supernatant was dialyzed (MW cut-off 3000 Da) against running water for two days and deionized water for one day and then freeze-dried to yield 9.1 g of the crude polysaccharide (S-CPP).

The crude polysaccharides, redissolved in distilled water, were forced through a filter (0.45 μm), and then applied to a DEAE cellulose column (2.6 cm × 30 cm) and eluted with different concentrations of stepwise NaCl solution elution (0.2, 0.4, 0.8 and 1.0 M NaCl) at a flow rate of 1.0 mL/min, leading to the isolation of three sub fractions S-CPPA, S-CPPB and S-CPPC, respectively. A 0.2 mL sample from each collected fraction (8 mL) was analyzed for carbohydrate content by a phenol–sulfuric acid assay (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Among them, fraction S-CPPA showed the relatively high content. Therefore S-CPPA was repeatedly subjected to size-exclusion column chromatography on a Sepharose CL-6B gel filtration column (2.6 cm × 100 cm) and eluted with 0.9% (w/v) NaCl at a flow rate of 0.5 mL/min. Collected fractions were dialyzed against distilled water and lyophilized to obtain a purified white polysaccharide, S-CPPA1 (1.65 g), which was subjected to subsequent analyses.

2.3. Chemical and structural analysis

2.3.1. Molecular weight determination

Molecular weights of the polysaccharide fractions were determined by high performance gel permeation chromatography (HPGPC). The samples were dissolved in distilled water, loaded into a Shimadzu HPLC system equipped with a TSK-GEL G3000 PWXL column (7.8 mm × 300 mm), eluted with 0.1 mol/L Na_2SO_4 solution at a flow rate of 0.5 mL/min and detected by a RID-10A Refractive Index Detector (RID). Dextran standards with different molecular weights (T-2000, T-70, T-40, T-20 and T-10) were used to calibrate the column and establish a standard curve.

2.3.2. Carbohydrate, uronic acid, protein content and optical rotation analysis

The total carbohydrate content of polysaccharides was determined by the reaction with phenol in the presence of sulfuric acid using glucose as a standard (Dubois et al., 1956). The total uronic acid content was measured by the m-hydroxydiphenyl assay using galacturonic acid as a standard (Filisetti-Cozzi & Carpita, 1991). The proteins in the polysaccharides were quantified according to the Bradford method using bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.3.3. Monosaccharide composition analysis

Gas chromatography (GC) was used for the identification and quantification of the monosaccharides. Samples were hydrolyzed and acetylated according to the method by Lehrfeld (1985), with some modifications. Polysaccharide sample (5 mg) was dissolved in 4 mL 2 mol/L trifluoroacetic acid solution (TFA) and hydrolyzed at 120 °C for 6 h. The resulting solution was concentrated under reduced pressure and the excess acid was removed by repeated codistillations with anhydrous ethanol. Then the hydrolyzed product was reduced with KBH_4 (30 mg), followed by neutralization with dilute acetic acid and evaporated at 45 °C after adding 1 mg myo-inositol and 0.1 M Na_2CO_3 (1 mL) at 30 °C with stirring for 45 min. Finally the reduced products (alditols) were added with 1:1 pyridine–propylamine at 55 °C with stirring for 30 min, and acetylated with 1:1 pyridine–acetic anhydride in a boiling water bath for 1 h. After cooled, approximately 1 μL of clear supernatant was submitted to GC analysis, performed on a Shimadzu GC-14C instrument (Shimadzu, Japan) equipped with a DM-2330 capillary column (30 m × 0.32 mm × 0.20 μm) and flame-ionization detector (FID). The operation was performed in the following conditions: N_2: 1.0 mL/min; injection temperature: 250 °C; detector temperature: 250 °C; column temperature programmed: 80 °C for 3 min, then increased to 250 °C at 10 °C/min and finally holding for 10 min at 250 °C.

2.3.4. Linkage analysis

The polysaccharides (10 mg) were methylated four times by the method reported by Needs and Selvendran (1993) with minor modifications. The methylated products was depolymerized with 90% formic acid at 100 °C for 6 h and further hydrolyzed with 2 M TFA at 100 °C for 2 h, respectively. The partially methylated residues were reduced and acetylated. The resulting products were analyzed by gas chromatography–mass spectrometry (GC–MS). GC–MS was run on the instrument HP5890 (II) (Hewlett-Packard Component, USA) with a HP-5 quartz capillary column (25 m × 0.22 mm × 0.2 μm), and at temperatures programmed from 120 °C to 240 °C at a rate of 5 °C/min. Partially methylated alditol acetates were identified by their fragments in EI-MS and by relative retention times in GC, and the molar ratios were estimated from the peak areas and response factors.

2.4. Animals

Adult male Wistar rats weighing 220 ± 9.3 g used for these experiments were obtained from the Animal Experimental Center of the Fourth Military Medical University. The animals were housed in standard cages with alternating light/dark cycles and were allowed free access to water and rat chow *ad libitum*. The experimental procedures were approved by the Institutional Animal Ethics Committee.

2.5. Acute toxicity studies

The rats, in groups of six each, were fed with S-CPPA1 at increasing dose levels of 10, 20, 50, 100, 200, 400 and 1000 mg/kg body weight. The animals were observed continuously for 2 h for gross behavioral changes and then intermittently once every 2 h and finally at 24 and 72 h.

2.6. Experimental design

Following one week's acclimation, the animals were divided into three groups each containing 10 mice as below: (1) sham: the animals were submitted to laparotomy without ischemia. (2) I/R: the animals were submitted to renal ischemia for 30 min. (3) IR + S-CPPA1: the animals received S-CPPA1 (10 mg/kg/day), orally, for

10 consecutive days before renal ischemia. The normal and control groups were given water, while the treated group was given S-CPPA1 with a gastric cannula daily.

Rats were anesthetized using ketamine hydrochloride (0.2 mL/100 g) in all experiments. The abdominal region was shaved, and the animal was placed on a heated table to maintain constant temperature. The abdominal area was prepared with Betadine, and sterile drapes were applied. To induce ischemia, kidneys were exposed through flank incisions and bilateral renal artery occlusion was then carried out for 30 min using nontraumatic microaneurism clamps for 30 min, as described previously (Park, Kramers, Vayssier-Taussat, Chen, & Bonventre, 2002). Following release of the occlusion, the abdomen was sutured, and the animal was returned to the cage to recover for 24 h. After this period, blood samples were taken and analyzed for renal function tests. Only laparotomy was performed in the sham group animals. The left kidney was subsequently removed from each animal with ice-cold physiological saline and immediately frozen and kept at −80 °C until analysis. At the end of the experiment, the animals were sacrificed according to guidelines for animal experimentation.

2.7. Assessment of renal function

The various serum samples collected from the animals were analyzed on various biochemical parameters, such as blood urea nitrogen (BUN), creatinine, lactate dehydrogenase (LDH), alanine transaminase (AST), and TNF-α. BUN, creatinine, LDH and AST activity were determined by standard methods (Varley & Alan, 1984) Serum TNF-α was evaluated by a radioimmunoassay–immunoradiometric assay (RIA–IRMA) method. The activity of radioactive assays was measured by a gamma counter (LKBWALLAC 1270 RACK Gamma Counter, Canada) and expressed as pg/mL.

The kidneys of the animals fixed in 10% formaldehyde were dehydrated, embedded in paraffin wax and serially sectioned (5-μm thick). Paraffin sections were dewaxed with xylene and gradually dehydrated with alcohol for hematoxylin and eosin (H&E) staining.

2.8. Histological score

Fifty tubules in the outer medullar of the kidneys were analyzed using the following scoring method: 0, no damage; 1, mild damage with the rounding of epithelial cells and dilated tubular lumen; 2, moderate damage with flattened epithelial cells, dilated lumen, and congestion of lumen; and 3, severe damage with flat epithelial cells lacking nuclear staining and the congestion of lumen. Four kidneys in each experimental animal group were used.

2.9. Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) and the Student's *t*-test. Differences were considered to be statistically significant if $P < 0.05$ and $P < 0.01$.

3. Results and discussion

3.1. Isolation and purification of polysaccharide

The crude polysaccharide S-CPP (9.1 g, 4.55% of crude material) was obtained from the stem of *C. pilosula* by the method of defatting, water-extraction, ethanol precipitation, deproteinization, dialysis and lyophilization. S-CPP was fractionated by ion exchange chromatography on a DEAE-cellulose column eluted with stepwise NaCl solution (0.2, 0.4, 0.8 and 1.0 M NaCl), and was separated into S-CPPA (eluted with 0.2 M NaCl), S-CPPB (eluted with 0.4 M NaCl) and S-CPPC (eluted with 0.8 M NaCl) (Fig. 1), as detected by the phenol–sulfuric acid assay. Because of the high content of S-CPPA, this polysaccharide was further separated and sequentially purified through Sepharose CL-6B gel filtration column. Fractions containing one major polysaccharide peak (Fig. 2) were collected and then freeze-dried to yield S-CPPA1 (1.65 g, 0.82% of crude material).

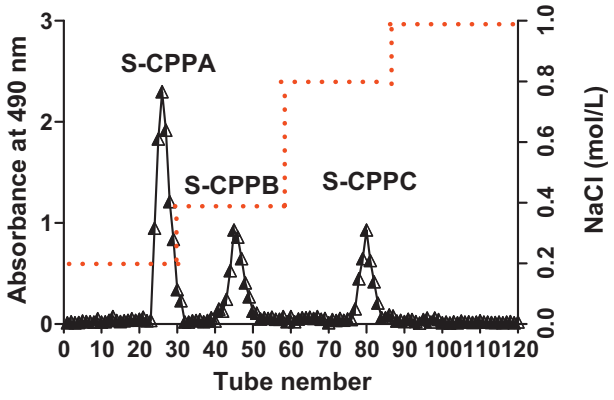

Fig. 1. The profile of different polysaccharide fractions from the stem of *C. pilosula* on a DEAE-cellulose column eluted with stepwise NaCl aqueous solutions (0.2, 0.4, 0.8 and 1.0 M) at a flow rate of 1 mL/min.

3.2. Structural characteristics

The HPGPC (Fig. 3) profile of S-CPPA1 showed a single and nearly symmetrical signal, indicating that S-CPPA1 was a homogeneous polysaccharide. In addition, HPGPC has been shown to be an effective method for the polysaccharide molecular weight determination. According to the retention time, the average molecular weight (Mw) of S-CPPA1 was approximately 133.2 kDa. There was an obvious absorption at 210 nm for S-CPPA1 and the absorbance at 280 nm for S-CPPA1 was very limited as detected by UV spectrophotometer. S-CPPA1 under investigation contained 93.35% of total carbohydrate, 3.24% of uronic acid, and 2.35% of protein (Table 1).

The monosaccharide composition of S-CPPA1 was determined by GC analysis of the alditol acetates. The result showed that S-CPPA1 contained glucose, galactose, and arabinose with a molar ratio of 10.5:3.4:1.7, along with a trace of mannose (Table 1).

The sugar linkages of S-CPPA1 were deduced from the methylation analysis and five partially methylated alditol acetates were

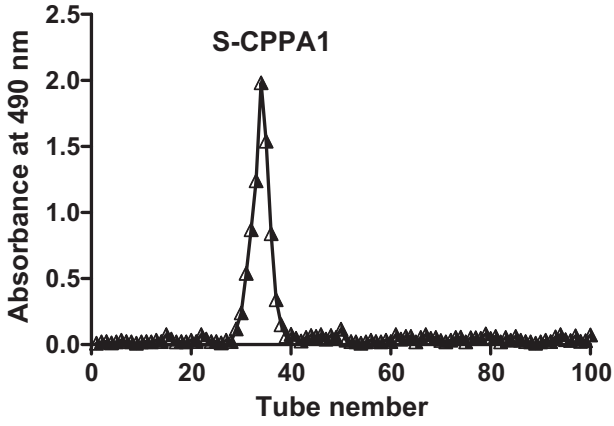

Fig. 2. The profile of the polysaccharide S-CPPA1 from the stem of *C. pilosula* on a Sepharose CL-6B gel filtration column eluted with 0.9% (w/v) NaCl at a flow rate of 0.5 mL/min.

Table 1
General chemical properties and molecular weight of the polysaccharide S-CPPA1.

Sample	Neutral sugar (mol%)				Uronic acid (wt%)	Total carbohydrate (wt%)	Protein (wt%)	Molecular weight (kDa)
	Glucose	Galactose	Arabinose	Mannose				
S-CPPA1	10.5	3.4	1.7	Trace	3.24	93.35	2.35	133.2

Table 2
Results of the methylation analysis of the polysaccharide S-CPPA1.

Residue	Methylated sugars	Type of linkage	Molar ratio	Mass fragments (*m*/*z*)
A	2,3,6-Me_3-Glc	(1→4)-linked Glc*p*	6.3	43, 45, 71, 87, 99, 101, 113, 117, 129, 161, 233
B	2,3,4-Me_3-Gal	(1→6)-linked Gal*p*	3.4	43, 45, 71, 87, 99, 101, 117, 129, 161, 189, 233
C	3,4-Me_2-Glc	(1→2,6)-linked Glc*p*	2.2	43, 87, 99, 129, 189, 233
D	2,3-Me_2-Ara	(1→5)-linked Ara*f*	1.7	43, 71, 87, 99, 101, 117, 129, 189
E	2,3,4,6-Me_4-Glc	(1→)-linked Glc*p*	2.1	71, 87, 101, 117, 129, 145, 161

mainly produced as below: 2,3,6-Me_3-Glc; 2,3,4-Me_3-Gal; 3,4-Me_2-Glc; 2,3-Me_2-Ara; and 2,3,4,6-Me_4-Glc. Based on these results, S-CPPA1 correspondingly contained five glucosidic linkage forms, *i.e.*, (1→4)-linked Glc*p* (residue A), (1→6)-linked Gal*p* (residue B), (1→2,6)-linked Glc*p* (residue C), (1→5)-linked Ara*f* (residue D), and non-reducing terminal (1→)-linked Glc*p* (residue E) (Table 2). These results suggested S-CPPA1 to be a branched polysaccharide. The detailed structural characterization for this polysaccharide is ongoing to be elucidated by the combination of both chemical and physical analysis.

3.3. Acute toxicity studies

The polysaccharide S-CPPA1 from *C. pilosula*, when orally administered in the dose range of 10–1000 mg/kg body weight, did not produce any significant changes in the autonomic or behavioral responses, including death during the observation period.

3.4. Effect of the polysaccharide on renal function in rats exposed to I/R injury

As mentioned in Table 3, serum levels of BUN and creatinine were significantly higher in I/R rats compared to sham animals ($P < 0.001$), suggesting a significant decrease of glomerular function caused by renal I/R. And S-CPPA1 administration to I/R group reduced these values ($P < 0.001$).

TNF-α could attract inflammatory leukocytes and more macrophages to sites of tissue injury and stimulates leukocytes to release chemokines and more inflammatory cytokines, thus leading to the tissue deterioration (Wojcikowski, Wohlmuth, Johnson, & Gobe, 2008). In untreated I/R group, serum TNF-α level showed a significantly increase compared to that of the sham-operated control group ($P < 0.001$). However, I/R induced rise in serum TNF-α level was significantly reduced by S-CPPA1 treatment ($P < 0.001$).

Both, LDH and AST are regarded as nonspecific markers of extensive cellular deterioration or systemic tissue injury. During renal tubular cell cold hypoxic preservation, considerable amounts of LDH are released (Wingenfeld et al., 1996). As a marker of cellular disruption and necrosis, AST is present within the proximal tubule and can be released following renal injury (Stogdale, 1981). In this study, both LDH and AST activities in renal tissue were higher in the I/R group than in the sham group and the differences were statistically significant ($P < 0.001$). Furthermore, LDH and AST activity in renal tissue were markedly reduced in the treatment group than in the IR group ($P < 0.001$), suggesting a protective effect of S-CPPA1 on renal cells against I/R.

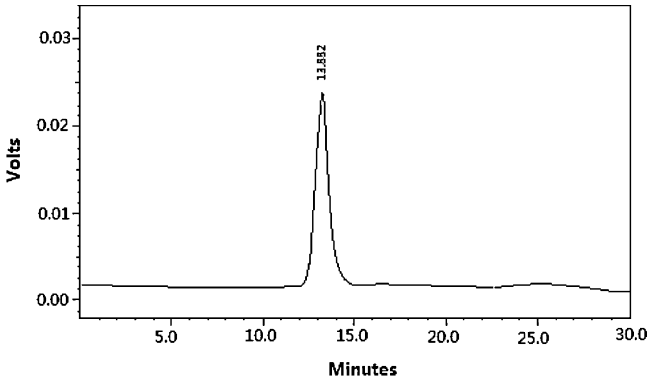

Fig. 3. Profile of the polysaccharide S-CPPA1 on HPGPC. The sample was analyzed by a TSK-GEL G3000 PWXL column (7.8 mm × 300 mm) and eluted with 0.1 mol/L Na_2SO_4 at 0.5 mL/min.

3.5. Post-ischemic histological change

Upon histopathological evaluation, conventional H&E staining of paraffin sections revealed no significant changes in the morphology of renal glomerular and tubular cells in the sham group (Fig. 4A). In the I/R group (Fig. 4B), histological examination of the kidneys subjected to I/R process showed the distinctive pattern of ischemic renal injury, characterized by widespread degeneration of tubular architecture, loss of brush border, lumen dilatation or collapse, cellular detachment from tubular basement membranes and tubular cell necrosis, especially in the outer medulla. In the treatment group (Fig. 4C), there were relatively mild swelling of the renal tubular epithelial cells, with a very small number of narrowed lumens, and limited inflammatory cell infiltration, interstitial congestion and edema. In addition, levels of histological-damage scores were higher in the I/R group than the S-CPPA1-administered mice (data not shown), which was consistent with the respective levels of renal function parameters.

Table 3
Effect of the polysaccharide S-CPPA1 on serum BUN, creatinine, TNF-α levels, as well as LDH and AST activities in rats exposed to I/R.

Parameters	Sham	I/R	IR + S-CPPA1
BUN (mg/dL)	18.21 ± 0.86	97.32 ± 4.30**	32.22 ± 2.17*,***
Creatinine (mg/dL)	0.64 ± 0.08	1.96 ± 0.26**	0.87 ± 0.12***
TNF-α (pg/mL)	2.43 ± 0.21	19.54 ± 0.76**	3.82 ± 0.34***
LDH (U/L)	1576 ± 231	3453 ± 345**	1685 ± 258***
AST (U/L)	412 ± 32	798 ± 56**	437 ± 53***

* $P < 0.01$.
** $P < 0.001$: compared with sham group.
*** $P < 0.001$: compared to saline-treated I/R group.

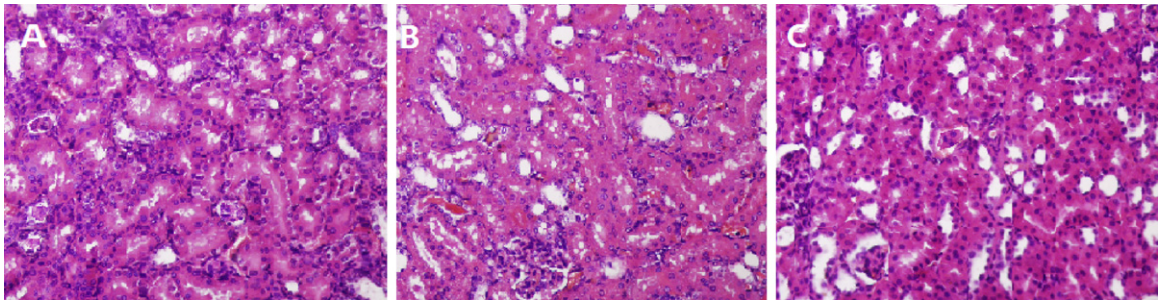

Fig. 4. The morphologic changes in renal tissue 24 h after ischemia and reperfusion (I/R). (A) Sham group; (B) I/R group and (C) IR + S-CPPA1 group.

4. Conclusion

Renal ischemia–reperfusion (I/R) injury is the major cause of morbidity and mortality in diseases such as acute renal failure, renal transplantation, trauma, and major surgery (Uz et al., 2009). The mechanisms underlying I/R damage to kidneys are likely multifactorial and interdependent, involving hypoxia, free radical (FR) damage and inflammatory responses (Paller, 1994). Despite a growing body of research, knowledge on the treatment of renal I/R injury remains limited. Renal ischemia initiates a series of events that can ultimately lead to cellular dysfunction and necrosis (Sivarajah et al., 2003). However, reperfusion can paradoxically worsen tissue injury by initiating the complex cellular events that result in renal injury and the eventual death of renal cells due to a combination of apoptosis and necrosis (Wang, Pei, Ji, & Xing, 2008).

The present study reported the purification and structural features of a water-soluble polysaccharide S-CPPA1 from the stem of *C. pilosula*. Furthermore, S-CPPA1 treatment improved I/R-induced impairment in kidney function and decreased I/R-induced elevations in serum LDH and AST activities, as well as BUN, creatinine and TNF-α levels. In conclusion, our results indicate that administration of S-CPPA1 has the potential to reduce kidney susceptibility to I/R injury and the precise protective mechanisms on kidney I/R injury require more study, including identification of its effective components in detail.

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