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Compositional features of carbohydrate compound from rhizoma ligustici wallichii and ethanol extract of danshen and its bioactivity

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ABSTRACT

Water extraction was applied to prepare carbohydrate compound of rhizoma ligustici wallichii. Four main fractions, fraction-I, fraction-II, fraction-III, and fraction-IV, were obtained by membranes of 1.0×10^{-4} mm pore size and normal molecular-weight cut-off of 50 kDa. The resulting four preparations were further analysed by capillary gas chromatography method. Thin layer chromatography (TLC) analysis showed that carbohydrate compound of rhizoma ligustici wallichii was composed of five types of monosaccharides, namely glucose, rhamnose, mannose, galactose and arabinose. Gas chromatography (GC) analysis showed that fraction I of rhizoma ligustici wallichii was composed of four types of monosaccharides, namely glucose, mannose, galactose and arabinose at a molar ratio of 521:1:4.6:3.3. Furthermore, the protective effect of the Rhizoma ligustici wallichii polysaccharides and ethanol extract of danshen against ischemia–reperfusion (IR) induced renal injury were evaluated. The findings imply that carbohydrate compound of the Rhizoma ligustici wallichii and ethanol extract of danshen play a causal role in IR-induced renal injury probably by the radical scavenging and antioxidant activities. Moreover, ethanol extract of danshen displayed stronger renoprotective effect than that of carbohydrate compound of the Rhizoma ligustici wallichii.

1. Introduction

Rhizoma Chuanxiong, derived from the rhizome of Ligusticum chuanxiong hort (Umbelliferae), is a well-known traditional Chinese medicine (TCM) with haemodynamic and analgesic effects. In the TCM practice, this herb is commonly prescribed for the treatment of migraine and various cardiovascular diseases, such as angina pectoris and ischemic stroke (Chen & Chen, 1992; Ju & Du, 1995).

Ligusticum chuanxiong contains various types of ingredients, including phthalides, alkaloids and organic acids. Because of the diverse therapeutic effects of LC, several pure compounds, such as ferulic acid, senkyunolide, ligustilide, and tetramethylpyrazine, have been purified from it (Yan, Li, Chung, Tam, & Lin, 2005), and their biological functions studied. Among these compounds, ferulic acid is ubiquitous in plants and is known for its free radical scavenging (Zhang et al., 2003) and anti-inflammatory activities (Sakai, Ochiai, Nakajima, & Terasawa, 1997).

Danshen, the root and rhizome of Salvia miltiorrhiza Bunge, is one of the earliest and also the most commonly used herbal drug in practice of traditional Chinese medicine. The earliest record of Danshen appears to come from the (Shen Nong Materia Medica, A.D. 102–200) E. Han Dynasty. The major active constituents of Danshen include tanshinones (Lee, Wu, Chang, Lin, & King, 1987), which have been reported to have anti-platelet (Chan, 2001; Yu, Chan, & Sanderson, 1997), cardio-protective (Au-Yeung, Zhu, & Siow, 2001; Fu et al., 2007; Wu et al., 1993), anti-inflammatory (Kim et al., 2002), hepatoprotective, vasodilatory effects (Lam, Yeung, Chan, & Or, 2008) and diminution of cancer cell proliferation (Lee, Chiu, & Yeung, 2008; Liu, Shen, & Ong, 2001) effects in preclinical studies.

One of the most important factors in pathophysiology of renal IR injury is reactive oxygen species (ROS), which especially increases in reperfusion phase (Paller, Hoidal, & Ferris, 1984; Weight, Bell, & Nicholson, 1996). The endogenous antioxidants which are responsible for defense against ROS during reperfusion have an important role in decreasing IR injury (Li & Jackson, 2002). Superoxide dismutase (SOD) and catalase (CAT) are the most important antioxidant enzymes of tissues. Glutathione (GSH), a free radical scavenger (Pincemail et al., 2000), plays a key role in maintenance of the cellular redox environment (Schafer & Buettner, 2001). Free radical ablation for the treatment of reperfusion injury has found its first clinical application in the prevention of postischemic tissue injury following organ transplantation (Amersi et al., 2002; Seo & Lee, 2002). Thus, agents proposed to be useful in the clinical settings of I/R damage include free radical scavengers.

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Therefore, the aim of the present research was to isolate and determine the structural features and bioactivity of the carbohydrate compound of rhizoma ligustici wallichii and ethanol extract of danshen. In this study, a water-soluble carbohydrate compound was isolated by anionexchange chromatography and gel permeation chromatography from the rhizoma ligustici wallichii. The present paper was concerned with the isolation, chemical characterization and evaluation of the protective effect of carbohydrate compound of rhizoma ligustici wallichii and ethanol extract of danshen against IR induced renal damage.

2. Materials and methods

2.1. Separation and purification of the carbohydrate compound

The crude polysaccharides from above were redissolved in ultrapure water, then applied to a DEAE-Sephadex A-25 column (2.4 cm × 60 cm) for separation. The column was coupled to an KTA Purifier 100 system (Amersham Pharmacia Biosciences). Detailed experimental conditions were as follows: concentration of crude extract, 3 mg/ml; injection volume, 4 ml; mobile phase, ultra-pure water; flow rate, 0.5 ml/min. Fractions of 5 ml were collected with a Pharmacia LKB Superfrac fraction collector, and the eluent (polysaccharide and protein elution) was monitored with a Shimadzu RID-10A Refractive Index Detector. At last, four fractions, named as fractions I, II, III and IV, were separated (Fig. 1).

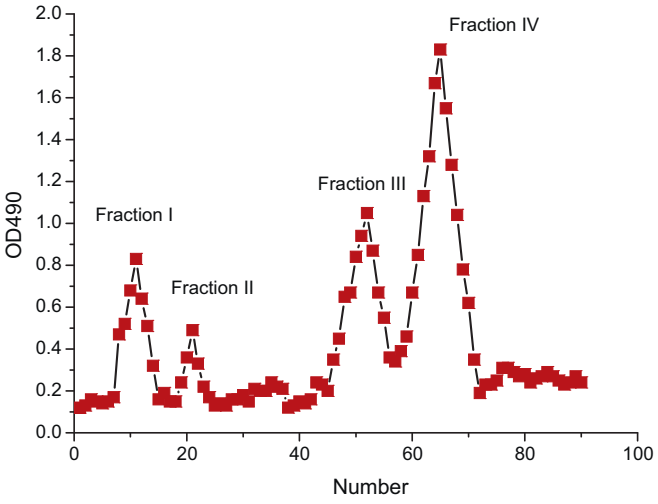

Fig. 1. Fraction of carbohydrate compound of rhizoma ligustici wallichii.

2.2. Determination of carbohydrate composition and content

The carbohydrate compound samples (1 mg) were subjected to methanolysis using 4 M HCl in anhydrous methanol at 80 °C for 24 h. Mannitol was added as internal standard. After the 24 h reaction time, the reagents were removed with nitrogen and the methylglycosides dried in vacuum over P_2O_5 for 1 h prior to conversion into the corresponding trimethyl silyl ethers (TMS-derivates). The samples were analysed by capillary gas chromatography on a Carlo Erba 6000 Vega Series 2 chromatograph with an ICU 600 programmer (Barsett et al., 1992; Chambers and Clamp, 1971).

2.3. HPLC–UV analysis

HPLC analyses were carried out on a Waters (Milford, MA, USA) 600-MS pump system, connected to a tunable UV–vis Waters 486 detector. A Diamonsil (Beijing, China) C18 column (5 μm, 200 mm × 4.6 mm I.D.) was used. The column was eluted with methanol–ammonium acetate (pH 6.0; 80 mM) (40/60, v/v) at a flow rate of 1 ml/min. Samples were filtered through a syringe filter (0.45 μm) prior to each injection.

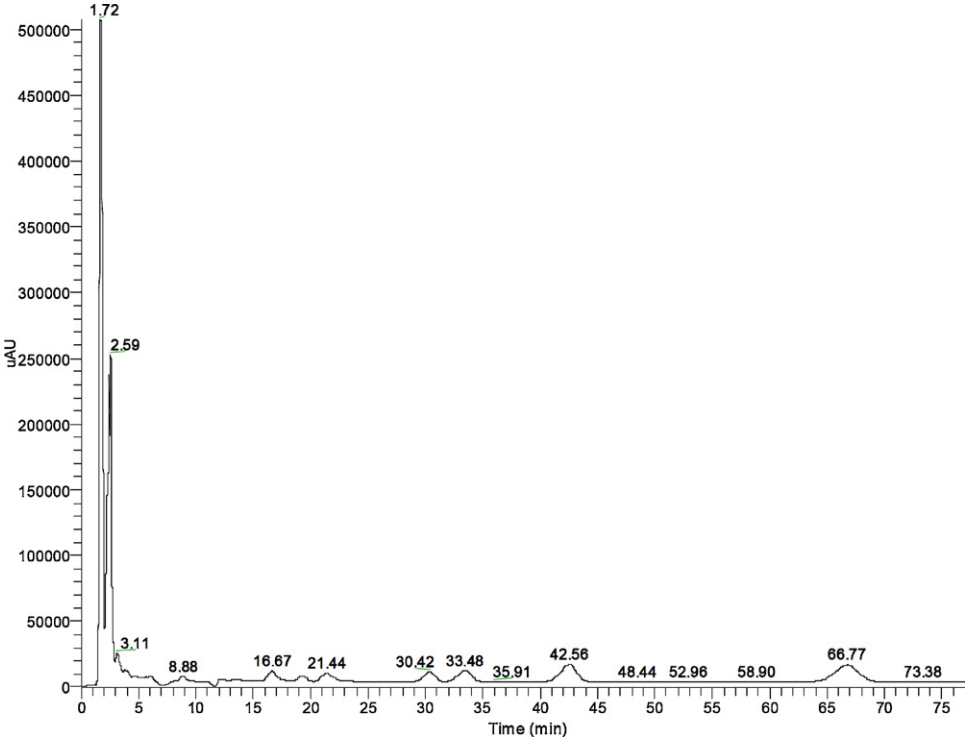

Fig. 2. HPLC chromatograph of ethanol extract of danshen.

2.4. Animals

Forty rats of body weight 235–260 g were used in this study. The rats were maintained under standard laboratory conditions (temperature 24 ± 2 °C; natural light–dark cycle). The rats had free access to drinking water and commercial standard pellet diet. The laboratory animal protocol used for this study was approved by the Institutional Ethical Committee for Animal Care and Use at Chongqing Medical University, Chongqing, China.

2.5. Experimental design

Forty animals were randomly divided into four groups: a sham group (I), IR group (II), two medicine-treated groups (III and IV).

Group I: sham rats were treated intragastrically with isotonic saline (2 ml/kg bw/day) for 4 weeks.
Group II: IR rats were treated intragastrically with isotonic saline (2 ml/kg bw/day) for 4 weeks.
Group III: rats were treated intragastrically with ethanol extract of danshen (150 mg/kg bw/day) dissolved in isotonic saline for 4 weeks using intragastric tube.
Group IV: rats were treated intragastrically with carbohydrate compound of Rhizoma ligustici wallichii (300 mg/kg bw/day) dissolved in isotonic saline for 4 weeks using intragastric tube.

On the day of experiment, under anesthesia (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine; intraperitoneally; ip), an upper abdominal midline incision was made and right nephrectomy was performed. The left renal pedicle was occluded for 45 min to induce ischemia and then subjected to reperfusion for 6 h (I/R groups). Another group of rats underwent only laparotomy, where the kidneys were manipulated without nephrectomy or occlusion. At the time of sacrifice, rats were anesthetized using the protocol described above and blood samples were collected and left kidney was removed. Three rats died (one for group I, one for group II, one for group IV) immediately after anesthesia, before undergoing ischemia–reperfusion experiment, and were not considered in data analysis.

2.6. Biochemical analysis

Blood urea nitrogen (BUN) and creatinine (Cr) levels were measured using clinical automated analysis (Hitachi 7600-10, Hitachi High-Technologies Corporation, Japan).

Interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF-α) levels in blood were measured using an ELISA kit (Biocompare, South San Francisco, CA, USA) according to the manufacturer's protocol. The data are presented as pg/ml.

Malondialdehyde (MDA) concentration was determined using the method described by Draper and Hadley (Draper & Hadley, 1990; Hammouda, Khalil, & Salem, 1995) based on TBA reactivity. Briefly, 2.5 ml of 10% trichloracetic acid and 0.5 ml of plasma were added into tubes and mixed. After incubating for 15 min at 90 °C and cooling with cold water the mixture was centrifuged at 3000 rpm for 10 min. Two milliliters of supernatant were taken and 1 ml of 0.675% TBA was added. The tubes were sealed and incubated at 90 °C for15 min and then cooled to room temperature. The optical density was measured at 532 nm by a spectrophotometer.

The catalase (CAT) activity was determined according to the Aebi method (1984). The rate of H_2O_2 decomposition was followed by monitoring absorption at 240 nm. One unit of CAT activity is defined as the amount of enzymes required to decompose 1 μmol of hydrogen peroxide in 1 min. The enzyme activity was expressed as μmol H_2O_2 consumed/min/mg protein. Superoxide dismutase (SOD) activity was estimated according to the method of Beauchamp and Fridovich (1971). The developed blue colour in the reaction was measured at 560 nm. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50% and the activity was expressed as U/mg protein. Activity of glutathione peroxidase (GSH-Px) was determined according to the method of Lawrence and Burk (1976). The assay mixture consisted of 2.0 ml of 75 mM phosphate buffer (pH 7.0), 50 μL of 60 mM glutathione, 0.1 ml of 30 units/ml glutathione reductase, 0.1 ml of 15 mM EDTA, 0.1 ml of 3 mM NADPH and the appropriate amount of tissue supernatant to a final volume of 3.0 ml. The reaction was started by the addition of 0.1 ml of 7.5 mM H_2O_2. The rate of change of absorbance during the conversion of NADPH to $NADP^+$ was recorded spectrophotometrically at 340 nm for 3 min. GSH-Px activity for tissues was expressed as μmoles of NADPH oxidized to $NADP^+$ min^{-1} mg^{-1} protein.

2.7. Immunohistochemical localization of intercellular adhesion molecule-1

Evidence of intercellular adhesion molecule (ICAM)-1 expression was determined using immunohistochemical protocols, as described recently (Chatterjee et al., 2004). Briefly, kidney sections were incubated overnight at 4 °C with primary anti-ICAM-1 (1:500, v/v in phosphate buffered saline, [PBS, 0.01 M, pH 7.4]). Separate sections were also incubated, with control solutions consisting of PBS alone. Specific labelling was detected using a biotin-conjugated goat antirabbit immunoglobulin G and avidin–biotin peroxidase. Samples were then viewed under a light microscope.

2.8. Histology

The kidney was fixed in 10% neutral-buffered formalin, paraffin embedded and sectioned at 4 μm thick according to the standard procedure. The sections were deparaffinized and hydrated gradually, and examined by HE staining. Morphological assessment was performed by an light microscopy.

2.9. Statistical analysis

SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis of results. All data are expressed as mean ± SD of the number of values corresponding to the same number of experiments and presented within parentheses for each experimental group and parameter measured in the different figures. Each value is the mean of data from an assay performed in duplicate. Minimum significance level was $P < 0.05$.

3. Result and discussion

3.1. Monosaccharide composition analysis

TLC analysis showed that carbohydrate compound of rhizoma ligustici wallichii was composed of five types of monosaccharides, namely glucose, rhamnose, mannose, galactose and arabinose. GC analysis showed that fraction I of rhizoma ligustici wallichii was composed of four types of monosaccharides, namely glucose, mannose, galactose and arabinose at a molar ratio of 521:1:4.6:3.3. GC analysis showed that fraction II of rhizoma ligustici wallichii was composed of three types of monosaccharides, namely glucose, galactose and arabinose at a molar ratio of 638:5.9:2.3. GC analysis showed that fraction III of rhizoma ligustici wallichii was composed of four types of monosaccharides, namely Glucose, mannose, galactose and arabinose at a molar ratio of 462:2.4:1:9.4. GC analysis showed that fraction IV of rhizoma ligustici wallichii was composed of five types of monosaccharides, namely glucose, rhamnose,

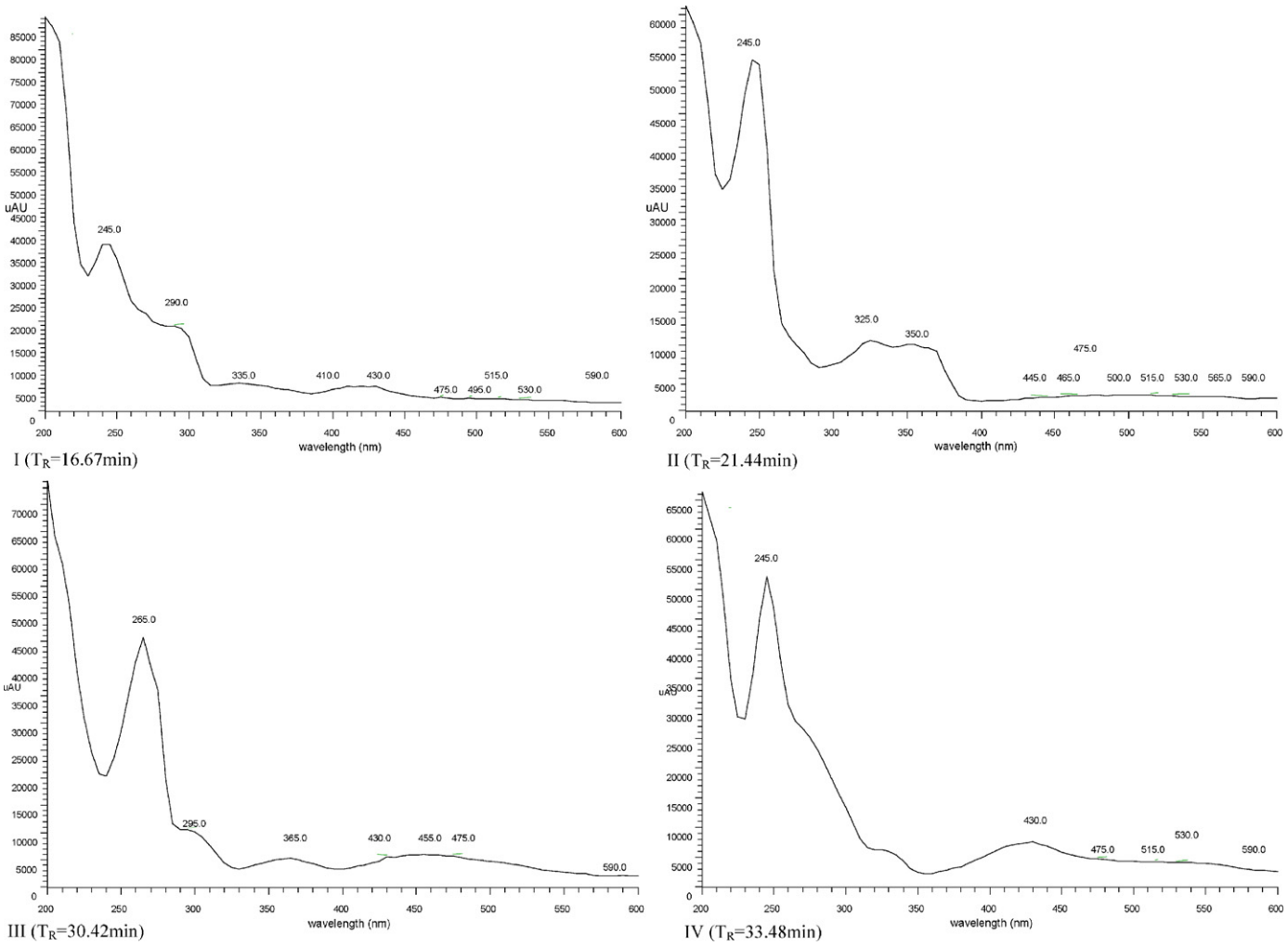

Fig. 3. UV spectrum analysis of ethanol extract of danshen.

mannose, galactose and arabinose at a molar ratio of 309:2.9:1:7.3:47 (Table 1).

3.2. Chemical composition analysis

HPLC analyses of ethanol extract of danshen were performed. A fractionation of the phenolic compounds was carried out according to the scheme of Figs. 2 and 3. Six fractions were obtained from the ethanol extract of danshen. HPLC analyses suggested that fraction II contains caffeic acid and caffeic and *p*-coumaric acid derivatives, and Fraction III contains flavonoids. The HPLC profile of fraction I was characterized by the occurrence of a hump in the chromatogram and a few compounds showing UV spectrum.

Table 1
Sugar composition of carbohydrate compound of rhizoma ligustici wallichii.

Heterosaccharide	Sugar composition	Molar ratio
Fraction I	Glucose:mannose:galactose: arabinose	521:1:4.6:3.3
Fraction II	Glucose:galactose:arabinose	638:5.9:2.3
Fraction III	Glucose:mannose:galactose: arabinose	462:2.4:1:9.4
Fraction IV	Glucose:rhamnose:mannose: galactose:arabinose	309:2.9:1:7.3:47

3.3. Bioactivities analysis

Compared with sham group (I), renal BUN and Cr contents were significantly ($P < 0.01$) increased in IR group (II). Similar results were obtained in previous studies (Erdogan, Fadillioglu, & Emre, 2006). Carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen pre-treatment decreased significantly ($P < 0.01$) IR-induced renal BUN and Cr contents in rats (groups III and IV) as compared to IR rats (group II) (Fig. 4). After pre-treatment with carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen, serum BUN and Cr levels were lower, indicating that IR-induced renal injury was improved.

Fig. 5 shows the values of TNF-α and IL-10 in kidney. Compared with the sham control (group I), the TNF-α and IL-10 levels of the IR group increased or decreased significantly in kidneys ($P < 0.01$). Pre-treatment of Carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen significantly produced significant decrease or increase ($P < 0.01$) in the renal TNF-α and IL-10 levels in groups III and IV.

The production of proinflammatory cytokines can increase the expression of adhesion molecules of endothelial cells as ICAM-1; this corresponds to an increase in the neutrophil recruitment. Hypoxic endothelial cells synthesize proinflammatory cytokines, which can up-regulate endothelial expression of the constitutive adhesion molecule ICAM-1 in aurocrine fashion (Jordán, Segura, Brea, Galindo, & Castillo, 2008). The expression of ICAM-1

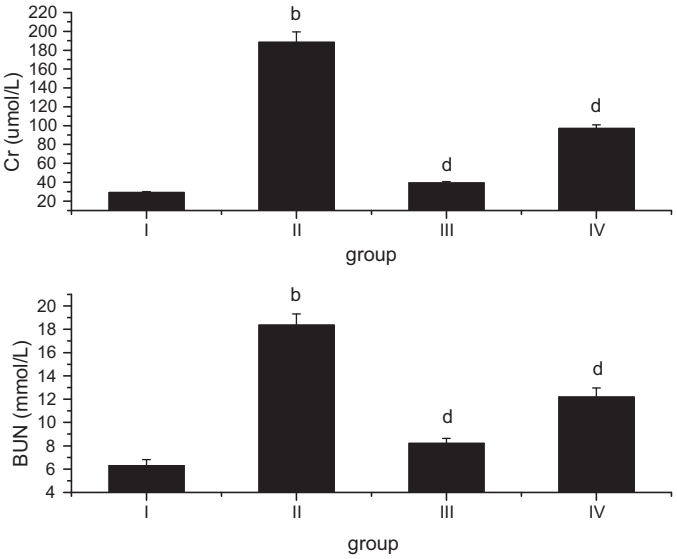

Fig. 4. Effect of carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen pre-treatment on blood BUN and Cr levels. $^{b}P < 0.01$, compared with group I; $^{d}P < 0.01$, compared with group II.

corresponds with the induction of neutrophil recruitment, which is maximal within the first hour of reperfusion, and persists at a lower rate in the late phase of reperfusion (Gauthier, Davenpeck, & Lefer, 1994; Hawkins et al., 1996).

The level of renal ICAM-1 expression was represented in Fig. 6. Compared with the sham control (group I), the ICAM-1 expression of the IR group was increased significantly in kidneys ($P < 0.01$). The level of renal ICAM-1 expression was significantly decreased ($P < 0.01$) in groups III and IV compared to group II.

It is also known that proinflammatory cytokines contribute to propagate the extension of the damage from I/R (Zingarelli, Cuzzocrea, Zsengeller, Salzman, & Szabo, 1997). Recently, various studies have demonstrated clearly that the ethanol extract of danshen reduced the expression of proinflammatory cytokines significantly (Kang et al., 2004). We thought that TNF-α and ICAM-1 may promote endothelial cells damage, and play synergy role with other cytokines in aggravating renal ischemia injury. Pre-treatment

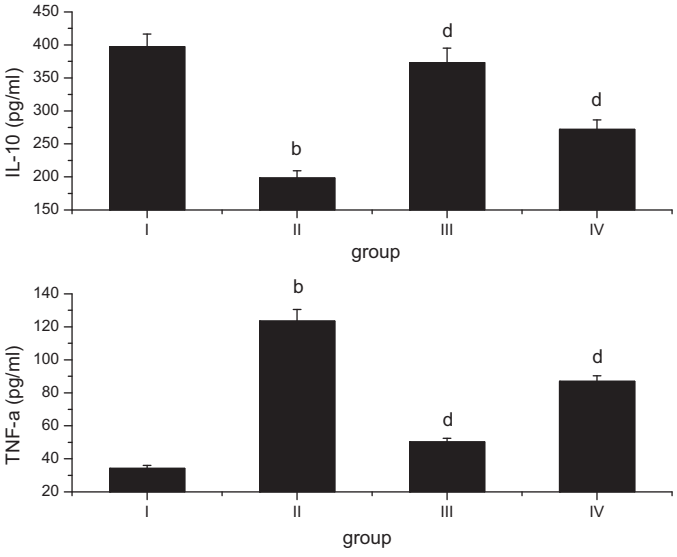

Fig. 5. Effect of carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen pre-treatment on blood TNF-α and IL-10 levels. $^{b}P < 0.01$, compared with group I; $^{d}P < 0.01$, compared with group II.

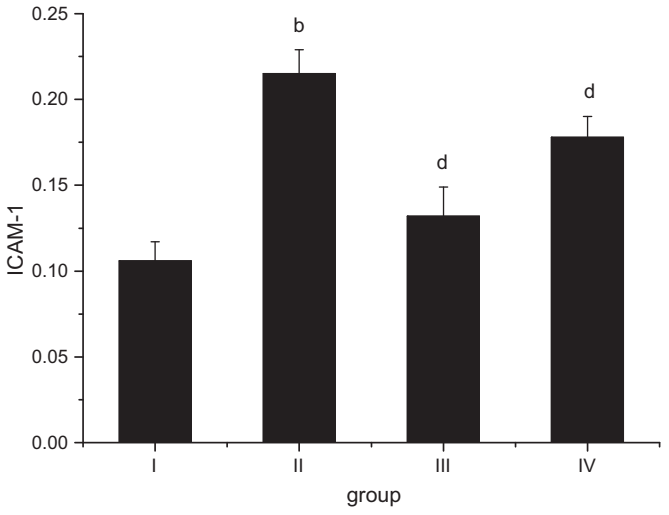

Fig. 6. Effect of carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen pre-treatment on renal ICAM-1 expression. $^{b}P < 0.01$, compared with group I; $^{d}P < 0.01$, compared with group II.

of carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen could reduce the TNF-α and IL-10 levels of the IR rats. We supposed that Carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen reduced capillary vessel bloodstream disorder, and endothelial cells damage possibly by inhibiting TNF-α secretion and ICAM-1 expression, and increasing flammatory cytokines IL-10 secretion.

Redox disturbances are known to negatively impact the body system through ROS generation, which destroy proteins, lipids and DNA by oxidation (Halliwell & Gutteridge, 1990). Elevation in free radicals and lipid peroxidation taking place after reperfusion played a key role in the injury associated with ischemia–reperfusion. Superoxide anion was generated during the period of reperfusion after ischemia, and free radical including superoxide and hydroxyl radicals was involved in the damage induced by ischemia–reperfusion injury (Chen et al., 2004). Ischemia–reperfusion injured renal tissue was particularly sensitive to oxygen radical-mediated injury due to its low concentrations of O_2 radical scavenging enzymes, and with high levels of polyunsaturated fatty acids that constitute the renal cell membrane and mitochondrial membrane.

Rats subjected to renal ischemia–reperfusion demonstrated significantly increased renal level of MDA and activities of SOD, CAT and GSH-Px compared with sham-operated rats (Table 2). Our present data support the notion that renal injury induced by I/R involves toxic oxygen metabolites. In order to determine whether anti-oxidative effects of Rhizoma ligustici wallichii or ethanol extract of danshen were involved in regulation of intracellular antioxidant enzymes, we examined the activity of antioxidant enzymes including catalase, SOD and GSH-Px activities and found that administration of Rhizoma ligustici wallichii or ethanol extract of danshen significantly attenuated post-ischemic decreases of these activities, leading to less lipid peroxidation and less production of hydrogen peroxide in the kidneys. Compared to rats subjected to renal ischemia–reperfusion, pre-administration of Carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen produced a significant reduction in renal MDA level and increase in SOD, CAT and GSH-Px activities (Table 2). This indicates that the resistance afforded by Rhizoma ligustici wallichii or ethanol extract of danshen pre-administration is associated with an anti-oxidative effect, resulting in less kidney functional and histological damage. It has been demonstrated that catalase or SOD pretreatment inhibits post-ischemic cell trafficking by enhancing

Table 2
Effect of carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen pre-treatment on renal MDA level, SOD, CAT, GSH-Px activities.

Group	MDA (nmol/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)	GSH-Px (U/mg protein)
I	7.02 ± 3.06	187.36 ± 12.76	42.09 ± 2.63	38.17 ± 1.85
II	18.37 ± 1.01[b]	83.17 ± 5.02[b]	21.68 ± 1.44[b]	20.77 ± 1.65[b]
III	8.96 ± 0.92[d]	180.32 ± 15.07[d]	40.29 ± 2.39[d]	36.04 ± 2.04[d]
IV	12.84 ± 1.11[d]	136.29 ± 11.49[d]	32.97 ± 1.92[d]	27.93 ± 1.83[d]

[b] $P < 0.01$, compared with group I.
[d] $P < 0.01$, compared with group II.

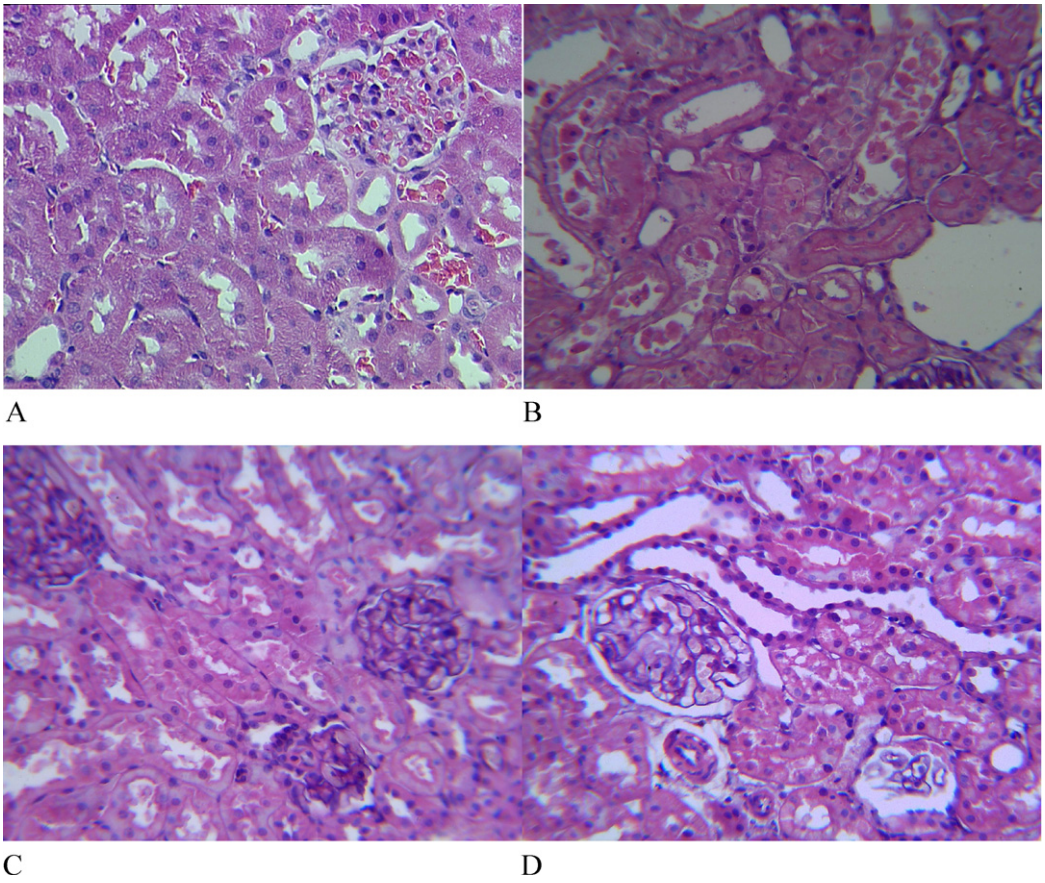

Fig. 7. Histopathological changes in rat renal tissue (H&E, ×400): (A) sham-operated rats, (B) IR control rats, (C) IR rats treated with ethanol extract of danshen (150 mg/kg bw/day), and (D) rats treated with carbohydrate compound of Rhizoma ligustici wallichii (300 mg/kg bw/day).

renal blood flow (Hansson et al., 1983) and that intravenous treatment with SOD in rats resulted in reduced apoptotic cell death and ROS production after kidney I/R (Chien et al., 2001).

Histopathological examination revealed severe lesions in the kidney of untreated acute renal failure rats. These changes were characterized by proteinaceous casts in tubuli in the inner zone of medulla, medullary congestion and hemorrhage in the outer zone inner stripe of medulla, and tubular necrosis in the outer zone outer stripe of medulla. Pre-treatment with Carbohydrate compound of Rhizoma ligustici wallichii and ethanol extract of danshen attenuated the development of all these lesions (Fig. 7).

4. Conclusion

In this study, Carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen pre-treatment significantly inhibits MDA production, implying a reduction in lipid peroxidation and cellular injury that protect the kidney against I/R-induced oxidative damage. In conclusion, the findings of the current study illustrate that Carbohydrate compound of Rhizoma ligustici wallichii or ethanol extract of danshen, with their potent free radical scavenging and antioxidant properties, seems to be a highly promising agent in protecting renal tissue against oxidative damage and in preventing renal dysfunction due to ischemia/reperfusion. Moreover, ethanol extract of danshen displays stronger protective effect on kidneys of IR rats that of carbohydrate compound of the Rhizoma ligustici wallichii.

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