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Full Length Research Paper

The protective effects of *Cordyceps sinensis* extract on extracellular matrix accumulation of glomerular sclerosis in rats

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The objective of the present study is to observe protective effects of *Cordyceps sinensis* extract on extracellular matrix (ECM) accumulation and explore its therapeutic mechanism. The glomerular sclerosis model was established by unilateral nephrectomy and injecting adriamycin. Then urinary protein, BUN and Scr levels were measured. Renal pathological changes were observed by light microscopy. Immunohistochemistry technique was used to examine expressions of CTGF, FN, Col-IV, PAI-1, and MMP-2. Compared with model group, the urinary protein level in *C. sinensis* extract group was markedly decreased. BUN and Scr levels in *C. sinensis* extract group were much lower than model group, and also lower than Fosinopril sodium group. *C. sinensis* extract produced obviously therapeutic effects on glomerular sclerosis. Immunohistochemical results presented that expressions of CTGF, FN and Col-IV in *C. sinensis* extract and Fosinopril groups were less than model group. The expression of PAI-1 in *C. sinensis* extract group was also less than model group, but MMP-2 was more expressed than model group. Taken together, *C. sinensis* extract could ameliorate glomerular sclerosis by reducing proteinuria, decreasing the expressions of FN, Col-IV, CTGF and PAI-1, and increasing the expression of MMP-2, Thus, *C. sinensis* extract might be one novel therapeutic drug for chronic kidney diseases.

Key words: *Cordyceps sinensis* extract, extracellular matrix, glomerular sclerosis.

INTRODUCTION

Glomerular sclerosis (GS) is the final stage of a variety of kidney diseases and its pathological demonstrations mainly are characterized by extracellular matrix (ECM) accumulation and intercapillary cells proliferation (Couser et al., 2006; Bijian et al., 2004; Zhang et al., 2005a). Extracellular matrix accumulation mainly results from the augmented synthesis and attenuated degradation of the extracellular matrix production and has been taken as an important risk factor for the development of the glomerular sclerosis (Pozzi et al., 2009; Martinez et al., 2009). However, lots of clinical evidence shows that conventional therapeutic methods did not significantly prevent and treat extracellular matrix accumulation in patients with glomerular sclerosis. It is very necessary for us to develop new drugs to treat glomerular sclerosis in chronic kidney diseases (CKD).

Cordyceps sinensis (also called *C. sinensis*, DongChongXiaCao, or Tochukaso) is one kind of traditional Chinese herbal and really is an insect parasitizing fungus belonging to the ascomycetes family, found at high altitudes on the Qinghai-Tibetan plateau. The record about this herbal medicine was firstly known written in the Ben-Cao-Cong-Xin (New Compilation of Materia Medica) in 1757 by Wu-Yiluo during the Qing Dynasty (Holliday et al., 2008). In the early medical books, *C. sinensis* was shown to use as a “Lung Protectorate” and as a “Yin/Yang double invigorant. Nowadays, *C. sinensis* has been demonstrated to inhibit inflammatory reaction and prevent

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ischemic injury in many organs (Shahed et al., 2001). However, whether *C. sinensis* can treat chronic kidney diseases remains unknown. The present study was therefore designed to study the protective effects of *C. sinensis* extract against rat glomerular sclerosis.

MATERIALS AND METHODS

Reagents

C. sinensis power was provided by Jiangxi Pharmaceuticals Limited Company. The methods to obtain the *C. sinensis* extract are just as described previously (Zhang et al., 2005b). In brief, the 100 g *C. sinensis* powder was heated at 90℃ for 2 h in 500 mL of distilled water and filtered. Adriamycin (ADR) was provided by Italian Pharmacia Company (Batch No. 6QLO11-D). Fosinopril was provided by China-USA Shiguibao tragacanth Limited Company (Batch No. 070511).

Experimental animals

The 40 male Wistar rats (200±20) g were provided by Experimental Animal Center of Basic College of Jilin University. After one week's adaptive feeding, experimental animals were randomly divided into four groups: (A) Sham operation group, 10 rats; (B) Model group, 10 rats; (C) *C. sinensis* extract group, 10 rats; (D) Fosinopril sodium group, 10 rats. The methods to establish the glomerular sclerosis model is just as described previously (5). In detail, Wistar rats were anesthetized by intraperitoneal injection of pentobarbital sodium 50 mg/kg.

Along the left arch of ribs, 1 - 1.5 cm incision was made on the back, and then left kidney was exposed and cut off. The rats in sham operation group were anesthetized by the same method, but the left kidney was not cut off. After one week, rats in group B, group C and group D were injected with ADR 3 mg/kg into vena caudalis for the first time, which was taken as the 1st day of 1st week. In 4 weeks after first injection, rats in group B, group C and group D were injected with ADR 2 mg/kg. Rats in group A were injected with same isotonic sodium chloride in 1 day and 4 weeks.

Administration of *C. sinensis* extract

After the first ADR injection, rats in group C were administrated every day with *Cordyceps sinensis* extract 5 $ml \cdot kg^{-1} \cdot d^{-1}$, rats in group D were administered with fosinopril sodium 7.5 $mg \cdot kg^{-1} \cdot d^{-1}$, and rats in group A and group B were treated with distilled water. Rats in each group were administrated with *C. sinensis* extract for 12 weeks.

Urine protein measurement

The urinary volume and urine protein level of rats were tested by a Coke Lui Phenol Red before the first ADR injection and in 6, 8, 10 and 12 weeks after the first ADR injection.

Biochemistry index examination

The blood sample was drawn from rat fossa orbitalis plexus venosus before the first ADR injection and in 8 weeks after the first ADR injection. The serum was separated from the blood and BUN and Scr levels were examined. In 12 weeks, rats were anesthetized by intraperitoneal injection of pentobarbital sodium 50 mg/kg and the abdominal aorta was bluntly separated. The blood serum was separated from collected blood and was used to detect biochemical index.

Renal pathological observation by HE staining

In 12 weeks, the kidney was quickly taken out and then was cut into two pieces from renal hilus with renal capsule striped. Renal samples then were fixed by 10% paraform, and cut into 4 μm renal tissue sections, which were placed on glass slides. Slides were deparaffinized in xylene for 5 min (3 times) and then rehydrated in 100, 95, 70% ethanol and PBS. HE (hematoxylin and eosin) staining protocol is as followed. In brief, slides were stained in hematoxylin for 3 min, rinsed in tap water dipped in acid-alcohol 8 - 12 times, and finally rinsed in tap water. Next, slides were stained with eosin for 30 s and then dehydrated with 95% ethanol, 100% ethanol and xylene. Images were observed with an Olympus Vanox-s equipped with an Olympus Japan 138132 objective and were captured using a Nikon Digital Camera System.

Immunological histochemistry examination

The expression of connective tissue growth factor (CTGF), fibronectin (FN), collagen-IV (Col-IV), plasminogen activator inhibitor-1 (PAI-1) and matrix metalloproteinase-2 (MMP-2) in kidney was examined by immunohistochemistry technique. Immunohistochemistry examination would be performed according to kit instruction under room temperature. We randomly chose 20 non-repetition glomerular and renal tubule-interstitium visual fields under the high-power microscope (× 200), and gave semi-quantitative score for five proteins expression according to its staining area and intensity. Staining intensity standards: no coloring is 0 points; the pale coloring is 1 points; moderate is 2 points; deep colored is 3 points. Staining area of standards: no staining or weak staining is 0 points; the stained area < 25% is 1 point; the stained area between 25 and 50% is 2 points; the stained area between 50 and 75% is 3 points; the stained area > 75% is 4 points. Staining area score and staining intensity score were added together. The total score 0 is regarded as 0; the total score between 1 and 2 is regarded as 1; the total score between 3 and 4 is regarded as 2; the total score between 5 and 6 is regarded as 3; the total score is 7 is regarded as 4.

Statistical methods

SPSS 13.0 is used for statistical analysis. Measurement data are presented as mean ± SD ($\overline{x} \pm s$). A paired Student's t-test was used to compare the different between various groups. $P < 0.05$ showed significant difference and $P < 0.01$ showed extremely significant difference.

RESULTS

Effects of *C. sinensis* extract on the urine protein level

After 6 weeks of the first ADR injection, the urine protein level in group B, group C and group D was significantly increased (Table 1). In 9 weeks, the urine protein level in group C was significantly lower than group B ($P < 0.01$). In 10 weeks and 12 weeks, the urine protein levels both in group C and group D were much lower than group B ($P < 0.01$). These results indicates that *C. sinensis* extract an fosinopril sodium both could markedly decrease the urine

Table 1. The comparison of the urine protein level among different groups.

Group	n	Urine protein (mg)				
		0 w	6 w	8 w	10 w	12 w
A	10	7.86 ± 0.82	8.23 ± 1.25	7.81 ± 0.63	9.86 ± 0.55	9.51 ± 0.71
B	10	8.12 ± 0.76	49.07 ± 13.90**	80.38 ± 24.29**	97.80 ± 19.50**	117.33±31.65**
C	10	8.32 ± 0.93	46.21 ± 15.81**	54.94 ± 18.10**##	67.44 ± 23.37**##	74. 21±26.11**##
D	10	7.79 ± 1.03	34.70 ± 12.55**#	49.27 ± 25.02**##	64.17 ± 21.33**##	71.88±29.91**##

Note: to compare with group A, **P<0.01; to compare with group B, #P<0.05 , ##P<0.01; to compared with group C, P < 0.05.

Table 2. The comparison of BUN and Scr among different groups.

Group	n	BUN (mmol/L)			Scr (μmol/L)		
		0 w	8 w	12 w	0 w	8 w	12 w
A	10	5.50 ± 1.23	6.47 ± 0.77**	6.63 ± 0.62**	38.31 ± 6.74	32.18 ± 5.26**	37.75 ± 7.58**
B	10	5.81 ± 0.86	13.73 ± 1.05	17.52 ± 3.01	33.11 ± 5.40	98.07 ± 5.90	156.92 ± 14.18
C	10	5.99 ± 1.11	9.07 ± 1.61**	9.35 ± 2.29**#	37.03 ± 6.50	79.79 ± 7.55**	93.33 ± 8.05**##
D	10	5.49 ± 0.95	9.41 ± 1.03**	12.80 ± 2.86**	35.78 ± 6.60	82.01 ± 6.52**	120.90 ± 11.21**

Note: to compare with group B, *P < 0.05 , **P < 0.01; to compare with group D, #P < 0.05 , ##P < 0.01

protein level. However, the urine protein level in group C was slightly higher than group D (P < 0.05).

Effects of *Cordyceps sinensis* extract on the BUN and Scr levels

There was no significant difference of BUN and Scr level among all groups before ADR injection. The BUN and Scr level in group B, group C and group D were gradually increased in 8 weeks and 12 weeks. Nevertheless, the level of BUN and Scr in group C and group D was much lower than that in group B in 8 weeks and 12 weeks (P<0.01). Furthermore, the level of BUN and Scr in group C was lower than group D (P < 0.01) (Table 2). These results suggest that *C. sinensis* extract and fosinopril sodium both could obviously reduce the BUN and Scr level in damaged kidney. However, *C. sinensis* extract presents more strong therapeutic effects than fosinopril sodium.

Effects of *C. sinensis* extract on the renal pathological changes

In 12 weeks, renal glomerular structure in group A was still normal and did not develop segmental glomerular sclerosis (Figure 1A). Renal tubular epithelial cells kept regularly arranged, with a small number of fibroblasts and inflammation cells in renal interstitial and without protein cast in renal tubule. The structure and proportion of renal capsule did not change. Nevertheless, in group B, obvious glomerular hypertrophy was observed, with broaden renal capsule, thickening capsule wall, and focal glomerular sclerosis (Figure 1B). About 50% glomerulus developed glomerular sclerosis with different stage, and the hardening glomerulus occupied about 10%. Protein cast was observed in broaden renal tubule, and plenty of fibroblasts and inflammatory cells existed in the renal interstitial. However, in group C, renal glomerulus only presented little alterations, with slight interstitial proliferation and glomerular capsule accretion (Figure 1C). Renal tubular epithelial cells were swollen, tube diameter became slightly narrow, the renal cavity was slightly expanded, protein cast could be occasionally observed, and there were less inflammatory cells in renal interstitial. In group D, we could find renal capsule basement membrane thickening (Figure 1D). Bowman's capsule was also expanded with partial glomerular sclerosis. Renal tubular epithelial cells swell, protein cast could be found in renal tubule, epithelial cell was granular degeneration, and inflammatory cells could be found in the renal interstitial. All these evidence shows that *C. sinensis* extract attenuated the pathological alteration in rat glomerular sclerosis.

Effects of *C. sinensis* extract on CTGF, FN and Col-IV

As illuminated in Table 3, there was less staining of CTGF, FN, Col-IV in mesangial region in group A, as well as in collecting tube, distal tubule and proximal tubule. The positive staining of FN and Col-IV existed in glomerular basement membrane (Figure 2 and Figure 3). The positive staining of CTGF was markedly increased in group B, compared with group A (P < 0.01) (Figure 4).

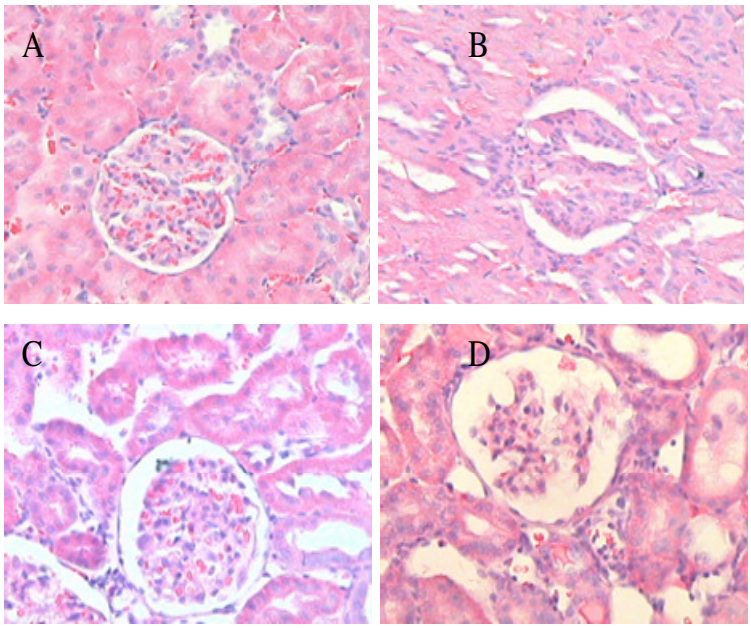

Figure 1. The HE staining picture of different groups (×200)

Table 3. The comparison of CTGF, FN and Col-IV among various groups.

Group	n	Glomerulus			Renal tubule and interstitial substance		
		CTGF	FN	Col-IV	CTGF	FN	Col-IV
A	10	0.73±0.46	0.50±0.45	0.61±0.76	0.75±0.66	0.57±0.44	0.52±0.54
B	10	3.42±0.47**	3.30±0.83**	3.34±0.71**	3.32±0.84**	3.78±0.81**	3.63±0.76**
C	10	2.61±0.33$^{**\#}$	2.52±0.78$^{**\#}$	2.58±0.90$^{**\#}$	1.40±0.30$^{*\#\#\geq}$	1.70±0.77$^{**\#\#}$	1.54±0.58$^{**\#\#}$
D	10	2.59±0.52$^{**\#}$	2.57±0.80$^{**\#}$	2.71±0.77$^{**\#}$	2.36±0.64$^{**\#}$	1.63±0.74$^{**\#\#}$	1.61±0.66$^{**\#\#}$

Note: to compare with group A, *P < 0.05，**P < 0.01; to compare with group B, #P < 0.05，##P < 0.01; to compare with group D, P < 0.05.

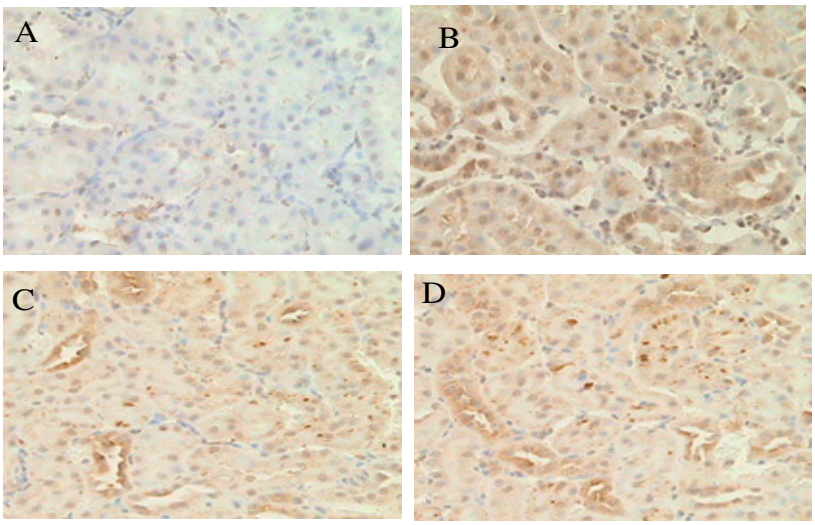

Figure 2. The immunohistochemistry staining of FN in different groups (× 200).

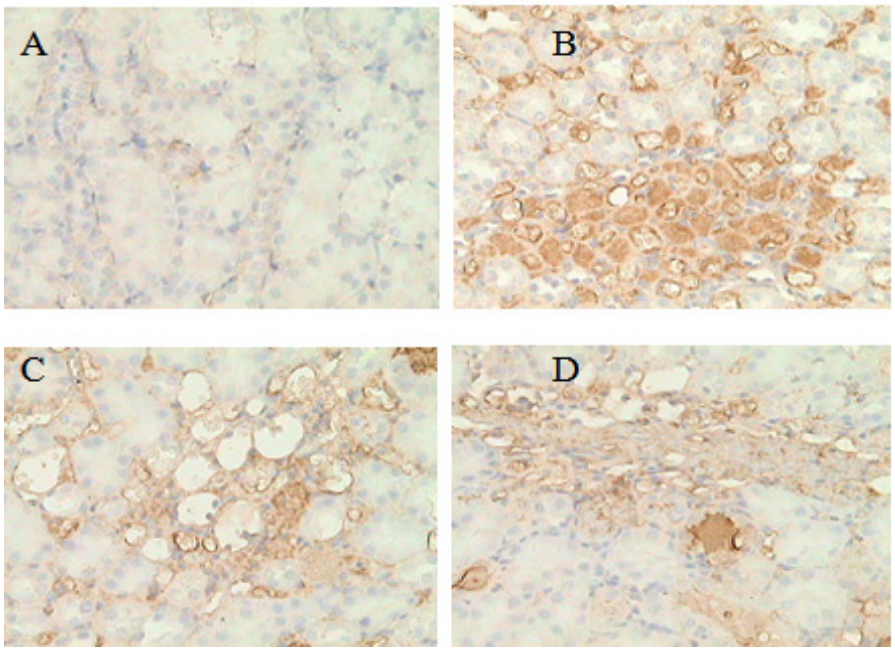

Figure 3. The immunohistochemitry staining of Col-IV in different groups (×200).

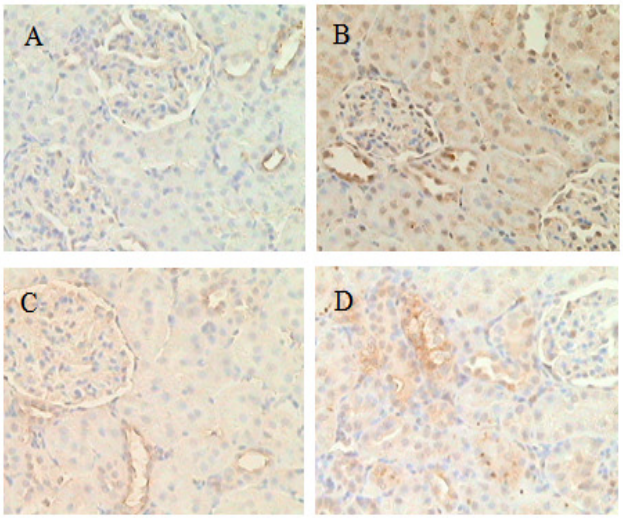

Figure 4. The immunohistochemistry staining of CTGF in different groups (× 200).

The positive staining of CTGF, FN and Col-IV in glomerulus was obviously reduced in group C and group D, compared with group B ($P < 0.05$). Meanwhile, the positive staining of CTGF, FN and Col-IV in renal tubule and interstitial was significantly reduced in group C, compared with group B ($P<0.01$). There was statistical difference of positive staining of CTGF between group C and group D ($P<0.05$). These findings indicate that *C. sinensis* extract could ameliorate the extracellular matrix remodeling in glomerular sclerosis.

Effects of *C. sinensis* extract on MMP-2 and PAI-1

As showed in Table 4, MMP-2 was more detected in glomerulus, renal tubule and interstitial substance in group A than group B ($P<0.01$) (Figure 5). The positive staining of MMP-2 in group C and group D were clearly increased, compared with group B ($P<0.01$). There was less staining of PAI-1 in glomerulus and even less in renal interstitial in group A (Figure 6). The positive staining of PAI-1 was obviously increased in group B ($P<0.01$). The positive staining of PAI-1 in group C and group D was more decreased than group B ($P<0.05$). These results suggest that C. sinensis extract could ameliorate the MMP-2 and PAI-1 remodeling in glomerular sclerosis.

DISCUSSION

The present study mainly demonstrated that *C. sinensis* extract could remarkably ameliorate the renal function injury in glomerular sclerosis, reverse the extracellular matrix accumulation by regulating CTGF, FN, Col-IV, PAI-1, and MMP-2, which indicates that *C. sinensis* extract possibly will be one therapeutic drug for chronic kidney diseases. *C. sinensis* is one kind of traditional Chinese herbal and has been used for a long time in traditional Chinese medicine (Shahed et al., 2001). *C. sinensis* has been demonstrated a variety of pharmacological effects, such as anti-inflammatory actions, anti-apoptotic effects, stimulating natural killer cells, anti-tumor activities, and so on (Shahed et al., 2001; Hamada, 1991; Zhang et al., 2005c; Chen et al., 2005;

Table 4. The comparison of MMP-2, PAI-1 and MCP-1 among various groups.

Group	n	Glomerulus		Renal tubule and interstitial substance	
		MMP-2	PAI-1	MMP-2	PAI-1
A	10	3.66 ± 0.76	0.42 ± 0.21	3.75 ± 0.66	0.47 ± 0.44
B	10	0.43 ± 0.65**	3.41 ± 1.09**	0.49 ± 0.84**	3.58 ± 0.81**
C	10	2.87 ± 0.40$^{*\#\#}$	2.67 ± 0.71$^{**\#}$	3.08 ± 0.30$^{*\#\#}$	1.55 ± 0.77$^{**\#\#}$
C	10	2.92 ± 0.19$^{*\#\#}$	2.74 ± 0.90$^{**\#}$	2.91 ± 0.64$^{*\#\#}$	1.63 ± 0.74$^{**\#\#}$

Note: to compare with group A, $^{*}P < 0.05$, $^{**}P < 0.01$; to compare with group B, $^{\#}P < 0.05$, $^{\#\#}P<0.01$; to compare with group D, $P < 0.05$.

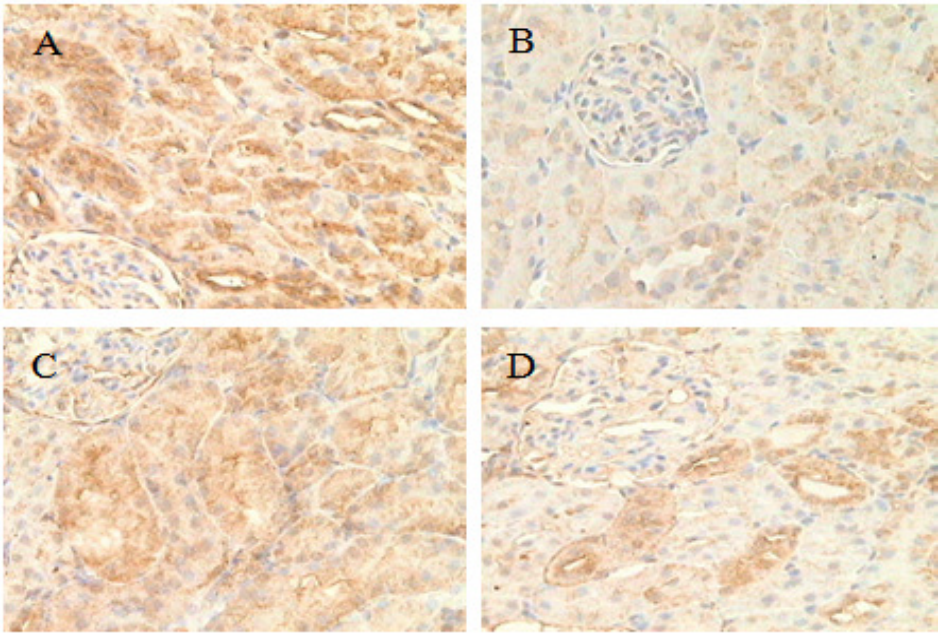

Figure 5. The immunohistochemistry staining of MMP-2 in different groups (×200).

Wu et al., 2007). For example, *C. sinensis* extract produced the anti-tumor effect on B16-induced melanoma in C57BL/6 mice, causing about 60% decrease of tumor size (Hamada, 1991). Furthermore, *C. sinensis* extract also produced beneficial effects on rat kidney following ischemia and reperfusion. It could significantly enhance renal functions and reduce the expression of inflammatory and apoptotic genes in rat subjected to ischemia and reperfusion. It implied that *C. sinensis* extract may play a potential therapeutic role in renal transplantation (Shahed et al., 2001). However, whether *C. sinensis* extract effectively reverses the extracellular matrix remodeling in chronic kidney diseases remains unknown.

Renal extracellular matrix is composed of some collagens (including I, III, IV collagen), glycoprotein (FN, LN) and proteoglycans (Qin et al., 2009). In the healthy kidney, the synthesis and degradation of extracellular matrix remain the dynamic balance. The disturbance of extracellular matrix metabolism due to the pathological factors stimuli will lead to renal fibrosis (Gaedeke et al., 2004). The plasminogen and the MMP system are showed the two key systems for regulating the extracellular matrix degradation. The accumulation of extracellular matrix makes contributions to the occurrence and development of glomerular sclerosis.

The excessive synthesis of extracellular matrix and the increasing fibrosis are the main reason of extracellular matrix accumulation. CTGF, FN and Col-IV play an important role in the development of glomerular sclerosis (Couser et al., 2006; Bijian et al., 2004; Zhang et al., 2005a). Our research showed that both *Cordyceps sinensis* extract and fosinopril sodium could reduce CTGF, FN and Col-IV, and play a therapeutic role in extracellular matrix accumulation. Through regulating CTGF, FN, Col-IV and MMP-2 so as to ameliorate the extracellular matrix remodeling, *Cordyceps sinensis* extract effectively treated the glomerular sclerosis induced by unilateral nephrectomy and Adriamycin injection. The renal function indexes such as the urinary protein level, BUN and Scr levels were significantly improved. As the main enzyme of

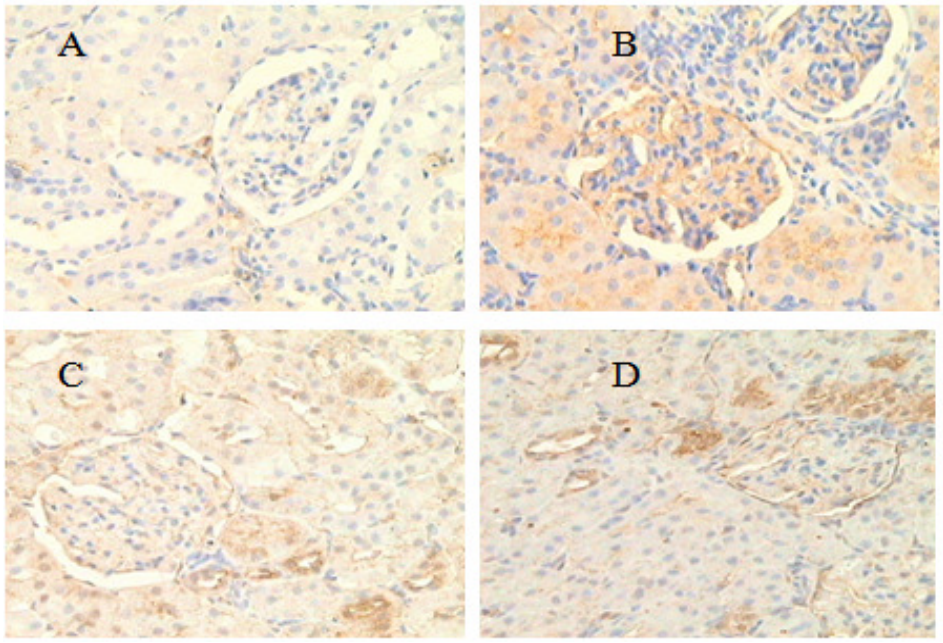

Figure 6. The immunohistochemistry staining of PAI-1 in diffe rent groups (× 200).

the Col-IV, MMP-2 is a very important factor for main-taining the balance of extracellular matrix metabolism (Qin et al., 2009). As we known, ACEI has regulatory influences in the extracellular matrix degradation by the MMP system and its suppression (MMPs/TIMPs). Our study revealed that the *C. sinensis* extract also could markedly increase MMP-2, which might be one of main mechanisms by which *Cordyceps sinensis* extract reduced the extracellular matrix accumulation and delayed glomerular sclerosis progress.

Activator of plasminogen and its inhibitor system (tPA/tPAI) is another major system to modulate extracellular matrix metabolism. In 12 weeks, the positive staining of PAI-1 was increased in model group, thereby inhibiting the Col-IV degradation of PA and breaking the original balance, and finally gave rise to the extracellular matrix accumulation, consistent with the previously reported (Gaedeke et al., 2004). *Cordyceps sinensis* extract could inhibit the PAI-1, regulate the plasminogen inhibitor system to alleviate the extracellular matrix accumulation, and then delay glomerular sclerosis development.

C. sinensis extract can reduce proteinuria, improve renal function, decrease the positive staining of FN, Col-IV, CTGF and PAI-1, and increase the positive staining of MMP-2, so that extracellular matrix accumulation in rat glomerular sclerosis was markedly inhibited. Of course, the mechanisms underlying *Cordyceps sinensis* extract treats extracellular matrix accumulation in glomerular sclerosis are multifaceted. Here we just selected the most important pathway-“extracellular matrix and its metabolic pathway”. The other mechanisms would be further studied in the future.

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