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Cordyceps cicadae extracts ameliorate renal malfunction in a remnant kidney model*

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Abstract: Background and Objectives: Chronic kidney disease (CKD) is a growing public health problem with an urgent need for new pharmacological agents. *Cordyceps cicadae* is widely used in traditional Chinese medicine (TCM) and has potential renoprotective benefits. The current study aimed to determine any scientific evidence to support its clinical use. Methods: We analyzed the potential of two kinds of *C. cicadae* extract, total extract (TE) and acetic ether extract (AE), in treating kidney disease simulated by a subtotal nephrectomy (SNx) model. Sprague-Dawley rats were divided randomly into seven groups: sham-operated group, vehicle-treated SNx, Cozaar, 2 g/(kg·d) TE SNx, 1 g/(kg·d) TE SNx, 92 mg/(kg·d) AE SNx, and 46 mg/(kg·d) AE SNx. Renal injury was monitored using urine and serum analyses, and hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stainings were used to analyze the level of fibrosis. The expression of type IV collagen (Col IV), fibronectin (FN), transforming growth factor-β1 (TGF-β1), and connective tissue growth factor (CTGF) was detected by immunohistochemistry. Results: Renal injury, reflected in urine and serum analyses, and pathological changes induced by SNx were attenuated by TE and AE intervention. The depositions of Col IV and FN were also decreased by the treatments and were accompanied by reduced expression of TGF-β1 and CTGF. In some respects, 2 g/(kg·d) of TE produced better effects than Cozaar. Conclusions: For the first time, we have shown that *C. cicadae* may inhibit renal fibrosis in vivo through the TGF-β1/CTGF pathway. Therefore, we conclude that the use of *C. cicadae* could provide a rational strategy for combating renal fibrosis.

Key words: Chronic kidney disease, *Cordyceps cicadae*, TGF-β, Traditional Chinese medicine
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1 Introduction

Chronic kidney disease (CKD) is a global public health problem with a high case fatality rate. Although remarkable advances have been made in intervention techniques, the incidence, prevalence, and cost of CKD are increasing (Trivedi *et al.*, 2002), which suggests that the exploitation of new pharmacological agents for treatment should be accelerated (Wojcikowski *et al.*, 2006). In contrast to the relatively recent period of new drug exploitation, many traditional Chinese medicines (TCMs) have been employed successfully for their diuretic and renal protective actions for centuries, and well-documented pharmacopoeias exist (Wu and Liang, 2007). However, the wide use of TCM has been largely restricted because of a shortage of pharmacological studies and well-designed clinical trials. More significantly, rigorous scientific research must be used to evaluate the safety and efficacy of TCM therapies, which may also provide information for the community and complement current treatments.

Cordyceps cicadae (Fig. 1), a caterpillar-shaped medicinal mushroom that derives its nutrients from larvae of *Cicada flammata* Dist., has been utilized in

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Chinese herbal medicinal prescriptions for thousands of years. *C. cicadae* belongs to genus *Cordyceps*, a genus of ascomycete fungi that includes about 400 described species (Paterson, 2008). All *Cordyceps* species are endoparasitoids, living mainly on insects and other arthropods. Since they were first used for treating multiple disorders in Chinese herbal medicinal prescriptions, there has been an accumulation of scientific evidence to support the use of *Cordyceps* for the treatment of kidney disease (Paterson, 2008; Wojcikowski *et al*., 2004; 2006). The best known species of the genus is *C. sinensis*, which has been used for nearly two thousand years as a kidney tonic (Wojcikowski *et al.*, 2006). However, its high price, restricted source, and low production have greatly limited its use. Several studies have demonstrated that *C. cicadae*, another useful member of the family, have various biological activities (Kuo *et al.*, 1994; 1996; 2001). Having been used in Chinese herbal medicinal prescriptions for relief of asthma for a long time (Kuo *et al.*, 2003), *C. cicadae* has recently received increasing attention for its effective renoprotective function (Jin *et al*., 2005; Jin and Chen, 2006; Wang and Chen, 2006). Compositional analysis has shown that the amino acid, *Cordyceps* polysaccharide, and mannitol contents of *C. cicadae* are similar to those of *C. sinensis* (Wang and Liu, 2004). Furthermore, *C. cicadae* may be safer than *C. sinensis* as it has lower contents of arsenic and lead, and no detectable mercury. However, in-depth research which could promote more usage is still limited.

Fig. 1 *Cordyceps cicadae* used for this study

In the present study, 5/6 subtotal nephrectomized (SNx) rats were used to determine whether *C. cicadae* extracts can effectively attenuate the renal fibrosis process and therefore, relieve kidney malfunction. We analyzed urine and serum biochemistry indexes to monitor renal injury following treatment with *C. cicadae* extracts. Hematoxylin and eosin (HE) and periodic acid-Schiff (PAS) stainings were used to study the dynamic histological changes in the kidneys 42 d after intervention. The expression of type IV collagen (Col IV), fibronectin (FN), transforming growth factor-β1 (TGF-β1), and connective tissue growth factor (CTGF) was analyzed using immunohistochemical staining. As expected, similar to Cozaar, the *C. cicadae* extracts alleviated the renal damage and decreased the deposition of Col IV and FN in SNx rats by reducing the expression of TGF-β1 and CTGF. For the first time, our findings revealed that *C. cicadae* could significantly retard the progression of renal malfunction induced by subtotal nephrectomy.

2 Materials and methods

2.1 Preparation of extracts from *C. cicadae*

Artificially cultured *C. cicadae* was kindly provided by Prof. Zhu-an CHEN (Zhejiang Subtropical Crops Institute, China). The preparation of extracts from *C. cicadae* was carried out according to the standard extraction procedure of Chinese medical herbs (Fig. 2). In detail, the dried fruiting bodies of artificially cultured *C. cicadae* were extracted twice with 200 ml of boiling water (100 °C) for 3 h. The brown-colored extracts were then filtered twice with 95% ethanol at room temperature, for 24 h each time. The solvent from all extractions was combined to obtain the total extract (TE) with extraction rate of 50%. The TE was further decompressed and extracted with acetic ether to obtain the acetic ether extract (AE), in which the extraction rate was 2.29% (Fig. 2).

The dosage of the drug used was calculated using the following formula: the classic adult dosage of *C. cicadae* mycelia (12 g/(60 kg·d))×20×extraction rate (50%)=2 g/(kg·d), as a single dose for one rat. To monitor the dose-related action of the drug, we further reduced the consumption to 1 g/(kg·d) as the lower dosage of the TE. Following the same method, we identified 92 mg/(kg·d) and 46 mg/(kg·d) as AE consumption doses. Consumption of 33 mg/(kg·d) of Cozaar (Losartan potassium; positive control) was identified from 200 mg/(60 kg·d) for the adults maximum dose.

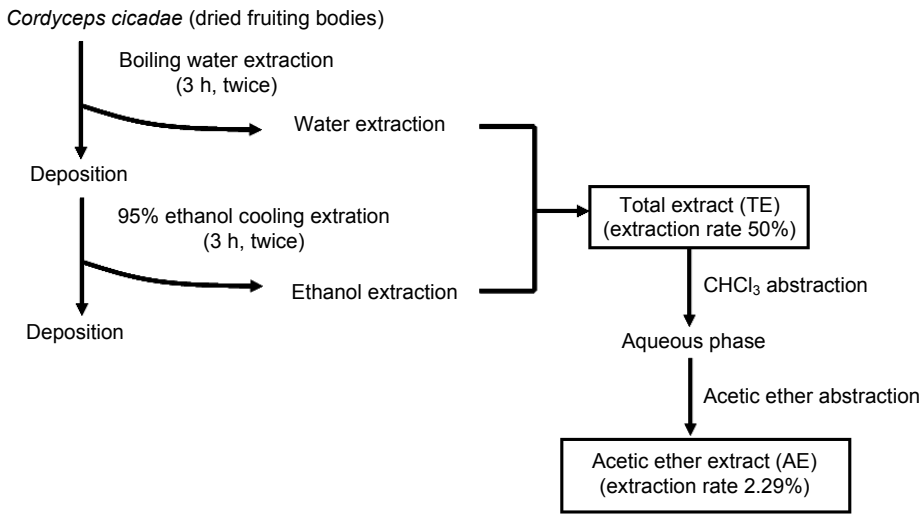

Fig. 2 Flowchart for preparation of TE and AE from the fruiting bodies of *C. cicadae*

2.2 Animals and experimental design

A total of 120 male Sprague-Dawley (SD) rats (8 weeks old, (265±30) g) were obtained from the Laboratory Animal Center of Shanghai University of Traditional Chinese Medicine. All experimental procedures were carried out according to the rules and regulations laid down by the Home Office (Animal Scientific Procedures Act, UK in 1986). The experimental protocol for this study was reviewed and approved by the National Kidney Research Fund (Animal Scientific Procedures Act, UK, in 1986). The rats underwent remnant kidney surgery (Kliem *et al.*, 1996). Caudal vein blood was collected at 14 d after secondary surgery. A serum creatinine (Scr) test was then performed to confirm the establishment of the expected model. Animals with Scr above 100 μmol/L were excluded from further research. All qualifying animals were randomly divided into six groups: SNx, Cozaar, 2 g/(kg·d) TE, 1 g/(kg·d) TE, 92 mg/(kg·d) AE, and 46 mg/(kg·d) AE (Fig. 3). Intragastric administration started at 14 d after secondary surgery on qualifying rats. The duration of therapy was 42 d. When the treatment was completed, blood was collected from the tail vein, then analyzed for hemoglobin (Hb), Scr, albumin (ALB), and alanine aminotransferase (ALT) using a Sysmex KX-21 automated hematology analyzer (Sysmex Co., Kobe, Japan) or a Hitachi 7170A biochemistry analyzer

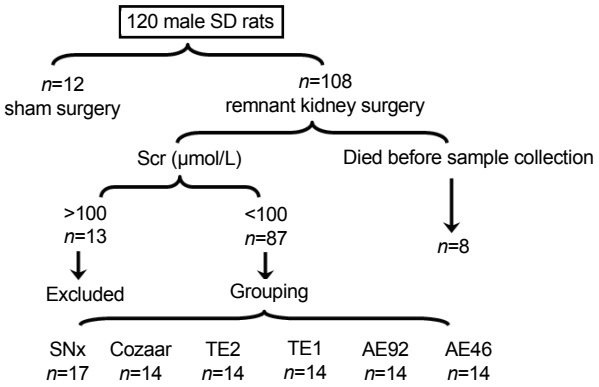

Fig. 3 Grouping information of the study
TE2: 2 g/(kg·d) TE; TE1: 1 g/(kg·d) TE; AE92: 92 mg/(kg·d) AE; AE46: 46 mg/(kg·d) AE

(Hitachi Co., Ltd., Tokyo, Japan). To reduce the influence of body weight on Scr level, we corrected the renal function according to the Cockcroft-Gault formula: $GFR=(140-a)\times W\times b/(72\times c)$, where GFR is glomerular filtration rate, a is the age (year), W is the body weight (kg), b is equal to 0.85 for female, and c is the concentration (μmol/L) of Scr. As the experimental animals shared a common age and gender, we corrected the Scr level using the ratio of body weight to Scr level. For urine collection, the rats were placed in metabolic cages for 24 h to determine protein excretion and other urinary parameters using a Hitachi 7170A biochemistry analyzer before they were

sacrificed. Finally, the rats were killed under isoflurane anesthesia (Webster Veterinary Supply, Bessemer, AL, USA), and the kidneys were collected, each of which was divided into four equal sections. One section was fixed in 10% neutral formalin solution while the others were frozen immediately in liquid nitrogen and preserved at −80 °C until used.

2.3 Histopathological examination

The fixed kidneys were embedded in paraffin, and then cut into 4-μm sections. All the sections were stained with HE and PAS to confirm glomerulosclerosis, which was defined by the collapse of the lumen of glomerular capillaries and/or hyalinosis. Sections stained with HE were used for general histology. The immunohistochemical microimage analysis system (East China University of Science and Technology, Shanghai, China) was used to identify the PAS-positive area and the total area of glomeruli, from which the ratio of the positive area to the matrix area was calculated. At least 50 glomeruli were evaluated under 400× magnification and results were averaged for each kidney.

The glomerulosclerosis index (GSI) was assessed in 50 glomeruli per rat. Injury score was evaluated with a scale of 1–4 as previously reported (Gadola *et al.*, 2004): 1, mesangial expansion/sclerosis involving 25% of the tuft; 2, 25% to 50%; 3, 50% to 75%; 4, >75%. The GSI for each rat was calculated as the mean value of all glomerular scores obtained.

A tubulointerstitial injury score (TIS) was assessed in 20 randomly selected cortical areas per sample. Lesions were graded from 0 to 3 (0, normal; 1, lesions involving <25% of the cortical area; 2, 25% to 50%; and 3, >50%) (Gadola *et al.*, 2004). The score index in each rat was expressed as the mean value of all scores obtained.

2.4 Immunohistochemical staining analysis

Paraffin-embedded sections (4 μm) were deparaffinized, then washed with phosphate-buffered saline (PBS). After 5 min of treatment with 3% (v/v) H_2O_2 and 1 h of blocking with 0.1 g/ml normal horse serum, the sections were incubated with primary antibodies, including antibodies to Col IV, FN, TGF-β1 and CTGF (Santa Cruz Biotechnology, CA, USA), at 4 °C overnight as described previously (Zuo *et al.*, 2009). Slides were incubated with appropriate biotin-conjugated secondary immunoglobulin G (IgG), and treated with reagents from a Vecta-Elite streptavidin-peroxidase kit (Vector Laboratories, Burlingame, CA, USA).

2.5 Statistical analysis

All statistical analyses were performed using the SPSS statistical software package (SPSS, Chicago, IL, USA). The data were expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used to perform comparisons between the different groups, and $P<0.05$ was considered as statistically significant.

3 Results

3.1 General data

Before surgery, the rats in all groups had similar body weight (data not shown) and average food intake was similar among groups. Although body weight increased in all groups throughout the study, SNx groups exhibited limited growth compared with the sham group (Table 1). However, there was no statistically significant difference in body weight before treatment among all SNx groups.

Table 1 Body weight differences between groups before and after treatments

Group	Body weight (g)	
	Day 0	Day 42
Sham	312.9±11.2	393.5±28.5$^{\#*}$
SNx	252.5±17.7	275.6±22.6$^{\#}$
Cozaar	244.5±17.7	274.8±20.5*
2 g/(kg·d) TE	251.4±15.7	335.4±32.9$^{\#*}$
1 g/(kg·d) TE	253.8±3.4	313.4±33.5$^{\#*}$
92 mg/(kg·d) AE	249.8±17.7	313.5±26.1$^{\#*}$
46 mg/(kg·d) AE	253.4±13.1	275.9±25.9*

Data: mean±SD. * $P<0.05$ vs. SNx group; $^{\#}$ $P<0.05$ vs. Cozaar group

Both TE and AE treatments were well tolerated: the animals had a similar mental status, activity, fur color, and appetite. There were significant differences in body weight between the groups according to the choice of drugs (Table 1). In fact, through exposure to the extracts of *C. cicada*, in both the TE and AE groups,

the reduction in body weight was reduced. Furthermore, the treatment effect appeared to be more significant in the TE and AE groups than in the Cozaar group. It may be that, compared with Cozaar, the *C. cicada* extracts provided better nutrition or attenuated disease-related malnutrition in the experimental animals.

3.2 Renal injury improvement after treatment shown by urine analysis

Measurement of protein excretion in a 24-h urinary protein (24HUP) test is the gold standard for the quantitative evaluation of proteinuria. SNx rats showed a significant increase in urinary protein excretion (Table 2), which was markedly reduced by Cozaar and *C. cicadae* extract intervention. However, the effect of *C. cicadae* extracts seemed weaker than that of Cozaar. The total protein-creatinine ratio (TPCR) is another parameter for analysis of renal function. As *C. cicada* extracts can elevate the urine creatinine (UC) level, they can also work as effectively as Cozaar in reducing the TPCR (Table 2). The most marked effect was obtained in the 2 g/(kg·d) TE group. Urine osmotic pressure (UOP) is also measured in research studies. Although SNx can greatly impair the renal concentration function, the UOP was only half of that of the sham group. *C. cicada* extracts and Cozaar can attenuate this impairment, leading to a significant upregulation in UOP (Table 2).

3.3 Renal function aggravation, and anemia and liver function attenuation by Cozaar and *C. cicadae* extract treatments

It is well known that renal function (usually reflected by the Scr) is impaired in SNx rats. In the current study, the SNx rats (receiving vehicle) developed proteinuria with higher Scr levels compared with sham-operated controls (Table 3). Treatment with intubation feeding of *C. cicadae* extract at a dose of 2 g/(kg·d) reduced the Scr level over 42 d by about 25%, to only (113.87±22.51) μmol/L. To diminish the influence of body weight, we corrected the Scr level values. *C. cicadae* extracts effectively reduced the corrected Scr levels. Moreover, this effect was stronger in both the large-dose group of TE and the AE group than in the Cozaar group (Fig. 4).

Anemia is an important concomitant symptom in renal disease, and the Hb level has a close relationship to the disease prognosis. Compared with the vehicle, *C. cicadae* extracts increased the Hb level considerably (Table 3). The Hb level in the Cozaar group was (131.75±5.00) g/L, which was dramatically lower than that in the 2 g/(kg·d) TE group.

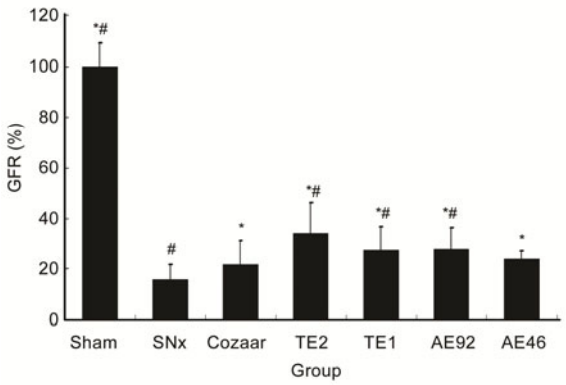

Fig. 4 Effect of *C. cicadae* on renal malfunction
C. cicadae extracts can effectively ameliorate renal malfunction, which is identified by high glomerular filtration rates (GFR; compared to the sham group). TE2: 2 g/(kg·d) TE; TE1: 1 g/(kg·d) TE; AE92: 92 mg/(kg·d) AE; AE46: 46 mg/(kg·d) AE. Data: mean±SD. $^{*}P<0.05$ vs. SNx group; $^{\#}P<0.05$ vs. Cozaar group

Table 2 Urine analysis showing that renal injury was improved after treatment

Group	UP (g/L)	24HUP (g)	UC (g/L)	TPCR (mg/g)	UOP (mOsm/kg H_2O)
Sham	$0.64\pm0.05^{\#*}$	$13.15\pm4.40^{\#*}$	$637.79\pm78.89^{\#*}$	$1.01\pm0.13^{\#*}$	$954.88\pm73.16^{\#*}$
SNx	$2.55\pm0.34^{\#}$	$77.24\pm11.92^{\#}$	$293.78\pm20.30^{\#}$	$8.68\pm1.92^{\#}$	$446.20\pm38.77^{\#}$
Cozaar	$1.34\pm0.10^{*}$	$21.37\pm8.49^{*}$	$374.79\pm39.62^{*}$	$3.60\pm0.42^{*}$	$611.25\pm47.06^{*}$
2 g/(kg·d) TE	$1.47\pm0.32^{\#*}$	$34.91\pm9.82^{\#*}$	$395.16\pm54.64^{\#*}$	$3.97\pm1.48^{\#*}$	$802.50\pm66.18^{\#*}$
1 g/(kg·d) TE	$1.83\pm0.50^{\#*}$	$58.96\pm6.34^{\#*}$	$319.86\pm57.12^{\#*}$	$5.80\pm1.63^{\#*}$	$663.50\pm37.82^{\#*}$
92 mg/(kg·d) AE	$1.81\pm0.52^{\#*}$	$50.49\pm8.49^{\#*}$	$354.86\pm33.06^{\#*}$	$6.02\pm1.63^{\#*}$	$727.80\pm59.18^{\#*}$
46 mg/(kg·d) AE	$1.86\pm0.27^{\#*}$	$60.48\pm9.08^{\#*}$	$294.00\pm44.30^{\#*}$	$6.63\pm0.98^{\#*}$	$630.20\pm48.60^{\#*}$

UP: urine protein; 24HUP: 24-h urinary protein; UC: urine creatinine; TPCR: total protein-creatinine ratio; UOP: urine osmotic pressure. Data: mean±SD. $^{*}P<0.05$ vs. SNx group; $^{\#}P<0.05$ vs. Cozaar group

Table 3 Renal function and anemia level measurements following Cozaar and *C. cicadae* extract treatments

Group	Scr (μmol/L)		Hb (g/L)
	Day 0	Day 42	Day 42
Sham	25.94±2.59$^{\#*}$	47.43±3.46$^{\#*}$	155.13±5.94$^{\#*}$
SNx	80.28±6.70	196.70±40.74*	106.50±3.59*
Cozaar	79.60±4.93	140.85±28.39$^{\#}$	131.75±5.00$^{\#}$
2 g/(kg·d) TE	80.78±6.46	113.81±22.51$^{\#*}$	143.80±9.01$^{\#*}$
1 g/(kg·d) TE	80.62±7.50	133.72±24.09$^{\#*}$	134.60±9.94$^{\#*}$
92 mg/(kg·d) AE	80.48±5.23	123.64±25.82$^{\#*}$	133.38±7.17$^{\#*}$
46 mg/(kg·d) AE	80.15±6.04	145.26±10.95*	130.50±7.17*

Scr: serum creatinine; Hb: hemoglobin; Data: mean±SD. * P<0.05 vs. SNx group; $^{\#}$ P<0.05 vs. Cozaar group

ALT is a specific clinical indicator of liver injury, which can indicate the presence of hepatotoxins in a drug. In our results, there was no obvious difference between the *C. cicadae* extract groups and Cozaar and vehicle-treated groups (Table 4), suggesting the the drugs were free of hepatotoxins. ALB is another parameter for the measurement of liver function, which indicates the synthetic function of the organ. The larger-dose TE treatment increased the level of serum ALB (Table 4), suggesting a better nutrition condition after treatment.

Table 4 Effect of *C. cicadae* extracts on liver function of SNx rats

Group	ALT (U/L)	ALB (g/L)
Sham	31.38±3.11	41.95±1.18$^{\#*}$
SNx	30.50±5.98	25.48±3.47$^{\#}$
Cozaar	29.00±1.83	30.68±3.74*
2 g/(kg·d) TE	29.40±3.85	38.84±0.86$^{\#*}$
1 g/(kg·d) TE	30.60±1.52	34.18±3.58$^{\#*}$
92 mg/(kg·d) AE	31.00±5.48	34.82±2.36$^{\#*}$
46 mg/(kg·d) AE	32.43±3.91	33.85±3.19$^{\#*}$

ALT: alanine aminotransferase; ALB: albumin. Data: mean±SD. * P<0.05 vs. SNx group; $^{\#}$ P<0.05 vs. Cozaar group

3.4 Effect of *C. cicadae* extracts on histological changes

On Day 42, the kidneys of rats subjected to 5/6 subtotal nephrectomy developed a conspicuous tubulointerstitial injury, which could be characterized by tubular dilatation and atrophy, interstitial inflammation, and obvious interstitial fibrosis. Renal injury was ameliorated after treatment, as indicated by reduced tubulointerstitial injury and interstitial fibrosis expansion (Fig. 5). For a more quantitative analysis, the PAS-positive region was examined using an image analysis system, which confirmed that the deposition of extracellular matrix (ECM) protein was significantly reduced by the *C. cicadae* extracts, especially the 2 g/(kg·d) TE treatment. There was a significant difference between the 2 g/(kg·d) TE and angiotensin receptor blocker (ARB) groups (Fig. 5). Glomerulosclerosis, indicated by the GSI, was a prominent component of renal injury after nephrectomy. Treatment with either Cozaar or *C. cicadae* extracts was associated with a less pronounced increment in the GSI. The 2 g/(kg·d) TE treatment produced better results than other groups. Renal injury was ameliorated after treatments with Cozaar and *C. cicadae* extracts as indicated by the lower TIS and interstitial fibrosis expansion in these groups. There was a significant difference between the Cozaar and *C. cicadae* extract groups (Table 5).

Table 5 Histological analysis of remnant kidney tissue after treatment

Group	GSI	TIS
Sham	0.11±0.06$^{\#*}$	0.05±0.17$^{\#*}$
SNx	1.78±0.03$^{\#}$	1.65±0.25$^{\#}$
Cozaar	0.99±0.41*	0.94±0.14*
2 g/(kg·d) TE	0.78±0.21$^{\#*}$	0.51±0.25$^{\#*}$
1 g/(kg·d) TE	0.94±0.28*	0.74±0.27$^{\#*}$
92 mg/(kg·d) AE	0.88±0.25$^{\#*}$	0.71±0.23$^{\#*}$
46 mg/(kg·d) AE	1.01±0.38*	0.85±0.34$^{\#*}$

GSI: glomerulosclerosis index; TIS: tubulointerstitial injury score. Data: mean±SD. * P<0.05 vs. SNx group; $^{\#}$ P<0.05 vs. Cozaar group

3.5 Altered expression of Col IV, FN, TGF-β1, and CTGF induced by *C. cicadae* extracts

There was also a significant increase in deposition of ECM in SNx rats (Fig. 6a), which was assessed by immunostaining of Col IV and FN. As expected, the expression of Col IV and FN was substantially lower in SNx rats treated with Cozaar or *C. cicadae* extracts. The positive-staining region was also quantified using the image analysis system. Clearly, 2 g/(kg·d) TE may provide more benefit than Cozaar or the lower concentration of extract.

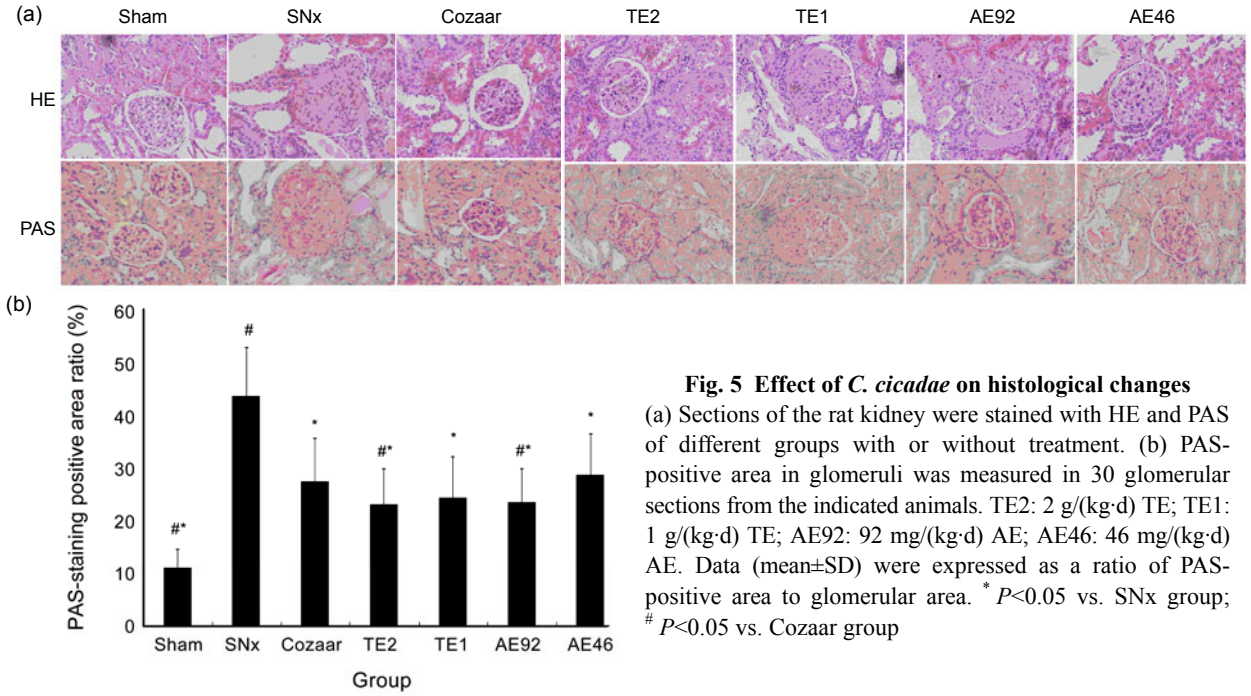

Fig. 5 Effect of *C. cicadae* on histological changes
(a) Sections of the rat kidney were stained with HE and PAS of different groups with or without treatment. (b) PAS-positive area in glomeruli was measured in 30 glomerular sections from the indicated animals. TE2: 2 g/(kg·d) TE; TE1: 1 g/(kg·d) TE; AE92: 92 mg/(kg·d) AE; AE46: 46 mg/(kg·d) AE. Data (mean±SD) were expressed as a ratio of PAS-positive area to glomerular area. * $P<0.05$ vs. SNx group; # $P<0.05$ vs. Cozaar group

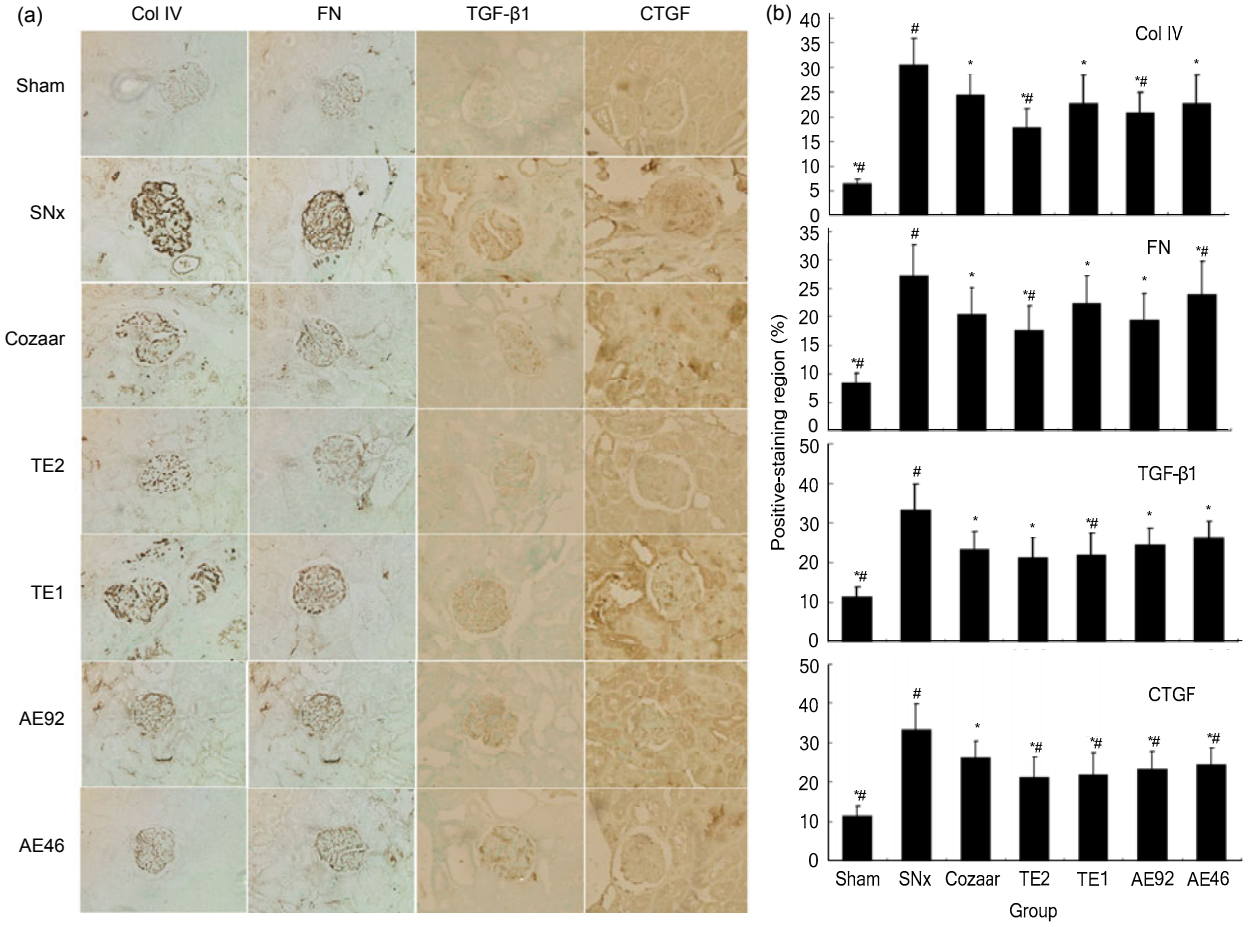

Fig. 6 Effect of *C. cicadae* extracts on Col IV, FN, TGF-β1, and CTGF expression by immunohistochemical staining
(a) Sections of the rat kidneys stained with Col IV, FN, TGF-β1, and CTGF in different groups with or without treatment. (b) The positive-staining regions of Col IV, FN, TGF-β1 and CTGF were quantified using image analysis system. Data: mean±SD. * $P<0.05$ vs. SNx group; # $P<0.05$ vs. Cozaar group

As TGF-β1 and CTGF are important cytokines in renal fibrosis, their expression was subsequently detected by immunohistochemistry for further analysis (Fig. 6b). As shown by the quantification of the positive-staining region, the expression of TGF-β1 and CTGF was obviously unregulated by SNx. After 42 d intervention, however, with Col IV levels and FN deposition conspicuously reduced by the 2 g/(kg·d) TE treatment, the increased expression of TGF-β1 and CTGF was significantly attenuated. Intriguingly, it seemed that similar trends appeared after treatment with Cozaar or the lower concentration of extract.

4 Discussion

While there are limited treatment options available, the incidence, prevalence, and cost of CKD are increasing rapidly (Trivedi *et al.*, 2002), suggesting that there is an urgent need for new pharmacological agents. Parasitic *Cordyceps* fungi, such as *C. sinensis*, have been used for medicinal purposes for centuries, particularly in China, Japan, and other Asian countries (Paterson, 2008; Zhou *et al.*, 2009). As an important member of the *Cordyceps* family, *C. cicadae* has been used generally in TCM for a long time. Column chromatography of the ethyl acetate soluble fraction of extracts identified seven compounds: ergosterol, ergosterol peroxide, bassiatin, bassiatin A, beauvericin, beauvericin A, and beauvericin B (Kuo *et al*., 2002). The current study aimed to determine whether there was any scientific evidence to support the clinical use of *C. cicadae* for complementary treatment of patients with CKD, or if any of these substances were worthy of further research.

In the present study, we demonstrated that extracts of *C. cicadae*, especially the 2 g/(kg·d) TE, significantly attenuated the biochemical and pathological changes in the kidney and subsequent renal failure induced by SNx. Cozaar was used as a positive control with proven efficacy to evaluate the efficiency of the treatment. Although the extracts seemed less effective at modifying some urinary chemical parameters (UP and UC), the Scr levels and corrected Scr levels of the larger-doses of TE and AE were significantly lower than those resulting from Cozaar treatment. Therefore, we suggest that *C. cicadae* may be beneficial for the protection of residual renal function, and that the underlying mechanisms of *C. cicadae* and ARB action may be different. In particular, we found that UOP was significantly increased by intervention with *C. cicadae* extracts, which may demonstrate that the noticeable alleviation occurred in the interstitial injury. However, further studies are necessary to determine whether *C. cicadae* extracts may provide more benefits for renal tubular injuries. We also noticed that the TE performed better than the AE, suggesting that some active components may have been lost or destroyed during the additional purification involved in AE.

A number of reasons may be put forward to explain the renoprotective effects of *C. cicadae* in SNx rats. Firstly, *C. cicadae* may provoke a direct antifibrotic response. By assessment of GSI and TIS (Table 5), the most direct factors for the evaluation of the status of renal injury, the *C. cicadae* extract treatments showed a significant relief of glomerular sclerosis and renal tubular injury. Furthermore, Col IV and FN, as the main components of ECM in fibrosis (Shweke *et al.*, 2008), were accumulated following nephrectomy. Different approaches have been proposed to combat renal fibrosis: (1) decreasing ECM protein synthesis, (2) promoting ECM protein degradation, or (3) preventing mesangial and fibroblast activation and tubular epithelial-mesenchymal transition (Shweke *et al.*, 2008). Obviously, the deposition of ECM, which contains the IV, V, andVI types of collagen and proteoglycan (hyaluronic acid, heparin sulfate, chondroitic acid, etc.), plays a central role in renal fibrosis. Therefore, we conclude that *C. cicadae* extracts may attenuate the deposition of ECM and subsequently relieve the renal fibrosis.

Secondly, *C. cicadae* extracts may modify some pro-fibrogenic cytokine activities. Among many profibrotic factors involved in renal fibrosis, TGF-β1 is a major pro-fibrogenic cytokine (Fan *et al.*, 1999; Shweke *et al.*, 2008). The cytokine TGF-β1 promotes wound healing and repair. Under pathological conditions, TGF-β1 orchestrates cross-talk between parenchyma, inflammation and collagen expression, and plays a key role in stimulating fibrosis. CTGF, a downstream mediator of TGF-β1 in the Smad3-depend manner, is another important fibrogenic factor (Qi *et al.*, 2008). In addition, CTGF regulates cellular apoptosis and fibroblast proliferation, angiogenesis, cellular adhesion, chemotaxis, and deposition of

ECM (Chen *et al.*, 2009). In our research, the expression of TGF-β1 and CTGF was unregulated by the surgery, and this trend was significantly attenuated by treatment with TE or AE, which confirmed the intervention of the TGF-β1/CTGF pathway. As inhibitors of TGF-β1 or CTGF have been considered as potential therapies for treating fibrotic diseases of the kidney (Liu, 2006; Phanish *et al.*, 2010), it is logical to consider *C. cicadae* as an efficient agent for combating fibrosis. An in-depth study of the function of *C. cicadae* extracts and the involvement of other cytokines is needed.

Thirdly, *C. cicadae* extracts may promote a better nutritional status. In renal disease, the ALB level is closely related to the prognosis and the quality of life. The larger-dose TE treatment increased the level of serum ALB (Table 4), indicating a better nutrition condition. The level of serum Hb was also significantly up-regulated by the reaction of TE, especially in the larger-dose group. The better nutrient status was also reflected in the body weight increase following *C. cicadae* extract treatments. It may suggest that compared with Cozaar, the extracts are likely to provide a better nutritional status or to attenuate disease-related malnutrition of experimental animals (Table 1). More importantly, they can effectively improve the prognosis.

Compared with chemical drugs, there are some advantages of using *C. cicadae* extracts to treat renal fibrosis. In general, TCMs often contain versatile biological functions. Although renal fibrosis is always the final common outcome in CKD, the pathogenic mechanisms underlying renal fibrosis are amazingly complicated and involve many mediators and cell types. Therefore, monotherapeutic approaches fail to completely stop the progression of renal fibrosis. The versatile bioactive components of *C. cicadae* extracts will provide multi-target therapeutic effects. In contrast to the relatively recent exploitation of new chemical drugs, herbal and natural "therapies" have been employed for their diuretic and renal protective actions for centuries and well-documented pharmacopoeias exist (Wu and Liang, 2007). Standardization and toxicological studies and well-designed clinical trials may effectively shorten the research and development period and greatly promote the "new uses of old drugs".

5 Conclusions

Although the precise mechanisms underlying the anti-fibrosis effects are incompletely understood, for the first time we have analyzed the effects of *C. cicadae* extracts in treating kidney disease. Our results suggest that *C. cicadae* extracts can inhibit renal fibrosis in vivo, and that this effect may be mediated through the TGF-β1/CTGF pathway. Finally, *C. cicadae* extracts could form the basis of a rational strategy for combating renal fibrosis.

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