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1H NMR spectroscopy analysis of metabolites in the kidneys provides new insight into pathophysiological mechanisms: applications for treatment with Cordyceps sinensis

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Abstract

Background. The number of patients with chronic kidney disease (CKD) is continuously growing worldwide. Treatment with traditional Chinese medicine might slow the progression of CKD.

Methods. In this study, we evaluated the renal protective effects of the Chinese herb Cordyceps sinensis in rats with 5/6 nephrectomy. Male Sprague–Dawley mice (weighing 150–200 g) were subjected to 5/6 nephrectomy. The rats were divided into three groups: (i) untreated nephrectomized group (OP group, n = 16), (ii) oral administration of C. sinensis-treated (4 mg/kg/day) nephrectomized group (CS group, n = 16) and (iii) sham-operated group (SO group, n = 16). The rats were sacrificed at 4 and 8 weeks after 5/6 nephrectomy, and the kidneys, serum, and urine were collected for 1H nuclear magnetic resonance spectral analysis. Multivariate statistical techniques and statistical metabolic correlation analyses were performed to evaluate any differences in metabolic profiles of these groups. Statistical analysis of the data was performed using canonical discriminant analysis.

Results. The renal protective effects of C. sinensis were confirmed using NMR spectroscopy. In the OP group, an accumulation of numerous metabolites involved in the regulation of inflammatory response, such as glutamine, glutamate, and arginine, was observed. On the other hand, in the CS group, the levels of these metabolites were significantly decreased.

Conclusion. The results of this study demonstrated that the renal protective effects of C. sinensis were due to the reduction of the accumulation of reactive nitrogen species. These effects may be due to the decrease in the levels of metabolites involved in the regulation of inflammatory response.
comparison analysis were performed to identify metabolic changes in aqueous kidney extracts between these groups.

**Results.** Significant differences between these groups were discovered in the metabolic profiles of the biofluids and kidney extracts. Pathways including the citrate cycle, branched-chain amino acid metabolism and the metabolites that regulate permeate pressure were disturbed in the OP group compared to the SO group; in addition, these pathways were reversed by *C. sinensis* treatment. Biochemistry and electron microscopic images verified that *C. sinensis* has curative effects on chronic renal failure. These results were confirmed by metabonomics results.

**Conclusions.** Our study demonstrates that *C. sinensis* has potential curative effects on CKD, and our metabonomics results provided new insight into the mechanism of treatment of this traditional Chinese medicine.

**Keywords:** 5/6 nephrectomy; *Cordyceps sinensis*; 1H NMR spectroscopy analysis; metabonomics; pathophysiology

**Introduction**

The number of patients with chronic kidney disease (CKD) continues to grow worldwide [1]. Treatment with traditional Chinese medicine might slow the progression of CKD; however, there is still a significant need for strategies for the treatment of CKD [2]. *Cordyceps sinensis*, one of the most valued traditional Chinese medicines, has been extensively used to prevent and cure human diseases for more than a millennium [3]. It consists of the dried fungus *C. sinensis* that grows on the larvae of caterpillars. It is prescribed as a tonic to increase strength after serious disease. More recently, other studies have shown its usefulness in treating (i) respiratory, renal, liver, nervous system and cardiovascular diseases and (ii) tumors, aging, hyposexuality and hyperlipidemia [4–6]. It has been officially classified as a drug in Chinese Pharmacopoeia since 1964 and has been suggested to play a significant role in the treatment of CKD [7–9] and amelioration of cyclosporin nephrotoxicity [10, 11]. One of the components of *C. sinensis* displays significant antioxidant effects, which have been proved to connect with repair of renal failure [12]. However, the mechanism of *C. sinensis* treatment on CKD is not yet clear at the systemic level. We applied metabonomics analysis combined with biochemical techniques and pathology examinations to explore the renoprotective effects of *C. sinensis* and its molecular mechanism of action.

Metabonomics-based 1H nuclear magnetic resonance (1H NMR) has been proven to very efficiently establish the metabolic consequences of complex biological responses to the challenge of both endogenous factors (such as genetic modification [13, 14]) and exogenous factors (such as disease [15, 16], surgery [17, 18] and drug effects [19, 20]). The commonly used multivariate statistical techniques in metabonomics include principal component analysis (PCA) and orthogonal projection to latent structure-discriminant analysis (OPLS-DA). PCA enables the visual investigation of the changes and trends of metabolic bioprofiles. OPLS-DA gives a better understanding of the relevant metabolite variations of different metabolic pro-files, providing insight into the molecular mechanisms of pathophysiological changes [21]. System statistical metabolic correlation analysis has been used in metabolomics to identify disturbed or recovered pathways [22, 23]. Recently, the application of metabolomics has been extended to characterize the molecular mechanisms of the treatment of traditional Chinese medicine [24–28].

The purpose of this study is to answer two questions: (i) does *C. sinensis* change energy metabolism in the kidney while as a tonic to increase strength and do these changes ameliorate to the pathogenesis of CKD? (ii) does *C. sinensis* exert a protective effect on proximal tubules; is it similar to the effects of calcium-channel antagonists on kidney [10, 11] or are there other reasons? Therefore, we compared the metabolic profiles of a 5/6 nephrectomy (5/6 Nx) rat model treated with *C. sinensis*. Significantly different metabolic profiles were observed in the 5/6 Nx rats compared to sham-operated rats, and the disturbed pathways were reversed with *C. sinensis* treatment. These studies suggest that metabonomics may be a potential tool for investigating the medicinal mechanisms of traditional Chinese medicine.

**Materials and methods**

**Animals and materials**

Male specific pathogen free Sprague–Dawley rats (130–150 g) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). Subtotal nephrectomy (5/6 SNx) in 3-week-old rats was performed by a two-step method as described by Wu et al. [29]. Renal decapsulation alone was performed in sham-operated rats as controls. Rats were randomly assigned into three groups as follows: (i) untreated nephrectomized group (OP group) (*n* = 16), (ii) oral administration of *C. sinensis*-treated (4 mg/kg/day) nephrectomized group (CS group) (*n* = 16) and (iii) sham-operated group (SO group) (*n* = 16). At 4 and 8 weeks after surgery, rats from each group (*n* = 8) were anaesthetized with diethyl ether (Changshu Chemical Co., Ltd, Jiangsu, China) and obtained 1000 IU/kg heparin i.v. (Ratiopharm, Ulm, Germany), serum was separated from blood samples and parts of the kidneys (~100 mg) were used for 1H NMR spectroscopy, parts for histopathology. Urine collection was performed on the day before euthanasia by housing the animals in metabolic cages to measure urinary protein excretion (Biuret assay).

*Cordyceps sinensis* was obtained from Jiminkexin Jiangxi, China. Chemicals were purchased from Sigma-Aldrich (St Louis, MO), unless stated otherwise. Animal use protocols were approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Materia Medica, China. The animals were maintained in climate-controlled conditions with a 12-h light/dark cycle, and they were fed standard rodent chow and water. The guidelines for the care of the animals were strictly followed throughout the studies.

**Preparation of aqueous kidney extracts and acquisition of 1H NMR spectroscopy**

Lyophilized aqueous kidney extracts were prepared using the methanol/chloroform/water system as previously described [30]. The powder of the extract was resolved in 600 μL of phosphate buffer [0.2 M Na2HPO4/0.2 M NaH2PO4 (pH 7.4)], vortexed and then centrifuged at 12 000 g for 10 min at 4 °C. Aliquots of the supernatant (500 μL) were transferred into 5-mm NMR tubes and then 50 μL of D2O containing 2.26 mM sodium 3-(trimethylsilyl) [2, 2, 3, 3-D4] propionate (TSP) was added. Solvent-suppressed 1D 1H NOESY spectra (NoesyPr1d) were acquired using the pulse sequence [RD–90–t1–90–tm–90–ACQ] with a mixing time (tm) of 120 ms. Water suppression was achieved by irradiation of the water peak during the recycle delay. A total of 256 free induction decays were collected into 32 768 data points at a flip angle of 90°, using a spectral width of 10 kHz, giving an acquisition time (ACQ) of 1.64 s,
with an additional relaxation delay (RD) of 5 s. All measurements were performed at 25°C.

To aid resonance assignments for 1D 1H NMR spectra, 2D pulsed field gradient COrrelation Spectroscopy (gCOSY) together with 2D homonuclear Total Correlation Spectroscopy with DIPSI spinlock and watergate (wgTOCSY) were acquired on selected samples.

Multivariate statistical techniques

To exploit the metabolic information embedded in the spectra, all 1D 1H NOESY spectra were multiplied by an exponential function of a 0.3-Hz line-broadening factor prior to Fourier transformation. The NMR spectra were manually phased, corrected for baseline distortion, referenced to the methyl group of TSP at 0.000 and carefully aligned using the software of MestReNova (Version 5.3.1; Mestrelab Research SL). The spectral region of δ 9.40–0.70 was segmented into 8542 bins with a width of 0.001 p.p.m. The residual integrals from the region of δ 5.20–4.60 in suppressed water resonance were excluded in all spectra, and the region of δ 1.15–1.21 including ethanol peak and the integrals of residual methanol (δ 3.34–3.38) were removed from the aqueous tissue extract spectra. The resulting 7945 integrals were normalized to the sum of the spectral integrals to compensate for differences in the concentrations of samples. Subsequently, the normalized integral values were mean centered for PCA and OPLS-DA by SIMCA-P+12.0 software package (Umetrics, Umeå, Sweden). The PCA score plots were visualized with the first principal component t[1] and the second principal component t[2], while OPLS-DA were visualized with the first principal component t[1] and the orthogonal component t[1]. The parameters of R2X(cum), R2Y(cum) and Q2(cum) [only R2X(cum) and Q2(cum) were extracted from PCA models] were computed to test the goodness of fit and model validity. R2X(cum) and R2Y(cum) are the fraction of the sum of the squares of the entire X's and Y's explained by the model, respectively. Q2(cum) represents the cross-validated explained variation. The reliability of models increases with R2Y(cum) and Q2(cum) approaching 1 [31]. Due to the small number of samples (CS:OP:SO = 7:7:8), the six-round cross-validation and permutation tests (200 cycles) were carried out to measure the robustness of the model [32].

Variable importance in the projection (VIP) derived from the OPLS-DA model ranks the importance of each variable for the classification, and those variables with VIP > 1.0 are considered statistically significant in this model [33]. The correlation coefficients of the variables relative to the predictive component (t[1]) in the OPLS-DA model were also calculated in a Java environment (the software environment was downloaded freely at the URLs http://www.eclipse.org/downloads/ and http://www.oracle.com/technetwork/java/javase/downloads/index.html). The cutoff values calculated with the Java platform are in agreement with the models’ degrees of freedom equal to n1 + n2 – 2, where n1 and n2 represent the samples numbers of two groups in the OPLS-DA models [34].

System statistical metabolic correlation analysis

A system statistical metabolic correlation analysis was further applied to display the relationships between the relative integrals of spectral peaks in a certain biological profile, as described previously [35, 36]. Metabolite intensities relative to the sum of the total spectral integral (δ 9.40–0.70 excluding δ 5.20–4.60, δ 1.15–1.21 and δ 3.34–3.38) were used as variables. For each rat, Pearson’s correlation coefficient was calculated among those variables in the above-mentioned Java environment, and a score plot was used to display the correlation matrices within the Spotfire Decision-

<table>
<thead>
<tr>
<th>Group</th>
<th>ALB, g/L</th>
<th>BUN, mmol/L</th>
<th>CREA, μmol/L</th>
<th>URIC, μmol/L</th>
<th>TG, mmol/L</th>
<th>TCH, mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO 4w (n = 8)</td>
<td>17.4 ± 0.5</td>
<td>4.7 ± 0.7</td>
<td>28.4 ± 3.9</td>
<td>59.8 ± 9.2</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>OP 4w (n = 8)</td>
<td>16.6 ± 1.1</td>
<td>9.9 ± 1.2</td>
<td>60.9 ± 6.5</td>
<td>78.9 ± 15.0**</td>
<td>1.7 ± 0.4*</td>
<td>1.3 ± 0.2*</td>
</tr>
<tr>
<td>CS 4w (n = 8)</td>
<td>17.8 ± 0.9#</td>
<td>9.6 ± 1.2</td>
<td>59.3 ± 5.9</td>
<td>64.6 ± 7.7#</td>
<td>1.2 ± 0.4#</td>
<td>0.9 ± 0.3#</td>
</tr>
<tr>
<td>SO 8w (n = 8)</td>
<td>16.8 ± 0.7</td>
<td>5.8 ± 0.5</td>
<td>29.5 ± 2.2</td>
<td>74.8 ± 12.4</td>
<td>1.2 ± 0.5</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>OP 8w (n = 8)</td>
<td>15.4 ± 1.4*</td>
<td>10.8 ± 1.4**</td>
<td>74.9 ± 7.7**</td>
<td>95.4 ± 14.2**</td>
<td>1.8 ± 0.5#</td>
<td>1.6 ± 0.3**</td>
</tr>
<tr>
<td>CS 9w (n = 7)</td>
<td>17.3 ± 0.3##</td>
<td>10.6 ± 1.8</td>
<td>72.7 ± 6.6</td>
<td>76.1 ± 10.7</td>
<td>1.3 ± 0.5#</td>
<td>1.0 ± 0.1##</td>
</tr>
</tbody>
</table>

*SO, sham-operated rats; OP, operated rats; CS, operated rats treated with C. sinensis; ALB, albumin; URIC, uric acid; TG, triglyceride.

### Results

#### Functional parameters of 5/6 SNx rats treated with C. sinensis

Rats with nephrectomy-induced CKD developed elevated serum creatinine concentrations (CREA) and blood urea...
nitrogen (BUN) within 4 weeks (60.9 ± 6.5 μmol/L; 9.9 ± 1.2 mmol/L) compared to the SO rats (28.4 ± 3.9 μmol/L; 4.7 ± 0.7 mmol/L) (Table 1). After 8 weeks, renal fibrosis was evident in the 5/6 SNx rats, and CREA was significantly increased compared to that in sham-operated animals (P < 0.01). Urinary albumin-to-creatinine ratios determined 4 and 8 weeks of OP rats gradually increased to 8.28 ± 3.34 μg/mg (n = 8 per group, P < 0.05 versus SO) and 13.40 ± 5.48 μg/mg (n = 8 per group, P < 0.01 versus SO), respectively. Treatment with C. sinensis significantly decreased the

*Chemical shifts are referenced to TMS-trimethyl singlet resonance at 0.000 p.p.m. and multiplicity definitions are: s, singlet; d, doublet; t, triplet; q, quartet; m, other multiplet. Ade* stands for the adenine partment signals in adenosine-5’-triphosphate, adenosine diphosphate, adenosine monophosphate and adenosine.

Table 2. Assignments of endogenous metabolites found in kidney aqueous extract samples from a typical 1H NMR spectrum in the CS-treated model

<table>
<thead>
<tr>
<th>Metabolite (abbreviation)</th>
<th>Group</th>
<th>δH (pp.m.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine (Leu)</td>
<td>-CH, β-CH₂, γ-CH₃, δ-CH₂, δ-CH₃</td>
<td>3.73 (m), 1.73 (m), 1.70 (m), 1.69 (m), 0.97 (d), 0.96 (d)</td>
</tr>
<tr>
<td>Isoleucine (Ile)</td>
<td>-CH, β-CH₂, γ-CH₃, half-γ-CH₂, δ-CH₃</td>
<td>3.67 (d), 2.00 (m), 1.01 (d), 1.42 (m), 1.21 (m), 0.94 (t)</td>
</tr>
<tr>
<td>Valine (Val)</td>
<td>-CH, β-CH₂, γ-CH₃</td>
<td>3.60 (m), 2.26 (m), 1.05 (m), 0.99 (d)</td>
</tr>
<tr>
<td>Lactate (Lac)</td>
<td>-CH, β-CH₂</td>
<td>4.13 (q), 1.34 (d)</td>
</tr>
<tr>
<td>Analine (Ala)</td>
<td>-CH, half-CH₂</td>
<td>3.79 (q), 1.49 (d)</td>
</tr>
<tr>
<td>Proline (Pro)</td>
<td>-CH, half-β-CH₂, half-β-CH₂, half-β-CH₂, γ-CH₂</td>
<td>4.13 (dd), 3.43 (dt), 3.34 (dt), 2.36 (m), 2.06 (m), 2.05 (m), 2.03 (m)</td>
</tr>
<tr>
<td>Glutamate (Glu)</td>
<td>-CH, half-β-CH₂, half-β-CH₂, half-β-CH₂, half-CH₂</td>
<td>3.78 (t), 2.13 (m), 2.06 (m), 2.34 (m), 2.37 (m)</td>
</tr>
<tr>
<td>Methionine (Met)</td>
<td>-CH, half-β-CH₂, half-β-CH₂, γ-CH₂, s-CH₃</td>
<td>3.85 (dd), 2.68 (t), 2.19 (m), 2.11 (m), 2.15 (s)</td>
</tr>
<tr>
<td>Succinate (Suc)</td>
<td>2×CH₂</td>
<td>2.41 (s)</td>
</tr>
<tr>
<td>Glutamine (Gln)</td>
<td>-CH, β-CH₂, γ-CH₂</td>
<td>3.78 (t), 2.44 (m), 2.14 (m)</td>
</tr>
<tr>
<td>Malate (Mal)</td>
<td>-CH, β-CH₂</td>
<td>2.68 (dd), 2.35 (dd)</td>
</tr>
<tr>
<td>Aspartate (Asp)</td>
<td>-CH, β-CH₂</td>
<td>3.89 (dd), 2.82 (dd), 2.69 (dd)</td>
</tr>
<tr>
<td>Creatinine (Cr)</td>
<td>-CH₂, N-CH₃</td>
<td>3.95 (s), 3.04 (s)</td>
</tr>
<tr>
<td>Carnitine (Car)</td>
<td>-CH₂, β-CH, γ-CH₂, N(CH₃)</td>
<td>2.45 (dd), 4.58 (br), 3.42 (m), 3.20 (s)</td>
</tr>
<tr>
<td>Choline (Cho)</td>
<td>1CH₂, 2CH₂, N(CH₃)</td>
<td>4.05 (t), 3.51 (dd), 3.21 (s)</td>
</tr>
<tr>
<td>Phosphorylcholine (PC)</td>
<td>1CH₂, 2CH₂, N(CH₃)</td>
<td>4.18 (m), 3.60 (t), 3.22 (s)</td>
</tr>
<tr>
<td>sn-Glycero-3-phosphorylcholine (GPC)</td>
<td>1CH₂, 2CH₂, N(CH₃), glycerol:halfCH₂, halfCH₂, halfCH₂, halfCH₂</td>
<td>4.33 (m), 3.68 (m), 3.23 (s), 3.68 (dd), 3.90 (m), 3.87 (m), 3.94 (m)</td>
</tr>
<tr>
<td>Betaine (Bet)</td>
<td>-CH₂, N(CH₃)</td>
<td>3.90 (s), 3.27 (s)</td>
</tr>
<tr>
<td>Taurine (Tau)</td>
<td>1CH₂, 2CH₂</td>
<td>3.43 (t), 3.27 (t)</td>
</tr>
<tr>
<td>myo-Inositol (m-Ino)</td>
<td>1CH₂, 2CH₂, 3CH₂, half1CH₂, half3CH₂</td>
<td>3.54 (dd), 4.07 (t), 3.54 (dd), 3.63 (t), 3.29 (t), 3.63 (t)</td>
</tr>
<tr>
<td>Glycine (Gly)</td>
<td>-CH₂</td>
<td>3.57 (s)</td>
</tr>
<tr>
<td>Allantoin (All)</td>
<td>CH</td>
<td>5.40 (s)</td>
</tr>
<tr>
<td>Adenosine* (Ade*)</td>
<td>Adenine moiety:2CH, 8CH, NH₂</td>
<td>8.58 (s), 8.27 (s), 6.13 (d)</td>
</tr>
<tr>
<td>Fumurate (Fum)</td>
<td>CH</td>
<td>6.52 (s)</td>
</tr>
<tr>
<td>Formate (For)</td>
<td>CH</td>
<td>8.46 (s)</td>
</tr>
<tr>
<td>Nicotinamide adenine dinucleotide (NAD)</td>
<td>Nicotinamide moiety:-CH₂, -CH₂, γ-CH₃, β-CH₂, NH(NH₂)</td>
<td>9.32 (s), 9.13 (d), 8.82 (d), 8.20 (m), 6.08 (s), 8.41 (s), 8.16 (s), 6.03 (d)</td>
</tr>
</tbody>
</table>

Fig. 2. The 600 MHz 1H NMR NoesyPr1d spectra (δ 0.5–4.6, 5.2–9.4) of kidney aqueous extract samples from the 8-week rat models of CS (A), OP (B) and SO (C). Metabolites’ assignments and abbreviation are seen in Table 2. Leu, leucine; Val, valine; Lac, lactate; Glu, glutamate; Pro, proline; Suc, succinate; Met, methionine; Mal, malate; Asp, aspartate; Cr, creatine; Car, carnitine; Cho, choline; Gln, glutamine; GPC, sn-glycero-3-phosphocholine; Bet, betaine; Tau, taurine; m-Ino, myo-inositol; Gly, glycine; FMA, fumarate; All, allantoin, For, formate; NAD, nicotinamide adenine dinucleotide.
from 1H NMR spectra of Week 8 kidney aqueous extracts from the OP and CS and OP models. R2X(cum) = 0.46, R2Y(cum) = 0.89, Q2(cum) = 0.55. (Figure 3B) and the OP and SO groups [R2X(cum) = 66%, Q2(cum) = 76%] (Figure 3C). A cluster of 200 permuted models from the first component were visualized using validation plots (Figure 3D and E). In the permutation test, all permuted R2 and Q2 values to the left were lower than the original point to the right and lower than the original values [39, 40], which indicated that the original models were valid.

According to VIPs, coefficient numbers (|r|) and metabolites variation statistical examination, several discriminatory metabolites were determined in the pairwise comparison (Figure 4A and B). At Week 8, the metabolites that dramatically declined in the OP model compared to the SO model included methionine, aspartate, creatine, carnitine, sn-glycero-3-phosphocholine, betaine, taurine, myo-inositol, adenine signals in ANP (ATP, ADP and AMP) and nicotinamide adenine dinucleotide, while certain metabolites were elevated, including amino acids (leucine, isoleucine, valine, proline and glycine), lactate and allantoin (Table 3). After C. sinensis administration, however, the trends of many metabolites were reversed. Compared with OP rats, the concentrations of amino acids (leucine, isoleucine, valine and proline) declined, and this was accompanied by increased levels of aspartate, creatine, taurine, myo-inositol and adenosine signals in ANP. The integration results for the above-mentioned metabolites were shown in the box charts (Figure 5), representing the maximum, median and minimum values of the concentration ranges.

In the 1H NMR spectra of kidney tissue aqueous extracts, we established the signal intensities of certain amino acids (leucine, isoleucine, valine, alanine, glutamine, methionine, glutamate, aspartate and glycine), choline metabolites [sn-glycero-3-phosphocholine (GPC), phosphocholine and choline], creatine, lactate, carnitine, organic osmolytes (betaine, taurine and myo-inositol), allantoin, adenine in ANP, fumarate, formate and nicotinamide adenine dinucleotide. The peaks of proline overlapped with those of glutamate; therefore, the signal intensities of proline and glutamate were marked as Pro+Glu. In total, we calculated the signal integrity of 26 metabolites and applied the calculation of system statistical metabolic correlation analysis. In the comparison correlation matrix, the branched-chain amino acids, including isoleucine, leucine and valine, were negatively correlated with GPC and positively correlated with myo-inositol and alanine in the OP model. These correlations are similar to those in the SO models. Fumarate was positively correlated with succinate and urinary albumin-to-creatinine ratios to 92.7% of the OP group at 4 weeks. This value further decreased to 42.6% at 8 weeks, indicating a significant difference (P < 0.05) (Figure 1).

Triglyceride (TG) and total cholesterol (TCH) levels also significantly increased in 5/6 SNx rats, indicating the development of a metabolic disturbance. Albumin levels in 5/6 SNx rats significantly decreased compared to the levels in sham-operated rats and rats treated with C. sinensis. Uric acid levels of CS rats were significantly lower than those of SNx rats throughout the entire experimental period after nephrectomy.

1H NMR spectroscopy studies of kidneys

Typical 1H NMR spectra of kidney tissue aqueous extracts from the three groups are shown in Figure 2. Spectral resonances of metabolites were assigned on the basis of NMR shifts, coupling patterns, coupling constants, 2D spectra such as TOCSY and gCOSY and previous studies [38]. The assignments of metabolites are listed in Table 2.

PCA score plots based on the resulting 7945 variables from the three groups using the first and the second components [R2X(cum) = 62%; Q2(cum) = 48%] presented a recovery tendency after C. sinensis administration (Figure 3A). The obvious separation between the OP model and the SO group was achieved along the first predictive component (PC1) axis; however, the CS group was closer to the SO group than the OP group, indicating the recovery effects of the metabolic profiles during C. sinensis treatment.

Application of OPLS-DA using the PC1 and one orthogonal component to the variables to optimize intergroup variation resulted in a clear biochemical distinction between the CS and OP groups [R2X(cum) = 46%, Q2(cum) = 55%] (Figure 3B) and the OP and SO groups [R2X(cum) = 66%, Q2(cum) = 76%] (Figure 3C). A cluster of 200 permuted models from the first component were visualized using validation plots (Figure 3D and E). In the permutation test, all permuted R2 and Q2 values to the left were lower than the original point to the right and lower than the original values [39, 40], which indicated that the original models were valid.

According to VIPs, coefficient numbers (|r|) and metabolites variation statistical examination, several discriminatory metabolites were determined in the pairwise comparison (Figure 4A and B). At Week 8, the metabolites that dramatically declined in the OP model compared to the SO model included methionine, aspartate, creatine, carnitine, sn-glycero-3-phosphocholine, betaine, taurine, myo-inositol, adenine signals in ANP (ATP, ADP and AMP) and nicotinamide adenine dinucleotide, while certain metabolites were elevated, including amino acids (leucine, isoleucine, valine, proline and glycine), lactate and allantoin (Table 3). After C. sinensis administration, however, the trends of many metabolites were reversed. Compared with OP rats, the concentrations of amino acids (leucine, isoleucine, valine and proline) declined, and this was accompanied by increased levels of aspartate, creatine, betaine, myo-inositol and adenosine signals in ANP. The integration results for the abovementioned metabolites were shown in the box charts (Figure 5), representing the maximum, median and minimum values of the concentration ranges.

In the 1H NMR spectra of kidney tissue aqueous extracts, we established the signal intensities of certain amino acids (leucine, isoleucine, valine, alanine, glutamine, methionine, glutamate, aspartate and glycine), choline metabolites [sn-glycero-3-phosphocholine (GPC), phosphocholine and choline], creatine, lactate, carnitine, organic osmolytes (betaine, taurine and myo-inositol), allantoin, adenine in ANP, fumarate, formate and nicotinamide adenine dinucleotide. The peaks of proline overlapped with those of glutamate; therefore, the signal intensities of proline and glutamate were marked as Pro+Glu. In total, we calculated the signal integrity of 26 metabolites and applied the calculation of system statistical metabolic correlation analysis. In the comparison correlation matrix, the branched-chain amino acids, including isoleucine, leucine and valine, were negatively correlated with GPC and positively correlated with myo-inositol and alanine in the OP model. These correlations are similar to those in the SO models. Fumarate was positively correlated with succinate and urinary albumin-to-creatinine ratios to 92.7% of the OP group at 4 weeks. This value further decreased to 42.6% at 8 weeks, indicating a significant difference (P < 0.05) (Figure 1). Triglyceride (TG) and total cholesterol (TCH) levels also significantly increased in 5/6 SNx rats, indicating the development of a metabolic disturbance. Albumin levels in 5/6 SNx rats significantly decreased compared to the levels in sham-operated rats and rats treated with C. sinensis. Uric acid levels of CS rats were significantly lower than those of SNx rats throughout the entire experimental period after nephrectomy.

1H NMR spectroscopy studies of kidneys

Typical 1H NMR spectra of kidney tissue aqueous extracts from the three groups are shown in Figure 2. Spectral resonances of metabolites were assigned on the basis of NMR shifts, coupling patterns, coupling constants, 2D spectra such as TOCSY and gCOSY and previous studies [38]. The assignments of metabolites are listed in Table 2.
negatively correlated with malate in the OP models; these relationships disappeared in CS and SO rats. The signals of branched-chain amino acids were positively correlated with Pro-Glu. Carnitine was positively correlated with methionine in the CS and SO models (Figure 6).

Discussion

The 5/6 nephrectomy rat is a classic model of progressive renal scarring characterized by both glomerulosclerosis and interstitial fibrosis, in which both glomerular and peritubular capillary endothelial injuries have been reported [41–43]. In this study, the levels of creatinine, BUN and proteinuria in the 5/6 Nx group were progressively elevated, which were also accompanied by typical pathological changes. Oral administration of C. sinensis was shown to ameliorate glomerulosclerosis and renal interstitial fibrosis in this study, which indicated that C. sinensis has the same renoprotective effects in the 5/6 Nx model as it was reported to ameliorate cyclosporin nephrotoxicity [10, 11].

The most important findings of this study are as follows. (i) As a tonic, C. sinensis can affect the energy metabolism in kidney, particularly in terms of the tricarboxylic acid (TCA) cycle. (ii) C. sinensis can help balance the disturbed osmotic pressure of the extracellular NaCl. (iii) C. sinensis
Fig. 5. Box charts representing the median, minimum and maximum of integral values of most significant metabolites for the CS, OP and SO models.

Fig. 6. Pearson’s correlation comparison scatter plots between the CS and OP models (A) or between the OP and SO models (B) of the quantities of the 26 metabolites measured by $^1$H NMR in Week 8 kidney aqueous extract samples. The cutoff values of correlation coefficients are marked by the color bar on the side.
might promote branched-chain amino acids degraded in the kidney. (iv) *C. sinensis* further decreased the concentration of triglycerides, lipoproteins and TCH in the kidney. This might contribute to the effect of *C. sinensis* on the amelioration of tubular and glomerular function.

In our metabolomics study on kidney tissue, there are some changes in energy metabolism in the 5/6 Nx group that were reversed in the CS group. Fumarate, succinate and malate are important intermediates in the TCA cycle. The two enzymes fumarate hydratase (FH) and succinate dehydrogenase (SDH) play a vital role in adenosine triphosphate (ATP) production via the mitochondrial respiratory chain. FH catalyzes the conversion of fumarate to malate, and SDH converts succinate into fumarate. Fumarate was positively correlated with succinate and negatively correlated with malate in the OP model. These relationships disappeared in the CS and SO rats, indicating that the functional changes in FH and SDH were induced by 5/6 Nx surgery and functionally recovered by *C. sinensis* treatment. The functional disorders of SDH and FH have been demonstrated to be involved in the control of cell proliferation and are associated with many tumors types including renal cancer [44, 45]. These mechanisms include pseudo-hypoxia, mitochondrial dysfunction, impaired apoptosis, oxidative stress and anabolic drive. Dramatically increased malate in the CS model may indicate that *C. sinensis* regulates the TCA cycle to produce more ATP, which was verified by the increased intensity of adenine signals in ANP. In the OP rats, succinate was positively correlated with lactate levels. In fact, lactate levels were increased in the OP model and decreased in the CS model, which indicates that the process of aerobic glycolysis was stimulated in the OP rats and that *C. sinensis* treatment downregulated glycolysis. Carnitine plays important roles in fatty acid metabolism [46]; its deficiency in the OP model indicates a dysregulation of lipid β-oxidation [47]. Although the concentration of carnitine did not significantly change with *C. sinensis* administration, the positive correlation between carnitine and methionine existed in both the CS and SO models, which were not observed in the OP rats. All things considered, energy metabolism and mitochondrial function were disturbed in OP rats, and *C. sinensis* treatment could modulate mitochondrial function to promote energy generation. These results are consistent with the altered number and structural changes in mitochondria observed by electron microscopy. The number of mitochondria in the OP models was reduced, and we observed the vacuolization and dissolution of the mitochondria in those models, while in CS rats, these changes in the mitochondria were partly reversed (data not shown).

The second change in metabolites was related to osmoregulation. *Cordyceps sinensis* exerted a protective effect on proximal tubules by balancing the disturbed osmotic pressure of the extracellular NaCl. Cells in the renal medullas of mammals contain large amounts of organic osmoles including GPC, myo-inositol, glycine, betaine and taurine [48]. In kidney aqueous extracts from OP rats, the concentration of GPC, myo-inositol, betaine and taurine significantly decreased, whereas glycine dramatically increased. This indicated a change in renal medullary osmolality [49]. The levels of GPC and betaine increased after *C. sinensis* administration to help balance the disturbed osmotic pressure of the extracellular NaCl [50, 51], which might contribute to the effect of *C. sinensis* on the amelioration of tubular function that also be confirmed by the change of urinary albumin-to-creatinine ratios.

The third change in metabolites concerns amino acid metabolism. The elevation of kidney valine, leucine and isoleucine in OP rats implies that the metabolism of these branched-chain amino acids had been suppressed. This might be a result of the inhibition of the branched-chain α-keto acid dehydrogenase complex, which is the unique dehydrogenase enzyme for each of the three branched-chain amino acids [52]. *Cordyceps sinensis* might protect kidney cells, promote efficient kidney function and activate the branched-chain α-keto acid dehydrogenase complex; subsequently, these branched-chain amino acids can be degraded in the kidney.

Other changes in metabolites occurred in the levels of lipids and apoptosis. Clinical chemistry serum measurements highlighted an increase in lipids, lipoproteins and TCH at Week 8 in the OP rats. CHe, eCHe, ePH2e lipid signals have been observed to be decreased in a rat glioma model undergoing apoptosis [53]. Al-Saffar et al. [54] found that apoptosis of Jurkat T-cells was associated with triacylglycerol accumulation. The mechanism for the relationship between increased lipid metabolites and apoptosis might result from high oxide stress induced by increased lipid peroxidation [55]. The observed accumulation of lipids, lipoproteins and TCH in serum might be associated with the 5/6 Nx-induced apoptosis; however, *C. sinensis* further decreased the concentration of triglycerides, lipoproteins and TCH. Therefore, *C. sinensis* plays a significant role in arresting the apoptosis of tubular epithelial cells. These results are consistent with the kidney pathology of the CS model.

In conclusion, based on the 1H NMR spectra of kidney tissue, biochemistry and pathophysiology examination, we could identify differences among the 5/6 Nx rats treated with or without *C. sinensis* and the sham-operated rats. Concerning the question whether *C. sinensis* is similar to the effects of calcium-channel antagonists that exert a protective effect on proximal tubules, there might be other mechanisms related to energy metabolism, osmoregulation, amino acid metabolism and the levels of lipids in kidney.

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Conflict of interest statement. We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled ‘1H NMR spectroscopy analysis of metabolites in the kidneys provides new insight into pathophysiological mechanisms: applications for treatment with Cordyceps sinensis’.

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Y27632 attenuates the aristolochic acid-promoted invasion and migration of human urothelial cancer TSGH cells in vitro and inhibits the growth of xenografts in vivo

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Abstract

Background. Aristolochic acid I (AAI) has been implicated in urothelial cell carcinoma (UCC) in humans. However, whether AAI promotes invasion/migration of UCC has not been established.

Methods. A study of human UCC TSGH cells cultured with AAI was conducted. Cell viability, the effects of AAI on the activity of matrix metalloproteinase (MMP)-9, the abilities of invasion/migration and the migration-related proteins (Ras, RhoA, ROCK1, PI-3K, pAkt and nuclear factor-kappaB) of the TSGH cells were assessed. The TSGH cells were subcategorized to 1-day or 30-day AAI exposure. An in vivo study using a nude mice xenograft model was employed to test the antitumor effects of Rho kinase inhibitor or Y27632.

Results. A time- and dose-dependent increase in both activity and messenger RNA (mRNA) level of MMP-9 were demonstrated. The mRNA level of urokinase-type plasminogen activator was increased and tissue inhibitor of metalloproteinase-1 was decreased in the cells with 30-day but not 1-day AAI exposure. A dose-dependent enhancement in wound-healing rate and cell migration was demonstrated, especially in the 30-day AAI-exposed cells. Expressions of Ras/RhoA and other migration-related proteins were increased after AAI treatment, which could be inhibited by Y27632. The in vivo results demonstrated that Y27632 was able to attenuate the speed of growth of the inoculated tumors in nude mice.

Conclusion. Clinically, the patients with prolonged AAI exposure are highly associated UCC, our results provided in vitro and in vivo evidence that prolonged AAI exposure enhances invasion and migration of human TSGH cells.

Keywords: aristolochic acid; invasion; matrix metalloproteinase; migration; urothelial cell carcinoma

Introduction

Chinese herb nephrotoxicity has been traced to aristolochic acid (AA), which was the major alkaloid extracted from Aristolochia fangchi [1, 2]. AA is a mixture of structurally related nitrophenanthrene carboxylic acids, with aristolochic acid I (AAI) and AAlI being major components.