Research Article

Cordyceps militaris Improves Chronic Kidney Disease by Affecting TLR4/NF-κB Redox Signaling Pathway

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Cordyceps militaris may show good promise in protecting against chronic kidney disease (CKD) but the molecular mechanism remains unclear. CKD risk is associated with the Toll-like receptor 4/nuclear factor-kappa B (TLR4/NF-κB) signaling pathway. Cordycepin is the main component of Cordyceps militaris and may affect the TLR4/NF-κB pathway. Cordycepin was prepared by preparative HPLC. CKD patients were assigned into Cordyceps militaris (COG, 100 mg daily) and placebo (CG) groups. Cordycepin activity was measured using human embryo kidney cells (HEK293T). Biochemical indices, the levels of TLR4, NF-κB, cyclooxygenase-2 (COX2), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β), were measured by real-time qRT-PCR, or ELISA kits and or Western blot. After 3-month treatment, cordycepin reduced the levels of urinal protein, blood urea nitrogen (BUN), and creatinine by 36.7%±8.6%, 12.5%±3.2%, and 18.3%±6.6%, respectively (P<0.05). Cordyceps militaris improved lipid profile and redox capacity of CKD patients by reducing the serum levels of TG, TC, and LDL-C by 12.8%±3.6%, 15.7%±4.1%, and 16.5%±4.4% and increasing the HDL-C level by 10.1%±1.4% in the COG group when compared with the CG group, respectively (P<0.05). The serum levels of cystatin-C (Cys-C), myeloperoxidase (MPO), and malondialdehyde (MDA) were reduced by 14.0%±3.8%, 26.9%±12.3%, and 19.7%±7.9% while nitric oxide (NO) and superoxide dismutase (SOD) were increased by 12.5%±2.9% and 25.3%±13.4% in the COG group when compared with the CG group, respectively (P<0.05). Cordycepin reduced the levels of TLR4, NF-κB, COX2, TNF-α, and IL-1β in HEK293T cells too (P<0.05). However, cordycepin could not affect the levels anymore if TLR4 was silenced. Cordyceps militaris protected against CKD progression by affecting the TLR4/NF-κB lipid and redox signaling pathway via cordycepin.

1. Introduction

Chronic kidney disease (CKD) is often caused by infections [1, 2], toxins [3, 4], and autoimmune diseases [5] and a major threat to public health [6]. CKD is involved with glomeruli [7, 8], tubules [9, 10], and interstitial tissue around the glomeruli and tubules [11]. CKD often results in glomerular injury because of the destruction of glomerular structure caused by high-level inflammatory cells [12, 13]. The result will prevent blood flow, resulting in the decrease in urine output and accumulation of uremic toxin. Subsequently, red blood cells may be released from injured glomeruli and hematuria will occur [14].

At present, there are many ways to treat CKD, including hypoglycemic [15], antihypertensive [16, 17], and control of urinary protein [18]. However, the treatment cost is high [19], the side effects are obvious [20], and the therapy is long-lasting [21] and ineffective [22]. Furthermore, there are some contraindications to the treatment of CKD [23]. Therefore, it is imperative to explore new anti-CKD drugs with few adverse effects.

Cordyceps militaris and its specific ingredient, cordycepin, have attracted much attention with multiple health-promoting properties, including anti-inflammatory, antitumor, antidiabetic, and antiobesity activities [24]. Cordycepin has been reported to exert antidiabetic and antinephritic
function [25]. However, the exact molecular mechanism for its function on CKD remains unknown. An evaluated level of TLR4 can cause renal fibrosis and result in CKD risk by activating inflammatory cytokines and dysregulating immune responses that are linked with CKD progression [26]. Significant reduction in the amounts of TLR4+ monocytes and impaired lipopolysaccharide are also linked with CKD development [27]. On the other hand, the increase in the level of nuclear factor-kappa B is also associated with CKD development [27]. On the other hand, the increase in the level of nuclear factor-kappa B is also associated with CKD development [27].

2. Materials and Antibodies.

2. Materials and Methods

2.5. Inclusion Criteria. All patients met the following criteria: (1) urine protein/creatinine ratio < 5; (2) blood pressure < 150/95 mmHg; (3) serum modified phosphorus and calcium for albumin and intact parathyroid hormone (PTH) < 100 pg/mL; (4) medically stable; and (5) signed a written informed consent.

2.6. Exclusion Criteria. The following patients were excluded: (1) took azathioprine, methotrexate, mycophenolate mofetil, or cyclophosphamide within 12 mon; (2) took calcium binder or supplements, vitamin D, or phosphate binders; (3) had renal thrombotic microangioplasty, preexisting chronic renal failure, pregnancy, previous malignancy, and diabetes mellitus; and (4) had anticipated poor compliance with the protocol.

2.7. Patient Groups. After the inclusion and exclusion criteria, 98 CKD patients were recruited and randomly assigned into cordycepin (COG, the patients received 100 mg of Cordyceps militaris/d) and control (CG, the patients received dried chickweed herb placebo/d) groups. The whole period was three months.

2.8. Measurement of Renal Function. Urinary protein in patient urine was determined using the kit from Beckman Coulter Inc. (South Kaemer Boulevard, Brea, CA, USA). Blood urea nitrogen (BUN) was measured using the kit from StressMarq Biosciences (Victoria, Canada) to determine kidney normal function. Urea nitrogen is a waste product and the kidneys filter out the waste, which is removed out from the body via urinating. The increase in BUN levels is supposed to be associated with CKD [42]. Creatinine is a chemical waste generated from muscle metabolism and is a reliable biomarker of kidney function [43]. Blood creatinine was measured by the kit from Enzo Life Sciences (Shanghai, China). eGFR was measured by using the modification of diet in renal disease study equation before and after 3-month therapy [44]. Interval
diagnosis of CKD and possible cause (these will have relevance to the ability of any therapeutic agent to alter the course of the disease. Specifically, they will affect the agent’s ability to affect the pathological changes in the kidney) was one month.

2.9. Biochemical Index Analysis. Serum lipid profiles, including triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C), have been reported to be linked with CKD development [45]. Serum TG was determined by using an immunomassay kit (Beijing Chemclin Biotech, Beijing, China). Serum TG was analyzed by using an automated kit (Biosino Biotech, Beijing). HDL-C was measured by an automated chemistry analyzer (Shanghai ChemDo International Trade Co. Ltd., Shanghai, China). Serum LDL-C was determined by using an LDL-C kit (Shanghai Kexin Institute of Biological Technology, Shanghai, China).

Serum Cys-C was measured by using the Behring system (BCS, Dade Behring, Marburg, Germany). MPO was measured using orthoiodobetate colorimetric assay at 450 nm [46]. MDA and SOD were measured using thiobarbituric acid reaction [47]. NO was measured by using the kit from Dojindo Laboratories (Kumamoto, Japan).

2.10. The Analysis of Renal Pathology. Renal tissues were isolated from all patients by using a noninvasive surgery [48]. 200 mg of kidney biopsy specimen was obtained from each subject using a laser capture microscope (Arcturus Engineering, Mountain View, CA, USA). 100 mg of renal tissues was iso-

2.11. Cell Culture and Treatment. Human embryo kidney cells (HEK293T) were purchased from Cell Bank, Chinese Academy of Sciences (Shanghai, China). HEK293T cells were cultured in DMEM with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO₂. Cordyceps was the main component of Cordyceps militaris, and the dose was referred to previous reported indicated concen-

2.12. shRNA Constructs for TLR4 Gene Silencing. The pTZU6+ vector was transfected with pTZU6+1-shRNA-TLR4 as treatment vectors. Transfection was performed in 50%-60% confluent cells in 6-well plates using 9 μL of Lipofecta-

2.13. Transfection of HEK293T Cells. The HEK293T cells were transfected with pTZU6+1-shRNA-TLR4 as treatment groups. The HEK293T cells were transfected with pTZU6+1 as control groups. Transfection was performed in 50%-60% confluent cells in 6-well plates using 9 μL of Lipofecta-

2.14. Real Time-PCR Analysis. 200 mg of renal tissues was isolated from all patients using a noninvasive surgery [49]. Total RNA was isolated from kidney tissues or cells using TRIzol. The concentration was determined by optical density measurement at 260 nm on a spectrophotometer. The total RNA was isolated with RNA purification kit according to the manufacturer’s instruction. The purity and concentration of RNA were measured by using a UV spectrophotometer. cDNAs were synthesized from purified RNA with reverse transcription kit. The mRNA levels of TLR4, NF-xB, COX2, IL-1β, and TNF-α were measured by using the primers as follows: TLR4, sense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3'. NF-xB, sense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3'. COX2, sense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3'. IL-1β, sense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3'. TNF-α, sense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3'. β-actin, sense primer, 5'-gctttttgcatacaag-3' and antisense primer, 5'-gctttttgcatacaag-3'. Beta-actin was used as a loading control. Relative unit was measured as 2-ΔΔCt where ΔΔCt equaled the difference between the Cts of target genes. The Cts of the target gene was counted as the difference between the cycle threshold of the target gene and β-actin.

PCR was performed with initial denaturation cycle at 94°C for 2 min, followed by 45 cycles consisting of 95°C for 6 sec, annealing at 58°C for 15 sec, and extension at 72°C for 25 sec. After the steps, a melting step was performed, consisting of 94°C for 6 sec, cooling to 43°C for 25 sec, and finally an increase in temperature to 90°C at a rate of 0.1°C per second with fluorescence decline.
2.15. Protein Concentration Measurement of TLR4-/NF-κB-Related Molecules. HEK293T cells were lysed in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% Triton X-100, 10 mM EDTA, 0.1% sodium dodecyl sulfate (SDS, Cat. No. L4509, Sigma-Aldrich, St. Louis, MO, USA), 50 mM sodium fluoride (NaF, Cat. No. S7920, Sigma-Aldrich, St. Louis, MO, USA), 100 μM phosphatase inhibitor sodium orthovanadate (Cat. No. S6508, Sigma-Aldrich, St. Louis, MO, USA), and 100 μM phenylmethylsulfonyl fluoride (PMSF, Cat. No. P7626, Sigma-Aldrich). Cellular proteins were measured using TLR4, NF-κB p65, COX2, IL-1β, and TNF-α ELISA kits.

2.16. Western Blot Analysis of TLR4/NF-κB-Related Molecules in HEK293T Cells. The supernatant was separated from cell lysate via centrifugation at 12,000 × g for 15 min at 4°C. Protein samples were separated by 12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (PVDF, Millipore, Bedford, MA USA). Membranes were blocked in TBST buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 0.1% Tween-20) with 5% nonfat dry milk for 1 h. The blots were then incubated with primary antibodies anti-TLR4, NF-κB, COX2, IL-1β, TNF-α, β-actin, phospho-TLR4, and phospho-NF-κB p65 (Ser529) (Sangon, Shanghai, China) overnight at 4°C. The blots were rinsed with TBST buffer and incubated with HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (at 1:5000 dilution, Sangon, Shanghai, China). Target proteins were visualized using chemiluminescence horseradish peroxidase (Millipore, Bedford, MA USA) and analyzed by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

2.17. Statistical Analyses. All number data were compared by using χ² values, and quantitative data were compared by using two-way ANOVA to explore the interaction between two factors. The data were analyzed by using SPSS 20.0 software package (SPSS Inc., IBM, NY, USA).

3. Results

3.1. Characterization of the Extracts of Cordyceps militaris. Figure 1 showed that Cordyceps militaris was rich in cordycepin, which may be useful for controlling CKD. Five main components (mg/100 g, carnine 10, HEA 15, adenosine 18, uridine 20, and cordycepin 37) were isolated from Cordyceps militaris. The above components were further identified by ESI MASS spectrometry produced mass spectra with [M + H]+. Figure 2 showed that the predicted masses of urine (Figure 2(a)), HEA (Figure 2(b)), cordycepin (Figure 2(c)), adenosine (Figure 2(d)), and carnine (Figure 2(e)) were 224, 311, 251, 267, and 161 Da, respectively.

3.2. Baseline Characters of Participants. For baseline characters of participants, there was no statistically significant difference for sex distribution, body mass index (BMI), age, diastolic blood pressure (DBP), and systolic blood pressure (SBP) (Table 1 P < 0.05).

3.3. Cordyceps militaris Improved Inflammatory Status and Thickness of Glomerular Filtration Membrane of Renal Tissues. PAS stain showed that inflammatory situation was obvious in renal biopsy specimens from the patients in the CG group (Figure 3(a)) when compared with the COG group (Figure 3(b)). On the other hand, H&E stain showed that the renal biopsy specimens were with thickening of glomerular filtration membrane as the arrow showed in the CG group (Figure 3(c)) while renal biopsy specimens were with normal glomerular filtration membrane in the COG group (Figure 3(d)).

3.4. Cordyceps militaris Reduced the Biomarker Levels of CKD. Before therapy, there was no statistically significant difference in the levels of urinary protein, BUN, and
creatinine between the COG and CG groups ($P > 0.05$).

After the three-month treatment, the levels of urinary protein, BUN, and creatinine were significantly reduced by 36.7%±8.6%, 12.5%±3.2%, and 18.3%±6.6%, respectively, in the COG group when compared with the CG group (Table 2, $P < 0.05$). The results suggested that *Cordyceps militaris* improved kidney function and controlled the blood levels of urinary protein, BUN, and creatinine.

3.5. *Cordyceps militaris* Improved the Chemical Indices of CKD Patients. Before therapy, there was no statistically significant difference for lipid profile (serum TG, TC, LDL-C, and HDL-C) between the COG and CG groups ($p > 0.05$). After the three-month treatment, the serum levels of TG, TC, and LDL-C were significantly reduced by 12.8%±3.6%, 15.7%±4.1%, and 16.5%±4.4%, while HDL-C was significantly increased by 10.1%±1.4% in the COG group when compared with the CG group, respectively (Table 3, $P < 0.05$). The results suggest that *Cordyceps militaris* improved the lipid profile of CKD patients by affecting serum levels of TG, TC, LDL-C, and HDL-C.
Table 1: Baseline characters of chronic kidney disease.

| Parameters         | CG         | COG        | Chi-square statistic/t-value | P values |
|--------------------|------------|------------|-----------------------------|----------|
| Cases (male/female)| 49 (28/21) | 49 (29/20) |                            | 0.042    | 0.837 |
| Age (years)        | 46.2 ± 13.6| 44.7 ± 11.2| -0.575                      | 0.288    |
| SBP (mmHg)         | 126.2 ± 11.5| 130.5 ± 12.7| -1.674                      | 0.076    |
| DBP (mmHg)         | 87.2 ± 7.1 | 86.5 ± 7.8 | -1.096                      | 0.156    |
| BMI                | 25.9 ± 1.7 | 24.5 ± 1.4 | -1.543                      | 0.094    |
| TC (mmol/L)        | 5.5 ± 0.6 | 5.7 ± 0.8  | -0.698                      | 0.214    |
| TG (mmol/L)        | 2.2 ± 0.8 | 2.3 ± 0.9  | -2.153                      | 0.106    |
| LDL-C (mmol/L)     | 2.0 ± 0.6 | 2.3 ± 0.8  | -1.865                      | 0.181    |
| HDL-C (mmol/L)     | 1.8 ± 0.4 | 1.6 ± 0.3  | -2.689                      | 0.078    |
| Cr (μmol/L)        | 85.2 ± 13.8| 87.0 ± 14.1| -1.214                      | 0.134    |
| HbA1C (%)          | 8.4 ± 0.7 | 8.7 ± 0.8  | -0.664                      | 0.241    |
| eGFR (mL/min)      | 32.9 ± 7.4| 33.1 ± 8.1 | -0.072                      | 0.345    |

Note: chi-square test and t-test were used to compare the significant difference between COG and CG groups. BMI: body mass index; eGFR: estimated glomerular filtration rate. All data were presented as mean value ± SD (standard deviation). There were statistically significant differences between the two groups if P < 0.05.

Figure 3: Histology analysis of renal biopsy specimens. (a) PAS stain of renal biopsy specimens with some neutrophils in the CG group. Red arrow: glomerular and renal interstitial fusion after rupture of basement membrane of Bauman’s sac; green arrow: inflammatory cell infiltration of renal interstitial tissue. (b) PAS stain of renal biopsy specimens in the COG group. (c) H&E stain of renal biopsy specimens with thickening of glomerular filtration membrane as the arrow showed in the CG group. Red arrow: glomerular capillary stenosis, occlusion; black arrow: glomerular basement membrane thickening. (d) H&E stain of renal biopsy specimens with normal glomerular filtration membrane in the COG group.

Table 2: The effects of Cordyceps militaris on the kidney functions of CKD patients.

| Parameters         | Before therapy | CG After therapy | P value | COG After therapy | P value |
|--------------------|----------------|-----------------|---------|------------------|---------|
| Urinal protein (g/24 h) | 2.77 ± 0.85 | 2.65 ± 0.73 | 0.65 | 2.83 ± 0.69 | 1.36 ± 0.45 | 0.001 |
| BUN (mmol/L)       | 9.67 ± 2.62 | 9.72 ± 2.38 | 0.78 | 9.38 ± 2.10 | 8.84 ± 2.36 | 0.026 |
| Creatinine (mmol/L)| 85.2 ± 13.8 | 81.6 ± 12.7 | 0.33 | 87.0 ± 14.1 | 59.63 ± 10.18 | 0.001 |

Note: BUN: blood urea nitrogen. All data were presented as mean value ± SD. There were statistically significant differences between the two groups if P < 0.05.
Before therapy, there was no statistically significant difference for Cys-C, MPO, NO, SOD, and MDA (Table 4, P > 0.05). After the three-month treatment, the serum levels of Cys-C, MPO, and MDA were significantly reduced by 14.0%±3.8%, 26.9%±12.3%, and 19.7%±7.9% while NO and SOD were significantly increased by 12.5%±2.9% and 25.3%±13.4% in the COG group when compared with the CG group, respectively (Table 4, P < 0.05). The results suggest that *Cordyceps militaris* improved redox properties of CKD patients by affecting serum levels of Cys-C, MPO, NO, SOD, and MDA.

### Table 3: The effects of *Cordyceps militaris* on the lipid profile of CKD patients.

| Parameters | Before therapy | CG After therapy | P value | COG After therapy | P value |
|------------|----------------|-----------------|---------|------------------|---------|
| TC (mmol/L) | 5.5 ± 0.6      | 5.9 ± 0.7       | 0.09    | 5.7 ± 0.8        | 4.9 ± 0.5 | 0.04 |
| TG (mmol/L) | 2.2 ± 0.8      | 2.4 ± 0.9       | 0.08    | 2.3 ± 0.9        | 2.0 ± 0.6 | 0.02 |
| LDL-C (mmol/L) | 2.0 ± 0.6       | 2.2 ± 0.6       | 0.12    | 2.3 ± 0.8        | 1.7 ± 0.5 | 0.01 |
| HDL-C (mmol/L) | 1.8 ± 0.4       | 1.7 ± 0.5       | 0.29    | 1.6 ± 0.3        | 1.9 ± 0.5 | 0.02 |

Note: TC: triglycerides; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol. All data were presented as mean value ± SD. There were statistically significant differences between the two groups if P < 0.05.

### Table 4: The effects of *Cordyceps militaris* on redox of CKD patients.

| Parameters | Before therapy | CG After therapy | P value | COG After therapy | P value |
|------------|----------------|-----------------|---------|------------------|---------|
| Cys-C (mg/L) | 1.1 ± 0.2       | 1.0 ± 0.3       | 0.07    | 1.2 ± 0.3        | 0.8 ± 0.2 | 0.01 |
| MPO (mg/L) | 25.1 ± 3.9      | 23.3 ± 4.3      | 0.13    | 24.7 ± 4.1       | 14.5 ± 3.4 | 0.01 |
| MDA (mmol/L) | 8.8 ± 2.1       | 8.1 ± 2.5       | 0.09    | 8.3 ± 2.0        | 5.4 ± 1.9 | 0.01 |
| NO (μmol/L) | 5.2 ± 1.9       | 5.8 ± 2.1       | 0.28    | 5.5 ± 2.2        | 6.8 ± 2.5 | 0.01 |
| SOD (U/L) | 521.4 ± 132.8   | 507.2 ± 131.2   | 0.31    | 509.7 ± 126.4    | 698.4 ± 145.6 | 0.01 |

Note: Cys-C: cystatin-C; MPO: myeloperoxidase; MDA: malondialdehyde; NO: nitric oxide; SOD: superoxide dismutase. All data were presented as mean value ± SD. There were statistically significant differences between the two groups if P < 0.05.

3.6. *Cordyceps militaris* Improved eGFR of CKD Patients.

Before *Cordyceps militaris* treatment, there was no significant difference for the eGFR of CKD patients between the COG and CG groups (P > 0.05). After the three-month therapy, the values of eGFR (28.3 ± 5.2) were reduced significantly when compared with the CG group (32.8 ± 9.2, P < 0.05).

3.7. *Cordyceps militaris* Reduced Relative mRNA Levels of TLR4/NF-κB in CKD Patients. In order to assess the properties of *Cordyceps militaris* on CKD patients, we first assessed the effects of each component on HEK293T cells. Real-time qRT-PCR showed that both *Cordyceps militaris* extracts and cordycepin had strong inhibitory effect for reducing the mRNA levels of TLR4 (Figure 5(a)), NF-κB p65 (Figure 5(b)), COX2 (Figure 5(c)), IL-1β (Figure 5(d)), and TNF-α (Figure 5(e)). Comparatively, carcine and adenosine could reduce relative mRNA levels of TLR4, NF-κB p65, COX2, IL-1β, and TNF-α too, but all of the changes were less than caused by cordycepin and the extracts (Figure 5, P < 0.05). Furthermore, cordycepin could not reduce these molecules anymore when TLR4 was silenced (Figure 5).

3.8. *Cordyceps militaris* Reduced the Concentrations of TLR4/NF-κB Signaling Pathway in Cells. In Figures 5 and 6, relative levels of mRNA and protein of TLR4 were lowest in TLR4-knockdown cells when compared with other groups, suggesting that the TLR4 gene was silenced. Similarly, ELISA analysis showed that extracts and cordycepin had strong inhibitory effect for reducing protein levels of TLR4 (Figure 6(a)), NF-κB p65 (Figure 6(b)), COX2 (Figure 6(c)), IL-1β (Figure 6(d)), and TNF-α (Figure 6(e)). Comparatively, carcine and adenosine could reduce protein levels of TLR4, NF-κB p65, COX2, IL-1β, and TNF-α too, but all of the changes were less than caused by cordycepin and the extracts (Figure 6, P < 0.05). Furthermore, the extracts and cordycepin could not reduce the levels anymore when...
Relative mRNA level of TLR4

Before therapy | After therapy

(a) $P < 0.01$

Relative mRNA level of NF-κB

Before therapy | After therapy

(b) $P < 0.05$

Relative mRNA level of COX2

Before therapy | After therapy

(c) $P < 0.05$

Relative mRNA level of IL-1β

Before therapy | After therapy

(d) $P < 0.05$

Figure 4: Continued.
The concentration of TLR4 (ng/mL)

The concentration of COX2 (ng/mL)

The concentration of IL-1\protect\textbeta (pg/mL)

Figure 4: Continued.
TLR4 was silenced (Figure 6). The results suggest that cordycepin may affect the NF-κB signaling pathway via TLR4.

3.11. Cordycepin Reduced the Relative Protein Levels of the Main Molecules in TLR4/NF-κB Signaling Pathway. Western blot analysis showed that extracts and cordycepin had strong inhibitory effect for reducing protein levels of p-TLR4 and TLR4 (Figures 7(a) and 7(b)), p-NF-κB and NF-κB (Figures 7(c) and 7(d)), COX2 (Figure 7(e)), IL-1β (Figure 7(f)), and TNF-α (Figure 7(g)). Comparatively, carmine could reduce these protein levels too. Furthermore, the extracts and cordycepin could not reduce the levels anymore when TLR4 was silenced (Figure 7). The results suggest that cordycepin may affect the NF-κB signaling pathway via TLR4.

4. Discussion

Moderate consumption of *Cordyceps militaris* was found to be associated with a lower incidence of kidney failure [50]. *Cordyceps militaris* reduced CKD severity and the progression of kidney failure in the present study (Figure 8). *Cordyceps militaris* increased kidney function and controlled the blood levels of urinal protein, BUN, and creatinine (Table 2, P < 0.05). According to an earlier report, the active constituents of *Cordyceps militaris* could downregulate the levels of phospho-AKT and phospho-GSK-3beta, decrease the oxidation in a urolithiasis animal model, and exert antinephritic activities [25]. *Cordyceps militaris* improved the lipid profile of CKD patients by affecting serum levels of TG, TC, LDL-C, and HDL-C (Table 3, P < 0.05). The lipid-improving results were only approved in the animal models by feeding a high-fat diet in a previous report before the present study [51]. Cordycepin may affect the serum lipid profile because it has been found to effect lipid deposition and improve lipid profiles by increasing the activity of lipoprotein lipase and hepatic lipase [51]. Meanwhile, *Cordyceps militaris* improved redox properties of CKD patients by affecting serum levels of Cys-C, MPO, NO, SOD, and MDA (Table 4, P < 0.05). The antioxidant properties of *Cordyceps militaris* were reported in the animal models with reproductive damage induced by bisphenol A by improving the SOD level and reducing the MDA level [52]. Comparatively, ascorbic acid has been well known to have strong antioxidant properties while a previous report showed that a significant protective effect of ascorbic acid was not observed and could not affect peak postoperative serum creatinine and the lowest postoperative creatinine clearance on the incidence of postoperative acute renal injury either [53].

Cordycepin is relatively abundant in *Cordyceps militaris* and has been associated with the removal of apoptotic cells by inducing autophagy [54, 55]. Autophagy is a highly evolutionally degradation process by which cytosolic materials and damaged organelles are degraded into basic components. Autophagy can get rid of some destructed materials and produce new components for cell normal cycle and stability. The association of organ autophagy and risks of kidney disease has been reported [56].

The cell culture studies were performed on healthy “untreated” cells, and the results could not be interpreted in association with biopsy results from “CKD” kidneys. However, the cell test showed that cordycepin may affect the NF-κB signaling pathway via TLR4 (Figures 6 and 7). In Figures 5–7, cordycepin reduces the mRNA expressions and concentration of IL-1β, TLR4, TNF-α, NF-kappaB,
Figure 5: The effects of different components of Cordyceps militaris extracts on relative mRNA levels of the main molecules in the TLR4/NF-κB pathway in HEK293T cells. (a) The relative mRNA level of TLR4. (b) The relative mRNA level of NF-κB. (c) The relative mRNA level of COX2. (d) The relative mRNA level of IL-1β. (e) The relative mRNA level of TNF-α. All data were presented as mean value ± SD. There were statistically significant differences if *P < 0.05 vs. the control group.
Figure 6: The effects of different components of *Cordyceps militaris* extracts on the concentrations of the main molecules in the TLR4/NF-κB pathway in HEK293T cells. (a) The concentration of TLR4. (b) The concentration of NF-κB. (c) The concentration of COX2. (d) The concentration of IL-1β. (e) The concentration of TNF-α. All data were presented as mean value ± SD. There were statistically significant differences if *P* < 0.05 vs. the control group.
Relative mRNA level of TLR4

0.5

value ± SD. There were statistically significant differences if *P < 0.05 vs. the control group.

Relative protein level of COX2. (f) Relative protein level of IL-1β

7: Western blot analysis of the relative protein levels of the main molecules in the TLR4/NF-κB signaling pathway in HEK293T cells. Lanes 1-9 stand for the extracts, cordycepin, uridine, carnine, HEA, adenosine, TLR4-, TLR4-/cordycepin, and control groups, respectively. (a) Relative protein level of p-TLR4. (b) Relative protein level of TLR4. (c) Relative protein level of p-NF-κB. (d) Relative protein level of NF-κB. (e) Relative protein level of COX2. (f) Relative protein level of IL-1β. (g) Relative protein level of TNF-α. All data were presented as mean ± SD.

and COX2 in both wild-type and TLR4-knockdown cells. We guessed that TLR4 promoted the expression of IL-1β, TNF-α, NF-kappaB, and COX2. Thus, cordycepin reduced the level of TLR4, resulting in the decrease in the expression of IL-1β, TNF-α, NF-kappaB, and COX2 in wild-type cells. Comparatively, TLR-4 knockdown also reduced the level of TLR4, also resulting in the decreased expression of IL-1β, TNF-α, NF-kappaB, and COX2 in TLR4-knockdown cells.

Normally, TLR4 mediates the NF-κB signaling pathway and is the upstream protein of NF-κB [57], and cordycepin as the main component of Cordyceps militaris can significantly inhibit lipopolysaccharide-induced TLR4 [49]. Cordycepin may affect TLR4 more directly than NF-κB. Cordycepin reduces the expression of TLR4 and will suppress the TLR4/NF-κB signaling pathway. However, the underlying mechanism responsible for cordycepin on CKD progression remains uncertain. Cordycepin could affect TLR4/NF-κB lipid and redox signaling pathway significantly. Activation of NF-κB p65 by TLR4 can promote the production of COX2, which results in the increase in the levels of cytokines IL-1β and TNF-α.

Cordyceps militaris still have an alternative therapeutics. For instance, the isolated polysaccharides (AE-PS) from Cordyceps militaris had a pyran-type polysaccharide with α- and β-configurations and exerted antioxidant and hypoglycemic functions on type 2 diabetes mellitus in an animal model [58]. Cordycepin and adenosine of Cordyceps militaris also have been reported to have protective effects on the liver disease by inhibiting proinflammatory factor and fibrosis-related factor expression [59]. Further work is highly needed to expand its application in various chronic diseases.
5. Conclusions

The present study provided the evidence that *Cordyceps militaris* negatively controlled CKD progression by regulating the TLR4/NF-κB redox signaling pathway via cordycepin. These findings provide further support for the current clinical trials aimed at assessing the effects of cordycepin administration against CKD progression.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

Authors’ Contributions

TS and WD conceived and designed the experiments and wrote the paper. GJ, JY, JL, and LZ contributed to the evaluation of the results and corrected the paper. PM contributed to the reagents and materials.

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