



# Cordycepin mitigates MPTP-induced Parkinson's disease through inhibiting TLR/NF- $\kappa$ B signaling pathway

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## ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease with movement disorder. PD is characterized by the loss of dopaminergic (DA) neurons in the substantia nigra. Cordycepin, a small molecule extracted from cordyceps sinensis, has neuroprotective, anti-inflammatory, antioxidant and anti-tumor properties. In this study, we explored its possible beneficial effects on PD. PD rat models and cell models were established via 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection and LPS treatment respectively, and cordycepin was administered. The motor functions of rats were examined, and the tyrosine hydroxylase (TH)-positive DA neurons and Iba1-positive microglia were detected by immunohistochemical and immunofluorescence staining. The expression levels of inflammatory and oxidative stress-related factors were also measured in vivo and in vitro. In addition, the TLR/NF- $\kappa$ B pathway was investigated to explore the mechanism. We found that in vivo, MPTP injection introduced motor disorders, the loss of DA neurons and the activation of TLR/NF- $\kappa$ B signaling pathway. Cordycepin treatment alleviated these MPTP-induced changes. In vitro, the results were confirmed in Lipopolysaccharide (LPS)-induced cells. Moreover, cordycepin mitigated the cytotoxic effects on PC12 cells produced by microglia. In conclusion, cordycepin alleviated PD symptoms by inhibiting TLR/NF- $\kappa$ B signaling pathway in vivo and in vitro.

## 1. Introduction

Parkinson's disease (PD) is a long-term neurodegenerative disease that happened in old people, featured with disabling dyskinesias and dopaminergic neuron loss [1]. Besides motor impairments, emotional and cognitive disorders, cardiovascular dysfunction and vision problems are observed in the later stage [2]. The primary feature of PD is dopaminergic neuron loss in the substantia nigra pars compacta [3]. Abnormal neuroinflammation and oxidative stress are considered to play principal roles in the loss of dopaminergic neurons [4,5]. Drugs with anti-inflammatory and antioxidant effects have been reported to alleviate the experimental PD symptoms [6,7].

Recently, many traditional Chinese medicines have attracted attention due to their neuron protective effects. Some of them are applied in corresponding treatments, including PD [8]. Cordycepin, extracted from a species of fungus named *Cordyceps militaris*, has anti-inflammatory, antioxidant and anti-cancer properties. Cordycepin was reported to upregulate interleukin-10 (IL-10) protein level in human peripheral blood mononuclear cells, and subsequently suppress the

release of inflammatory cytokines [9]. Cordycepin protected against NDEA-induced hepatocellular carcinomas via the Nrf2/HO-1/NF- $\kappa$ B pathway [10]. Moreover, it was shown that cordycepin exerted neuroprotective effects via mitigating oxidative damage, enhancing free radical scavenging activity and suppressing neuronal cell death [11–13]. However, whether cordycepin has effects on PD remains unknown. In this study, we aimed to investigate the role of cordycepin in PD and the possible underlying mechanisms.

In the present study, we demonstrated that cordycepin mitigated the motor disorders in PD rat models and exerted neuroprotective effects by alleviating inflammation and oxidative stress response. The modification of cordycepin on TLR/NF- $\kappa$ B signaling pathway was found to be involved in the mechanism. These findings suggested the potential application of cordycepin against PD in clinical.

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## 2. Materials and methods

### 2.1. Experimental animals

Adult male Sprague–Dawley rats weighing about 200 g (5–7 weeks) were randomly separated into four groups: control, MPTP, MPTP + Cor (L) and MPTP + Cor (H). Rats in MPTP, MPTP + Cor (L) and MPTP + Cor (H) groups were injected with MPTP (20 mg/kg, Sigma, USA) for 4 times with 2 h intervals. Thereafter, rats in MPTP + Cor (L) and MPTP + Cor (H) were administered cordycepin of 10 mg/kg and 20 mg/kg respectively [11,14] 1 h post the first MPTP injection. At the 15th day after injection, motor function tests were carried out. Then, all rats were sacrificed for the following experiments. Six individuals were used in each detection of animal experiments. The protocol was approved by the Ethic Committee of Heze Municipality Hospital.

### 2.2. Cell culture

Rat microglial cell line (BV2 cells) and rat adrenal pheochromocytoma cell line (PC12 cells) were purchased from the Cell Culture Centre at the Institute of Basic Medical Sciences (IBMS). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% Fetal Bovine Serum (FBS) and incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C.

### 2.3. Motor function tests

#### 2.3.1. Grasping test

Grasping test was performed as previously described [15]. In brief, the forelimbs of rats were suspended on the mental rod and the holding time was recorded.

#### 2.3.2. Pole-climbing test

According to previous studies [16], a foam ball about 2.0 cm in diameter was fixed on a stick, and a rat was placed on the peak of the ball. The time that a rat climbed from the peak to the bottom of the stick was recorded.

#### 2.3.3. Rotarod test

Rats were subjected to a rotarod test as previously described [17]. In brief, rats were placed on the rotating bar of the rotarod unit (DXP-2; Institute of Materia Medica, Chinese Academy of Medical Sciences) at a rotation speed of 25 rpm. The time each rat remained on the rotating bar was recorded.

### 2.4. Immunofluorescence and immunohistochemistry assays

After fixed with 4% paraformaldehyde for 24 h, the brain tissues were soaked in 0.05 M PBS containing 30% sucrose for cryoprotection. Frozen brains were cut into 25- $\mu$ m coronal sections. Anti-TH antibody (Abcam, USA) was used for immunofluorescence staining and Anti-Iba1 antibody (Abcam, USA) was used for immunohistochemical staining. Positive cells were observed by a microscope (Olympus, Tokyo, Japan), and the cell numbers of each group were recorded from six random visual fields.

### 2.5. Total RNA extraction and real-time quantitative PCR

Total RNA was isolated from cells or mid brain tissues using Trizol reagent (Invitrogen) following the manufacturer's instructions. After digested with DNase, RNA was reversely transcribed into cDNA via M-MLV Reverse Transcriptase (Promega, Madison, USA). The cDNA was used as templates for real-time quantitative PCR by SYBR green (TaKaRa, Tokyo, Japan).  $\beta$ -Actin was chosen as internal control. The sequences of primers were listed in Table 1.

**Table 1**  
Primers for Real-time PCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
TNF- $\alpha$	CGGGGTGATCGGTCCCAAG	GGAGGGCGTTGGCGCGCTGG
IL-1 $\beta$	CGCAGCAGCACATCAACAAG	TGTCTCATCTGGAAGGTC
IL-6	GAGATACAAAGAAATGATGG	ACTCCAGAAGACCAGAGGAA
$\beta$ -Actin	CTACAATGAGCTGCGTGTGG	CAGGTCCAGACGCAGGATGG

### 2.6. Measurement of reactive oxygen species (ROS)

For mid brain tissues, 20-fold volume of PBS was used to mix it. After freeze-thawed thrice and smashed by a homogenizer, the tissue was centrifuged at 1000<sup>5</sup>g for 8 min. Then, the supernatant was gathered for ROS detection via a ROS Detection Assay Kit (Sangon, Shanghai, China), according to the manufacturer's instructions. For cells, the ROS content was directly examined using the ROS Detection Assay Kit mentioned above.

### 2.7. Measurement of superoxide dismutase (SOD)

For mid brain tissues, 10-fold volume of PBS was used to mix it. After freeze-thawed thrice and smashed by a homogenizer, the tissue was centrifuged at 1000<sup>5</sup>g for 8 min. Then, the supernatant was gathered for SOD detection. For cells, RIPA lysis buffer was used to lysis the cells. After centrifuged at 1000<sup>5</sup>g for 8 min, the supernatant was gathered for SOD detection. The SOD activity in tissue or cell supernatant was examined using a SOD Activity Assay Kit (BioVision, Wuhan, China).

### 2.8. Measurement of myeloperoxidase (MPO)

For mid brain tissues, 8-fold volume of saline was used to smash it. For cells, lysis buffer was used to lysis them. After that, an MPO Activity Assay Kit (Abcam, Cambridge, UK) was used to detect the MPO activity in tissues or cells according to the manufacturer's instructions.

### 2.9. Western blot

RIPA lysis buffer (Beyotime, Shanghai, China) was used to extract the total protein from mid brain tissues or cells. Nucleus and Cytoplasm Protein Extraction Kit (Beyotime, Shanghai, China) was used to isolate the nuclear

or cytoplasmic protein. The concentrations of proteins were determined by a BCA Protein Assay Kit (CST, Shanghai, China). Then, equal amounts of proteins were resolved via SDS-PAGE and transferred onto the polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, USA). After blocked with 5% non-fat milk for 2 h, the membranes were incubated at 4 °C overnight with specific primary antibodies as follows: rabbit anti-TLR2 (1:1000; Santa Cruz, Dallas, USA), rabbit anti-TLR4 (1:1000; Santa Cruz, Dallas, USA), rabbit anti-NF- $\kappa$ B (1:500; Abcam, Cambridge, UK), mouse anti- $\beta$ -actin (1:1000; Cell Signaling Technology, Boston, USA) and rabbit anti-Histone H3 (1:500; Santa Cruz, Dallas, USA). After that, the membranes were washed with TBST thrice and incubated with the goat anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP for 1 h at room temperature. The signals were developed via Pierce ECL Western Blotting Substrate (Thermo Scientific, Shanghai, China) and analyzed with Image J software.

### 2.10. MTT assay

Cell viability was measured by MTT assay. BV2 cells were plated into 6-well plates for 4 groups: control, LPS, LPS + Cor (L) and LPS + Cor (H). Six hours later, cordycepin were added into LPS + Cor (L) CS and LPS + Cor (H) CS groups at final concentrations of 5  $\mu$ g/ml

and 10 µg/ml respectively [18,19], and 30 min later, LPS was added into LPS CS, LPS + Cor (L) CS and LPS + Cor (H) CS groups at a final concentration of 0.5 µg/ml [18]. After 24 h, the culture supernatants (CS) in different groups were collected as control CS, LPS CS, LPS + Cor (L) CS and LPS + Cor (H) CS. Then PC12 cells were plated into a 96-well plate. Six hours later, the culture supernatant was replaced with control CS, LPS CS, LPS + Cor (L) CS and LPS + Cor (H) CS. After another 24 h incubation, the viabilities of cells in different groups were detected with the MTT assay. In brief, MTT was added into medium at a final concentration of 5 mg/ml and followed with a 4-h-incubation. After then, the medium was removed and 200 µl DMSO was added into each well. A microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) was used to detect the optical density of the solution.

### 2.11. Flow cytometry

PC12 cells were plated into 6-well plates and incubated in different culture supernatants as previously described. After then, the apoptotic rate of PC12 cells was determined via Annexin V-FITC Apoptosis Detection Kit (Sigma St. Louis, USA) according to the manufacturer's instructions, and subsequently detected with a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with FlowJo software.

### 2.12. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) values. Student's two-tailed *t*-test was used to analyze the differences between two groups and one-way analysis of variance (ANOVA) was used for multiple groups. The results were considered statistically significant with *P*-value < 0.05 and very statistically significant with *P*-value < 0.01.

## 3. Results

### 3.1. Cordycepin alleviated the dyskinesia of MPTP-induced PD rats

The Pole-climbing test, grasping test and rotarod test were performed to determine the PD degree of rats with different treatments. On one hand, the results demonstrated that upon MPTP injection, the time in Pole-climbing test was increased 1.49-fold (Fig. 1A) and the time in grasping test (Fig. 1B) or rotarod test (Fig. 1C) was decreased by 67% and 61% respectively, confirming that the PD rat model was established successfully. On the other hand, the administration of cordycepin attenuated the changes (*P* < 0.01), indicating that cordycepin could alleviate the motor disorders induced by MPTP in rats.

### 3.2. Cordycepin prevented the dopaminergic neuronal loss and the microglia activation

The TH (a key enzyme of dopamine) and Iba1 (a marker of activated microglia) were detected by immunohistochemical and immunofluorescence staining. As shown in Fig. 2A and C, the amounts of TH-

positive cells were decreased by 65% in MPTP group, and were increased 1.46-fold and 2.15-fold post cordycepin administration. Moreover, the number of activated Iba1 was increased 3.3-fold after MPTP injection, and cordycepin alleviated the activation of Iba1-positive microglia by 36% and 57% (Fig. 2B, D).

### 3.3. Cordycepin mitigated inflammation and oxidative stress in MPTP-induced PD rats

The expression levels of inflammatory cytokines, Tumor Necrosis Factor α (TNF-α), Interleukin-1β (IL-1β) and Interleukin-6 (IL-6), in the mid brain tissues of MPTP-induced PD rats were increased 5.1-fold, 4.5-fold and 3.1-fold respectively, compared with that of control. The administration of cordycepin attenuated these changes (Fig. 3A–C). In addition, the expression levels of oxidative stress response related molecules, ROS, SOD and MPO, were also detected. The content of ROS and the activity of MPO were increased 1.4-fold and 3.4-fold post MPTP injection, and cordycepin treatment alleviated the increases (Fig. 3D and F). Further, cordycepin administration mitigated the reduction of SOD activity induced by MPTP (*P* < 0.01, Fig. 3E). All the results indicated that cordycepin could alleviate the inflammation and oxidative stress response in MPTP-induced PD rats.

### 3.4. Cordycepin inhibited the TLR/NF-κB pathway activation in MPTP-induced rats

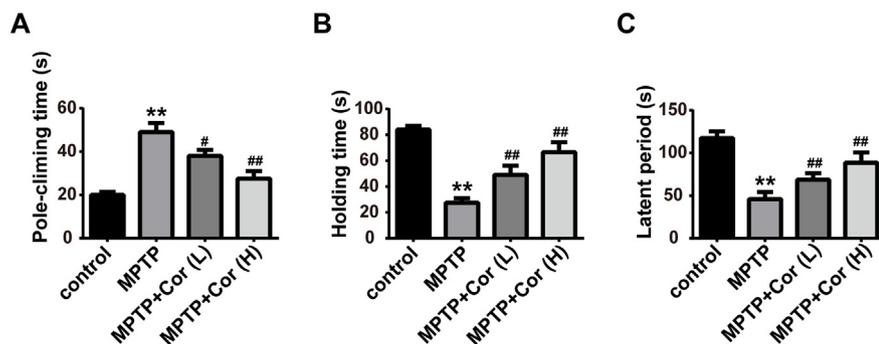
We further investigated whether the TLR/NF-κB signaling pathway, an important transduction pathway related to inflammation and oxidative response, was involved in the mechanism. The results showed that the protein levels of TLR2 and TLR4 were upregulated by 3.1-fold and 1.7-fold post MPTP injection (Fig. 4A). The nucleus accumulation of NF-κB was also enhanced 1.9-fold by MPTP (Fig. 4B–C). Treatment of cordycepin attenuated these MPTP-induced changes markedly (*P* < 0.01).

### 3.5. Cordycepin mitigated inflammation and oxidative stress in LPS-induced BV2 cells

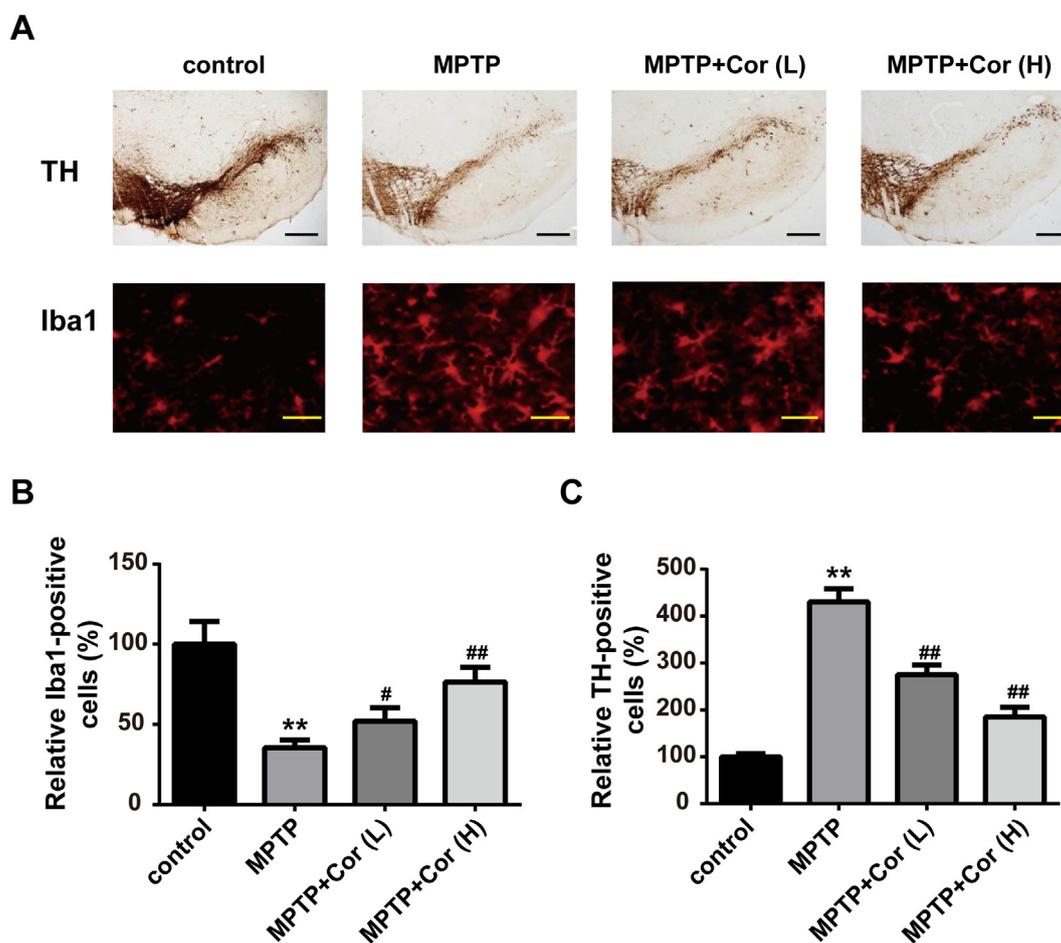
In vitro study was further performed to confirm the beneficial effects of cordycepin on PD symptoms. Similarly, LPS treatment increased the expression levels of TNF-α, IL-1β, IL-6 and ROS by 8-fold, 6.5-fold, 5.25-fold and 1.2-fold, decreased the activity of SOD by 58% and enhanced the MPO activity by 3.1-fold in BV2 cells. Administration of cordycepin suppressed these changes in inflammatory factors and oxidative stress response related molecules (*P* < 0.01, Fig. 5).

### 3.6. Cordycepin inhibited the TLR/NF-κB pathway activation in LPS-induced BV2 cells

Similar to the study in vivo, western blot results demonstrated that the protein levels of TLR2, TLR4 and nuclear NF-κB were increased 3.5-fold, 3.1-fold and 3.6-fold by LPS treatment, and the cytoplasmic NF-κB



**Fig. 1.** Cordycepin mitigated the movement disorder in MPTP-induced PD rats. (A) Pole-climbing test (A), grasping test (B) and rotarod test (C) were carried out to examine the motor deficits in MPTP-induced PD mice. Cor, cordycepin. \*\* < 0.01, compared with control group; # < 0.05, compared with MPTP group; ## < 0.01, compared with MPTP group.



**Fig. 2.** Cordycepin showed neuroprotective effects in MPTP-induced PD rats. (A) Immunohistochemistry of TH and immunofluorescence of Iba1 in the SNpc. Quantitative analysis of TH-positive (B) and Iba1-positive (C) cells. Black bar represented 500  $\mu\text{m}$ ; yellow bar represented 50  $\mu\text{m}$ . Cor, cordycepin. \*\* < 0.01, compared with control group; # < 0.05, compared with MPTP group; ## < 0.01, compared with MPTP group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression level was decreased by 65%. Cordycepin administration attenuated these changes in LPS-induced BV2 cells ( $P < 0.01$ , Fig. 6).

### 3.7. Cordycepin mitigated the microglia-mediated toxicity *in vitro*

In neurodegenerative diseases, microglia play key roles in inflammatory and oxidative stress response. We have demonstrated that cordycepin could mitigate the inflammation and oxidative stress response in LPS-induced BV2 cells, a murine microglial cell line (Fig. 5). Next, we investigated whether cordycepin could attenuate the microglia-mediated cytotoxicity to neurons. The culture supernatants (CS) collected from the BV2 cells that treated with nothing, LPS, LPS + Cor (L) and LPS + Cor (H) were used to incubate PC12 cells. The MTT assay revealed that the viability of PC12 cells was decreased by 44% after LPS CS incubation, and restored post the LPS + Cor (L) CS and LPS + Cor (H) CS incubation by 30% and 47% respectively (Fig. 7A). Moreover, cordycepin decreased the apoptotic rate of PC12 cells induced by LPS CS incubation by 48% (Fig. 7B–C). All the results indicated that cordycepin could mitigate the microglia-induced cytotoxic effect on neurons.

## 4. Discussion

PD is a long term neurodegenerative disease in the old people, characterized by disabling dyskinesias and dopaminergic neuron loss, associated with abnormal inflammation and oxidative stress response [1]. In this study, we demonstrated that cordycepin mitigated the

MPTP-induced motor deficits and dopaminergic neuron loss. In addition, we showed that the protective effects of cordycepin were mediated by inhibition of TLR/NF- $\kappa\text{B}$  signaling pathway.

Existing studies have demonstrated that abnormal neuroinflammation and oxidative stress response contributed to the dopaminergic neuron loss, leading to PD [20]. Degenerated neurons were reported to be surrounded by activated microglia, which could release an excess of inflammatory cytokines and oxidative stress response-related factors, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and ROS. In this study, we found that Iba1-positive microglia were activated in MPTP-induced PD rats and a series of inflammatory cytokines and oxidative stress response-related factors were upregulated *in vivo* and *in vitro*. These changes were ameliorated by cordycepin treatment. Moreover, cordycepin mitigated microglia-mediated cytotoxicity to PC12 cells.

Cordycepin, a traditional Chinese medicine, has anti-oxidation, anti-inflammation, anti-cancer and neuroprotective properties [9]. For instance, it was reported that cordycepin conferred substantial neuroprotection against A $\beta$ 25–35-induced neurotoxicity in the hippocampal neurons [21]. Cordycepin exerted neuronal protective effects in mouse models of intracerebral hemorrhage through inhibiting NLRP3 inflammasome activation [14]. Lei J et al. found that cordycepin could mitigate LPS-induced acute lung injury by alleviating inflammation and oxidative stress [22]. Cordycepin prevented oxidative stress-induced inhibition of osteogenesis [23]. In addition, the study performed by Peng J et al. showed cordycepin mitigated the impairments of brain by suppressing the NF- $\kappa\text{B}$  pathway activation [19]. In the present study, we pretreated with cordycepin and found that it could attenuate the

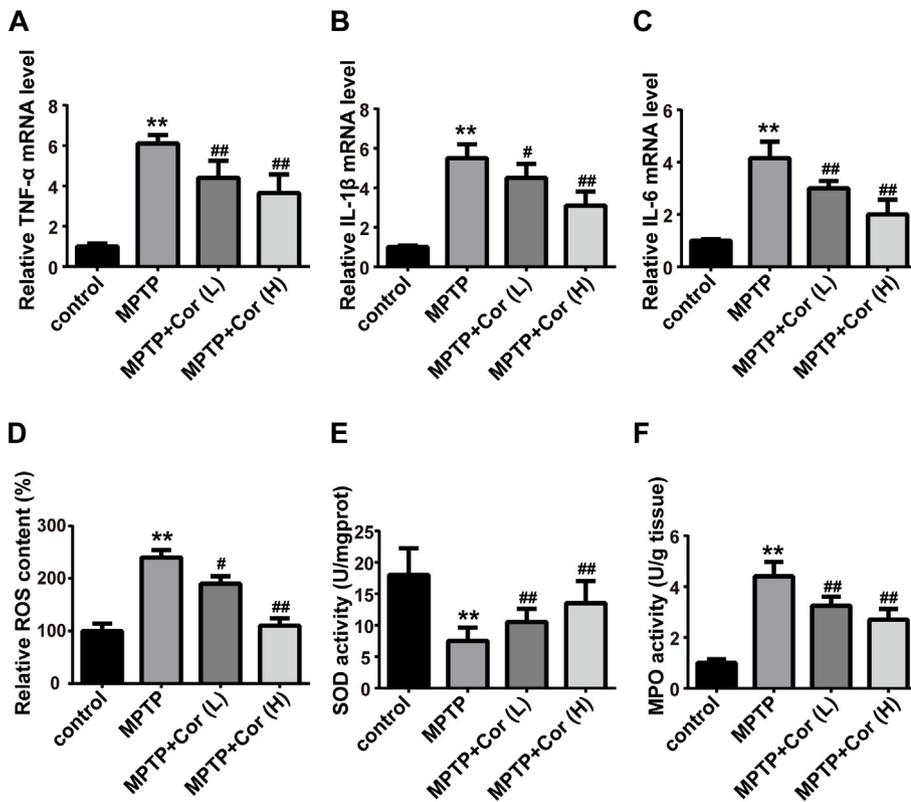


Fig. 3. Cordycepin attenuated inflammatory and oxidative stress response in mid brain tissues of MPTP-induced PD rats. The expression levels of inflammatory cytokines TNF- $\alpha$  (A), IL-1 $\beta$  (B) and IL-6 (C) were examined by qPCR. The activities of oxygen stress response related molecules ROS (D), SOD (E) and MPO (F) were detected. Cor, cordycepin. \*\* < 0.01, compared with control group; # < 0.05, compared with MPTP group; ## < 0.01, compared with MPTP group.

MPTP induced toxicity, motor impairment, inflammation and oxidative stress. However, how cordycepin may attenuate MPTP effects if given after the lesion has been administered is not known, and we will

investigate that in the further study.

NF- $\kappa$ B, a protein complex that controls DNA transcription, can modulate the inflammatory response, viral infection and improper

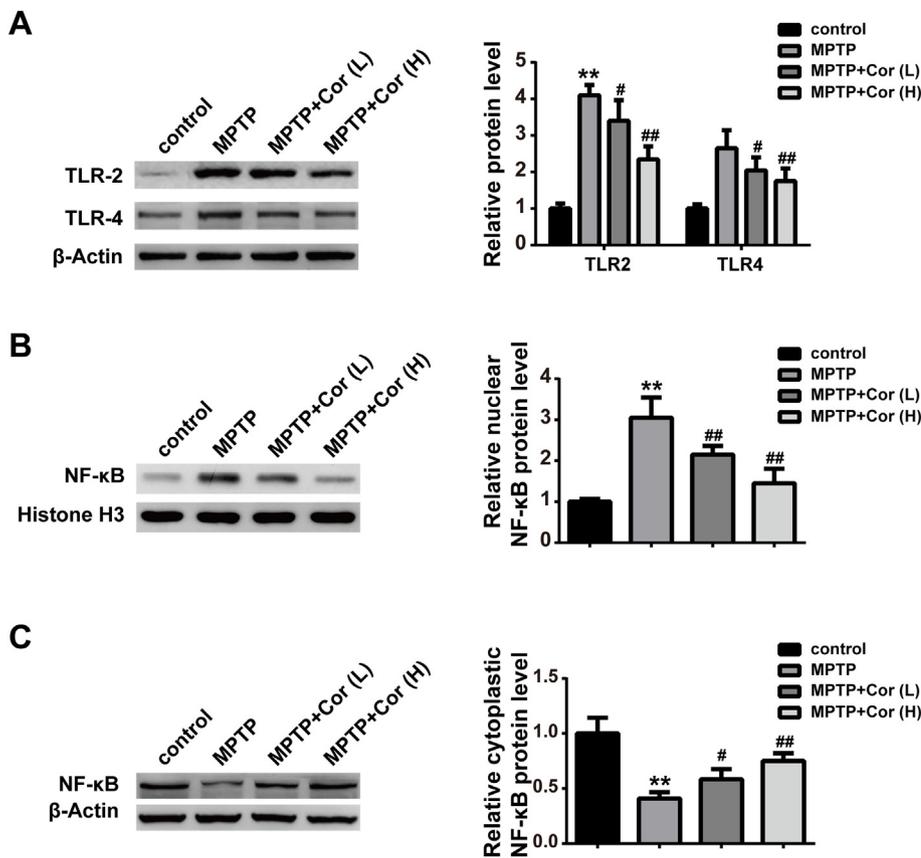


Fig. 4. Cordycepin inhibited TLR/NF- $\kappa$ B signaling pathway in mid brain tissues of MPTP-induced PD rats. The expression levels of TLR-2 (A), TLR-4 (B), nuclear NF- $\kappa$ B (C) and cytoplasmic NF- $\kappa$ B (D) were detected by western blot. Cor, cordycepin. \*\* < 0.01, compared with control group; # < 0.05, compared with MPTP group; ## < 0.01, compared with MPTP group.

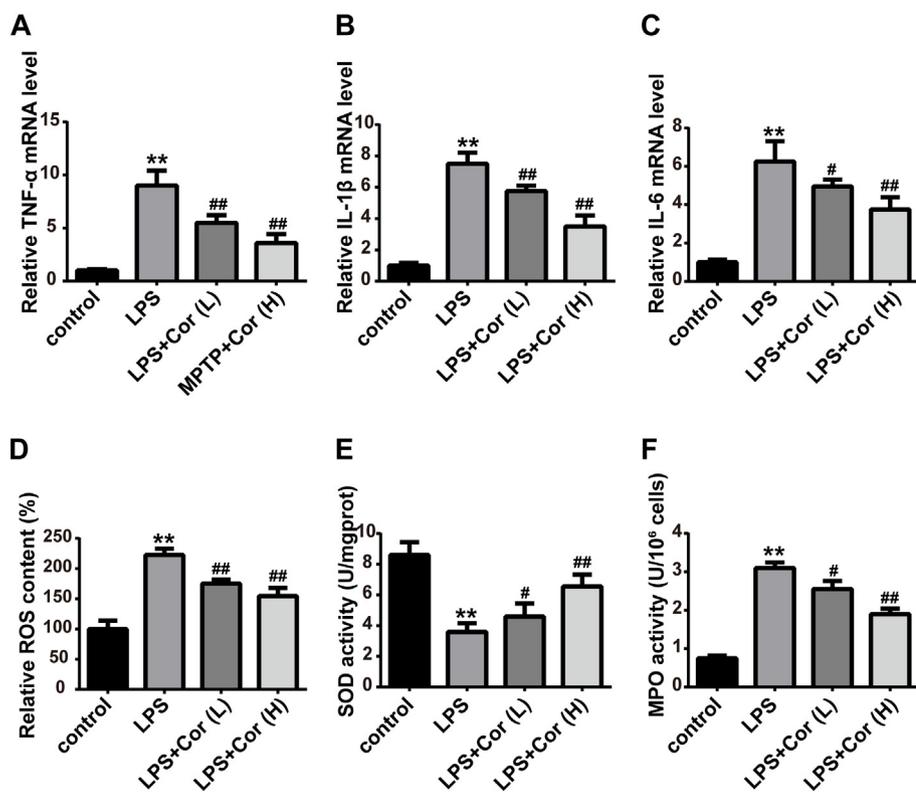


Fig. 5. Cordycepin attenuated inflammatory and oxidative stress response in LPS-induced BV2 cells. The expression levels of inflammatory cytokines TNF-α (A), IL-1β (B) and IL-6 (C) were examined by qPCR. The activities of oxygen stress response related molecules ROS (D), SOD (E) and MPO (F) were detected. Cor, cordycepin. \*\* < 0.01, compared with control group; # < 0.05, compared with LPS group; ## < 0.01, compared with LPS group.

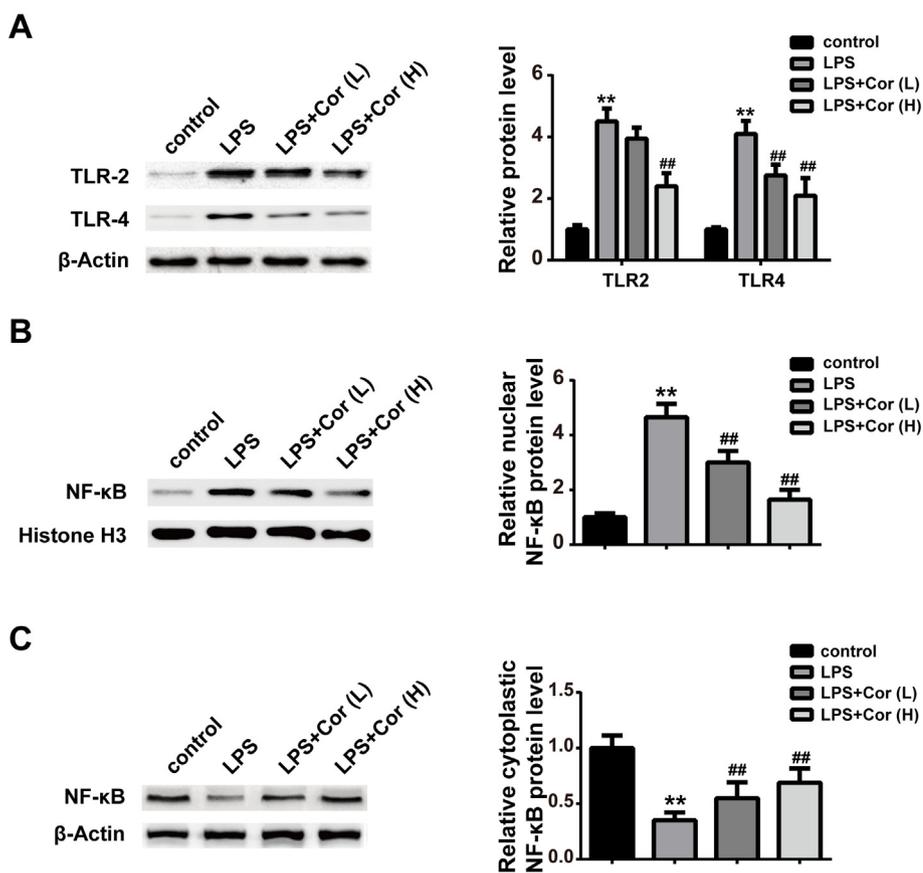
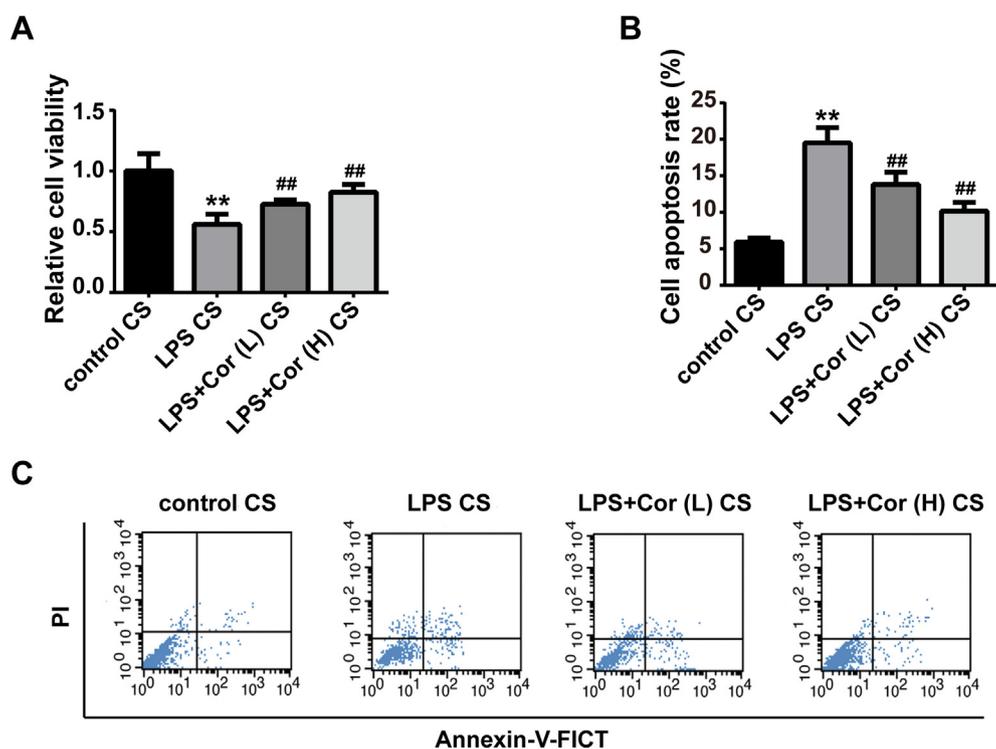


Fig. 6. Cordycepin inhibited TLR/NF-κB signaling pathway in LPS-induced BV2 cells. The expression levels of TLR-2 (A), TLR-4 (B), nuclear NF-κB (C) and cytoplasmic NF-κB (D) were detected by western blot. Cor, cordycepin. \*\* < 0.01, compared with control group; ## < 0.05, compared with LPS group; ## < 0.01, compared with LPS group.



**Fig. 7.** Cordycepin alleviated microglia-mediated cytotoxic effect on PC12 cells. PC12 cells were cultured in BV2 cell culture supernatant with/without cordycepin. (A) The viabilities of PC12 cells with different treatments were detected by MTT. (B, C) The apoptotic rates of PC12 cells with different treatments were examined by flow cytometry assay. Cor, cordycepin; CS, culture supernatant. \*\* < 0.01, compared with control CS group; ## < 0.01, compared with LPS CS group.

immune development [24–26]. In resting cells, NF- $\kappa$ B stays in the cytoplasm. Upon external stimuli, NF- $\kappa$ B is freed from I $\kappa$ B to enter the nucleus, where it can regulate the expression of target genes, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8. The activation of these genes then leads to corresponding reactions, like inflammatory response [27,28]. Hunot S et al. found that the proportion of NF- $\kappa$ B localized in nuclei was more than 70-fold in PD patients, compared with healthy ones [29]. NF- $\kappa$ B was also found activated in 6-hydroxydopamine-induced PD rats [30]. TLRs function as pattern-recognition receptors (PRRs) and participate in intracellular inflammatory responses [31]. Previous studies have demonstrated that inflammatory cytokines could induce TLRs upregulation, and further activated NF- $\kappa$ B [32,33]. In this study, we found that TLR/NF- $\kappa$ B signaling pathway was activated in MPTP-induced PD rats and LPS-induced BV2 cells. Cordycepin treatment attenuated these changes. Thus, we speculated that cordycepin mitigated PD symptoms by suppressing TLR/NF- $\kappa$ B pathway. Reverse experiments will increase the credibility of this speculation, and we will do it in our next work.

## 5. Conclusion

Taken together, we showed that cordycepin ameliorated motor disorders in MPTP-induced PD rats, and mitigated the inflammation and oxidative stress response in vivo and in vitro. These effects may be via suppressing TLR/NF- $\kappa$ B pathway. These results suggested that cordycepin might serve as a promising therapeutic candidate against PD.

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## Authors' contributions

Xiaoying Zhu designed the whole research and revised the manuscript. Chunyan Cheng conducted the whole experiments and wrote draft of the manuscript. Both authors have reviewed the draft and approved the final manuscript before submission.

## Conflict of interest

The authors declare there are no conflicts of interests.

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