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Cordycepin (3′-deoxyadenosine) promotes remyelination via suppression of neuroinflammation in a cuprizone-induced mouse model of demyelination

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ABSTRACT

Multiple sclerosis (MS) is an inflammatory demyelination disease characterized by autoimmune damage to the central nervous system. In this disease, failure of remyelination could cause persistent disability. Cordycepin, also known as 3′-deoxyadenosine, exerts anti-inflammatory, anti-oxidic, anti-apoptotic and neuroprotective effects. The cuprizone (CPZ) model has been widely used to study MS as it mimics some characteristics of demyelination disease. To determine whether cordycepin promotes remyelination and functional recovery after CPZ-induced demyelination, we administered cordycepin to the CPZ-induced demyelination mice. Cordycepin reversed CPZ-induced loss of body weight and rescued motor dysfunction in the model mice. Cordycepin effectively promoted remyelination and enhanced MBP expression in the corpus callosum. Cordycepin also inhibited the CPZ-induced increase in the number of Iba1-positive microglia, GFAP-positive astrocytes and Olig2-positive oligodendroglial precursor cells in the corpus callosum and cerebral cortex. Pro-inflammatory cytokine expression (IL-1β and IL-6) was inhibited while anti-inflammatory cytokine IL-4 and neurotrophic factor BDNF release was elevated in the corpus callosum and hippocampus after cordycepin treatment. In addition, we also found that cordycepin ameliorated CPZ-induced body weight loss, motor dysfunction, demyelination, glial cells activation and pro-inflammatory cytokine expression in the corpus callosum and hippocampus. Our results suggest that cordycepin may represent a useful therapeutic agent in demyelination-related diseases via suppression of neuroinflammation.

1. Introduction

Accumulating evidence strongly suggest that multiple sclerosis (MS) is primarily an autoimmune disease [1,2] characterized by motor dysfunction, neuroinflammation, glial cells activation, mature oligodendrocyte loss and axonal injury [3–5]. Innate immune response to demyelination has important roles in remyelination, and the key function of proinflammation factors, such as TNF-α and IL-6, is to prepare damaged tissue for reparative processes. Anti-inflammatory factors, such as IL-4 and IL-10, play a crucial role in the remyelination process to create a suitable environment for remyelination [6]. Given that, remyelination attracts biologists and clinicians alike since given the lack of remyelination-promoting therapies in the clinic that directly address this condition by promoting remyelination [7]. In the course of demyelinating pathology, this innate immune response is mediated by microglia and other immune-related cells [6].

The experimental autoimmune encephalomyelitis (EAE) model as well as cuprizone (CPZ) and virus-induced demyelination models have

Abbreviations: MBP, myelin basic protein; Iba1, ionized calcium-binding adapter molecule 1; GFAP, glial fibrillary acidic protein; Olig2, oligodendrocyte lineage transcription factor 2; IL-1β, interleukin-1 beta; IL-6, interleukin-6; BDNF, brain derived neurotrophic factor; IL-4, interleukin-4; TNF-α, tumor necrosis factor-alpha; IL-10, interleukin-10; TGF-β, transforming growth factor-β

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been widely used to study MS diseases. The CPZ model has been widely used to investigate de- and remyelination in the corpus callosum [8]. CPZ is a copper-chelating compound that selectively acts on mature oligodendrocytes and causes demyelination in several brain regions, such as the corpus callosum, hippocampus, cerebellum and cortex [9–11]. Therefore, we use the CPZ model to evaluate demyelination and remyelination processes in the corpus callosum, cerebral cortex and hippocampus. The CPZ model is characterized by early and severe oligodendrocyte damage with concomitant microglia activation followed by severe astrocyte proliferation [10,12].

Cordycepin (3′-deoxyadenosine) as the main bioactive ingredient of *Cordyceps militaris*, has been used to treat different diseases for thousands of years [13–15]. Cordycepin can also suppress lipopolysaccharides (LPS)-induced inflammation in microglial cell lines [16]. Our previous studies confirmed that cordycepin is transported from peripheral blood to the brain across the blood-brain barrier (BBB) via an adenosine transporter [17]. Recently, cordycepin has been proven to be a neuroprotective agent in central nervous system (CNS) disorders, such as cerebral ischemia/reperfusion [18], traumatic brain injury-induced impairments of blood-brain barrier integrity [15,19] and intracerebral hemorrhage [20]. Cordycepin modulates the macrophagic phenotype transition in the peripheral system, downregulates gene expression of pro-inflammatory factors (IL-1β, TNF-α, and IL-2) and upregulates anti-inflammatory factors (IL-4, IL-10, and TGF-β) peripherally in macrophages and mononuclear cells [21,22]. Published papers have also shown that cordycepin can induce apoptosis and suppress proliferation in different cancer cells [23]. Although it seems toxic to cancer cell, in fact it was benefit for cancer patients. Cordycepin plays a protective role in various pathological conditions. These pharmacological properties suggest that cordycepin may be a useful agent to treat MS during remyelination. However, the effect of cordycepin on remyelination remains unclear. The present study aimed to determine whether cordycepin promotes remyelination in CPZ-induced demyelination mice model. As such, we investigated the effects of cordycepin on motor dysfunction, myelin repair, glial cell activation, and pro-inflammatory or anti-inflammatory cytokine variation.

2. Materials and methods

2.1. Animals and drugs

Thirty-six of male C57BL/6 mice (7–8 weeks old, from Vital River, Beijing, China) were housed in groups of 4 in clear plastic cages with free access to food and water in a room with constant temperature and humidity. The animals were maintained on a 12-h/12-h light/dark cycle. The mice were acclimated to the housing conditions for one week before experiments. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (ISBN: 0-309-05377-3) and approved by the Institutional Animal Care and Use Committee at School of Medicine, Yunnan University (IACUC: MS201605).

After acclimation for 1 week, the mice were randomly assigned into three groups, including control group (CON, N = 12), CPZ only group (CPZ, N = 12), and CPZ plus cordycepin group (CPZ + C, N = 12) based on body weight. CPZ group mice were fed standard rodent chow containing 0.2% CPZ (Sigma-Aldrich, Cat#: 14690) for 5 weeks to induce acute demyelination [24–26]. Control group mice were fed standard rodent chow without CPZ. Mixed chows were produced by Beijing Keao Xieli Feed Co., Ltd. All mice were changed to normal standard chow before cordycepin or vehicle treatment (Fig. 1). During remyelination, the mice were administered vehicle or cordycepin intraperitoneally, and the volume used for intraperitoneal treatment was 0.1 ml/10 g. Cordycepin (MW: 251 g/mol), was purchased from Shanghai Yuanye Biology Co. (Cat. No.: 73-03-0, Lot No.: N12M7W14489) and dissolved in 0.9% saline (Lot: A16030752, Kunming Nanjiang Pharmaceutical Co., Ltd.). Our previous study demonstrated that 12.5 mg/kg cordycepin provided a better protective effect on the CNS [17]. In this study, cordycepin was administered at a dose of 12.5 mg/kg by intraperitoneal injection once daily for 7 days during remyelination.

2.2. Rotarod test

We used an accelerating rotarod treadmill for mice (Model No.: YLS−4C, Jinan Yi Yan Science and Technology Development Co., Ltd.) to measure motor balance and coordination after 7 days of cordycepin or vehicle treatment; Longer times were equated with better coordination and balance. The diameter and the length of rod were 30 and 60 mm respectively, the surface of the rotating rod was covered with rubber. All mice were given a practice at the beginning of the study at 10 rpm for 5 min for 3 days. After the training, all mice received a test at 10 rpm at 3 min. Mice that finished the test were adapted to study. All mice were tested on the rod at 30 rpm for 3 min at the end of the study. The time each mouse was able to stay on the rod (locomotion time) was recorded by a trip switch. The time required for a mouse to fall off from rotating drum to the floor was recorded, and the maximum time was 180 s.

2.3. Histology

For histological analysis, the mice were deeply anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg) at 0.01 ml/g body weight (bw). Then, the mice were transcardially perfused with 40 ml ice-cold 0.1 M Na_2HPO_4/NaH_2PO_4 (0.1 M PB) buffer followed with 4% paraformaldehyde in 0.1 M PB buffer. Brains were removed after perfusion and post fixed in the same fixative at 4 °C overnight. The brains were transferred into different concentrations (10%, 20% and 30%) of sucrose in 0.1 M PB for dehydration. Then, the brain tissues were embedded in Tissue-Tec O.C.T. compound after dehydration for sectioning. Coronal brain sections (20 μm) were cut in a cryostat (LEICA CM1950, Germany) and mounted on gelatin-coated glass slides. After the sections were baked in a constant 50 °C dryer for 1 h, the slides were transferred into an −80 °C freezer and stored until use.

2.4. Luxol fast blue (LFB) staining

For LFB staining, the sections were removed from −80 °C freezer and allowed to stand at room temperature for 15 min. Then, the sections were rinsed with PBS for 3 min, immersed in 95% ethanol for 5 min at room temperature, and transferred into 0.1% LFB solution (containing 0.15 g Solvent Blue 38, 150 ml 95% ethanol and 0.5 ml glacial acetic acid) (Solvent Blue 38, Cat: 1328-51-4, Lot: MKCC9169, Sigma) for 30 min in an oven at 60 °C. Sections were then differentiated using a 0.5% lithium carbonate and 70% ethanol solution. Finally, sections were dehydrated and mounted with cover-slips.

2.5. Immunofluorescent staining

For immunofluorescence assay, the sections were taken out from −80 °C freezer and allowed to stand at room temperature for 15 min. Then, the sections were rinsed thrice with PBST for 5 min. Sections were blocked with 1% BSA in PBST for 1 h and then incubated with primary anti-MBP (MBP, Cat No.: ab40390, Lot: GR3197159-1, 1:200, Abcam), Iba1 (Cat No.: ab5076, Lot: GR237928-2, 1:100, Abcam), GFAP (Cat No.: 16825-1-AP, 1:200, ProteinTech) or Olig2 (ab109186, 1:200, Abcam) antibodies at 4 °C overnight. Then, the sections were rinsed thrice with PBST on a shaker at approximately 60 rpm for 10 min. Appropriate secondary antibodies labeled with Alexa Fluor488 (Cat No.: 805-545-180, Lot:139171, Jackson) or Alexa Fluor 594 (Cat No.: 711-585-152, Lot: 136429, Jackson) were prepared in PBST containing 5% BSA. The sections were transferred into second antibody solution for 1 h at 4 °C and rinsed with PBST thrice. Finally, cell nuclei

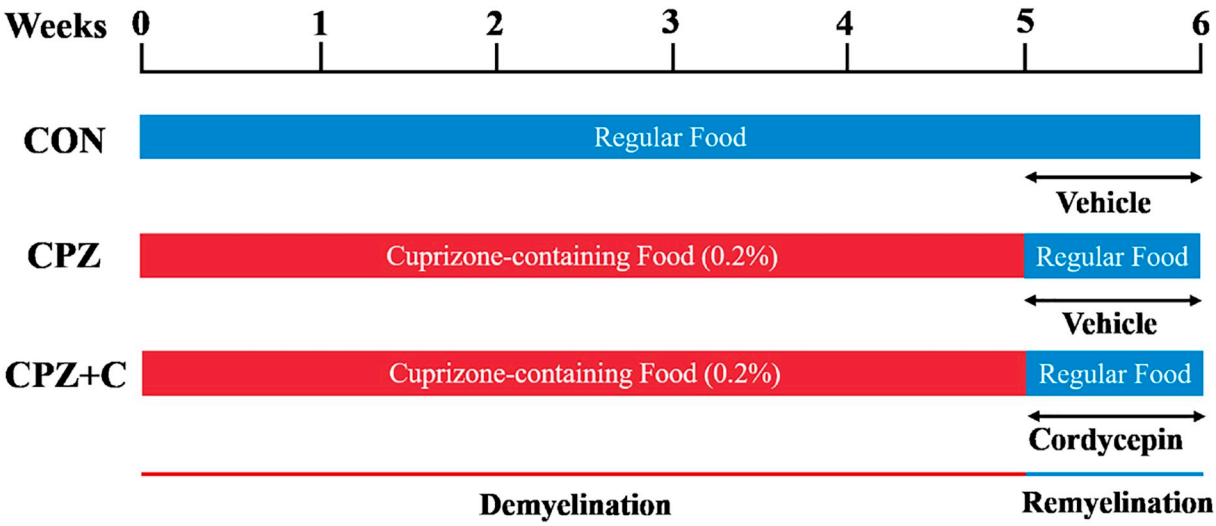

Fig. 1. Timeline of experimental procedures. Male C57BL/6 mice were fed with chow either supplemented with or without 0.2% CPZ for 5 consecutive weeks. After 5 weeks, all mice were changed to normal chow for 1 week. Cordycepin or Vehicle was administrated to mice per day during this week. All mice were performed for behavioral test 24 h after last injection, then executed for western bot or pathological study.

were stained with 4′,6-diamidino-2-phenylindole (DAPI) solution. After being mounted with mounting media, the sections were transferred into a dark box for storage until photographs were obtained.

2.6. Image analysis

Three sections from each mouse (N = 4–5) were photographed and digitized using a video camera mounted on a Leica microscope (Leica DM2500, Germany). Pictures were further processed using Image-Pro Plus software (Media Cybernetics, Rockville, USA) and Adobe Photoshop CS5 (Adobe Systems Software, Ireland). Data were presented by mean value of integrated optical density (IOD) or related positive cell numbers in a total area of 1000 μm × 1000 μm field.

2.7. Western blot

For Western blotting (WB) analysis, mice were sacrificed by cervical vertebrae dislocation. Then, corpus callosum and hippocampus tissues were harvested on ice. Briefly, gross coronal cuts were sectioned at approximately Bregma 0.25 mm and 1.25 mm. Sagittal cuts were made through the cingulum, medial to each lateral ventricle, followed by cuts above and below the corpus callosum to remove most of the cortex and fornix [25]. Brain tissues were transferred into an −80 °C freezer and stored until use. The tissues were lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer (Cat No.: P0013B, Beyotime) plus a Protease Inhibitor Cocktail Tablet (Cat No.: 04 693 132 001, Lot: 24348600, Roche, Germany) in a tissue grinder (Wheaton, Millville, USA). Protein concentrations were determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, USA). Equal amounts of proteins (10 μg) were subjected to 10–12.5% SDS-PAGE gel electrophoresis and transferred to 0.22-μM polyvinylidene difluoride (PVDF) membranes (Cat No.: ISEQ00010, Lot: R55A8398C, Merck Millipore Ltd). Antibodies against IL-1β (Goat pAb, Cat No.: AF-401-NA, 1:1000, R&D), IL-6 (Rabbit pAb, Cat No.: ab208113, 1:1000, Abcam), BDNF (Rabbit mAb, Cat No.: ab108319, 1:2000, Abcam), and IL-4 (Rabbit pAb, Cat No.: PA5-25165, 1:1000, Thermo Fisher) were used as primary antibodies. Secondary antibodies, including Donkey anti-Goat IgG (H + L) HRP (Cat No.: A15999, 1:10000, Invitrogen) and Goat anti-Rabbit IgG (H + L) HRP (Cat No.: S0001, 1:10000, Affinity). The anti-GAPDH antibody (Rabbit pAb, Cat No.: PA1-987-HRP, 1:5000, Thermo Fisher) was applied for loading calibration. Immunoreactive bands were visualized using the ECL detection system (Millipore, Billerica, USA). The images were acquired by the chemiluminescent imaging system (Amersham Imager 600, GE) and quantified using Image Pro Plus version 6.0 software (Media Cybernetics, Rockville, USA). The relative integrated optical density for IL-1β, IL-6, BDNF and IL-4 was normalized to that of GAPDH in the same sample.

2.8. Statistical analysis

All data were analyzed with one-way ANOVA followed by Turkey post hoc test. All data were analyzed using Graph Pad Prism Ver. 5.0 (Graph Pad Software, Inc., San Diego, CA) and expressed as the mean ± SEM. Any experimental data value greater than mean plus 2 × standard deviations (SDs) from a group was considered an outlier and was not considered in the analysis. *P*-values < 0.05 were considered statistically significant. Figures were generated by GraphPad Prism version 5 software.

3. Results

3.1. Cordycepin ameliorated CPZ-induced motor dysfunction

For this study, mice were fed chow with or without 0.2% CPZ for 5 weeks and then 12.5 mg/kg cordycepin or the vehicle alone for 1 week (Fig. 1). We established demyelination animal models using CPZ-treated mice. CPZ-exposed mice exhibited reduced food intake (Fig. 2A). To investigate the effect of cordycepin on remyelination in CPZ-treated mice, we assessed locomotor coordination using a rotarod apparatus. The mice developed a progressive disease that manifested by motor dysfunction, which was revealed by a reduction in the locomotion time by 51.1 s in the rotarod test and weight loss. Compared with the control group, CPZ-exposed mice exhibited a decline in locomotion time, which was rescued by cordycepin (Fig. 2C). During remyelination, the cordycepin treatment group showed a trend to improve CPZ-induced body weight loss; however, the result did not reach statistical significance (Fig. 2B).

3.2. Cordycepin rescued CPZ-induced demyelination

To investigate the effects of cordycepin on remyelination, the myelin content was quantified in the corpus callosum by LFB staining (Fig. 3A) under various conditions. The level of coronal sectioning of the brain was well controlled approximately at the optic chiasm.

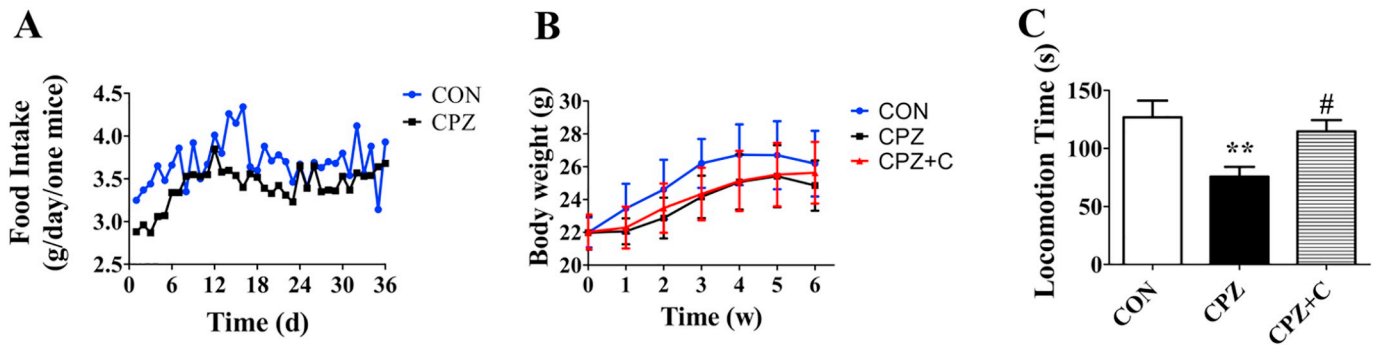

Fig. 2. Food intake, body weight and motor performance in demyelination animal models after cordycepin treatment.
(A) Food intake was monitored daily after 5 weeks of CPZ exposure. Data are presented as average food consumption per mouse per day. (B) Body weight was measured weekly after 5 weeks of CPZ exposure and during the week of cordycepin treatment. (C) Motor performance was evaluated on the rotarod after 1 week of consecutive cordycepin treatment. All data were presented as Mean ± SEM, N = 9–11 per group. Statistical analysis was performed using one-way ANOVA followed by post hoc Turkey test ($^{**}P < 0.01$ vs. CON, $^{\#}P < 0.05$ vs. CPZ).

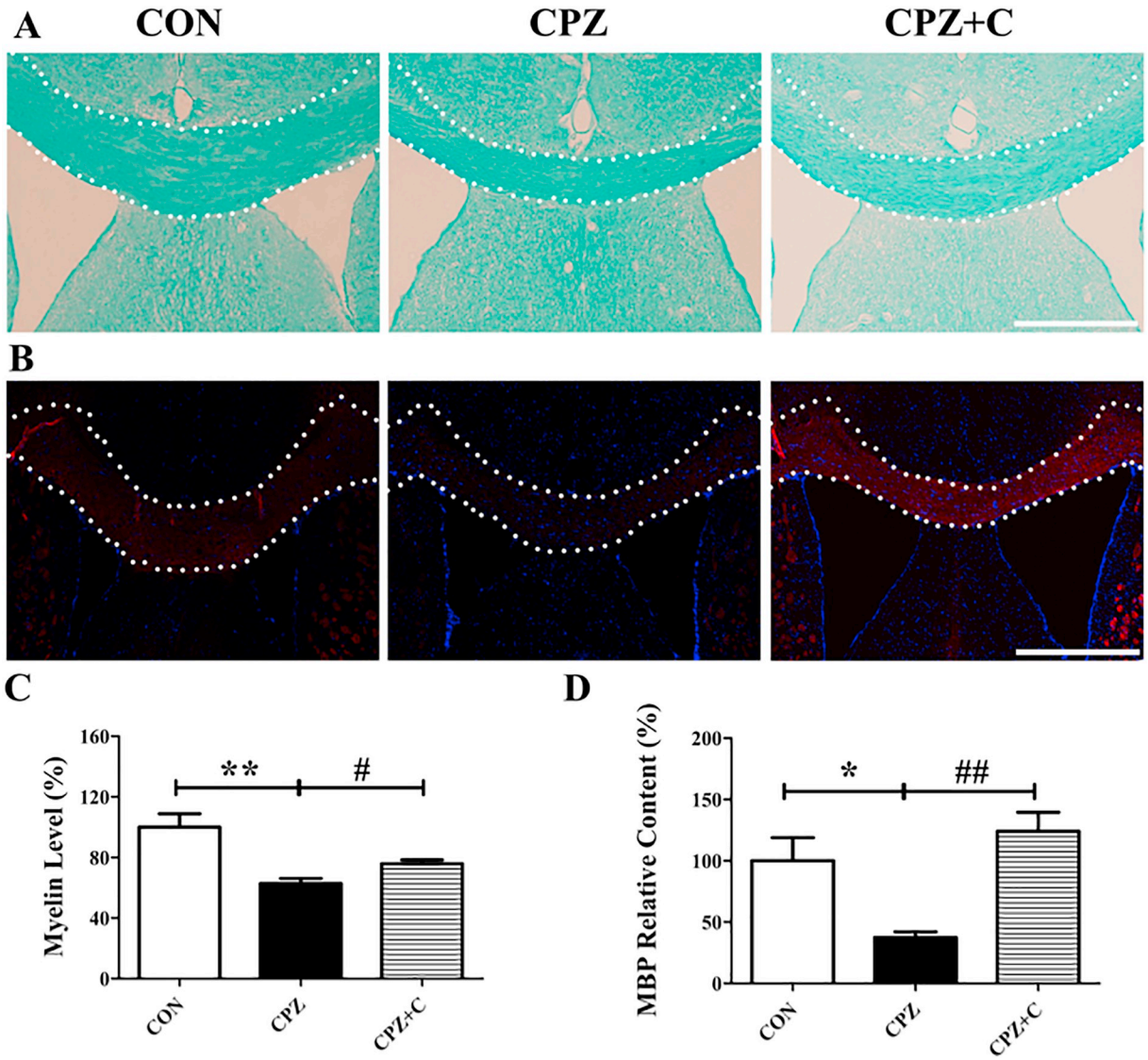

Fig. 3. LFB staining and MBP immunofluorescence staining of myelin.
Representative photographs of coronal brain sections showed that cordycepin ameliorated CPZ-induced demyelination at the corpus callosum during the re-myelination phase. (A) LFB staining of the corpus callosum in the CON, CPZ and CPZ + C groups. (B) MBP immunofluorescence staining of the corpus callosum in the CON, CPZ and CPZ + C groups. (C) Myelin densities in the corpus callosum were determined as a percentage of the control value using the Image Pro Plus analysis program. (D) Relative MBP expression levels were quantified using the Image Pro Plus analysis program. Scale bar equals 500 μM. Data are presented as Mean ± SEM. N = 4–5. ($^{**}P < 0.01$, $^{*}P < 0.05$ vs. CON; $^{\#\#}P < 0.01$, $^{\#}P < 0.05$ vs. CPZ).

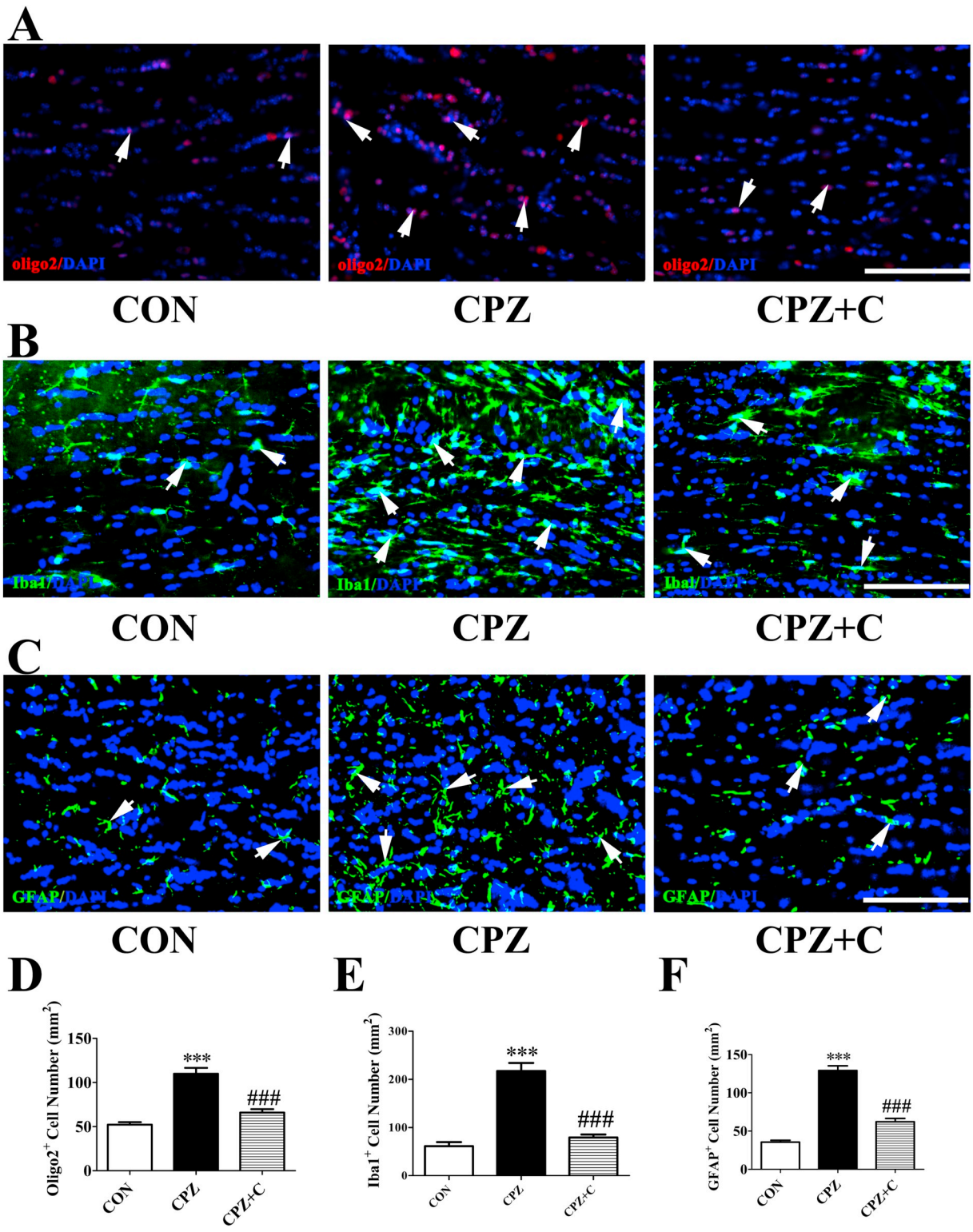

Fig. 4. Immunofluorescence staining of the markers for oligodendrocyte precursor cells, microglia and astrocytes in the corpus callosum brain region in CPZ-exposed mice with or without cordycepin treatment.

After establishment of demyelination animal models with CPZ and remyelination with cordycepin treatment, immunofluorescence staining with anti-Olig2 (oligodendrocyte precursor cell marker, A), anti-Iba1 (microglial marker, B) and anti-GFAP (astrocyte marker, C) antibodies was performed. The number of cells positive for Olig2 (D), Iba1 (E), and GFAP (F) was determined using the Image Pro Plus analysis program in an average area of 1 mm^2, and the center and lateral regions of the corpus callosum were included. Data were presented as Mean ± SEM. Scale bar equals 200 μM. N = 4–5. ($^{***}P < 0.001$ vs. CON; $^{\#\#\#}P < 0.001$ vs. CPZ). Statistical analysis was performed using one-way ANOVA followed by post hoc Turkey tests.

Compared with the control group, CPZ-exposed mice showed a significant decrease in myelin content measured by integrated optical density with Image Pro Plus software, and this decrease was statistically improved after cordycepin treatment (Fig. 3A and C). To further confirm the LFB staining result, MBP immunofluorescence staining (Fig. 3B) was performed in the corpus callosum to evaluate myelin recovery in CPZ-exposed mice with or without cordycepin treatment. We found that the MBP relative content showed a significant decrease in CPZ-exposed mice as measured by the MBP fluorescent intensity, which was statistically rescued after cordycepin treatment (Fig. 3B and D).

3.3. Cordycepin attenuated CPZ-induced activation of oligodendrocyte precursor cells, microglia and astrocytes in the corpus callosum

To investigate the cell-specific effect of cordycepin on CPZ-induced demyelination in mice, we performed immunostaining in the corpus callosum brain region using microglial marker (Iba1, Fig. 4A), astrocyte marker (GFAP, Fig. 4B) and oligodendrocytes precursor cell marker (Olig2, Fig. 4C) antibodies. All photos were captured at a constant condition based on exposure time, saturation, gain and gamma value. These images were used to calculate the integrated optical density or related number of positive cells in a relatively impartial manner. Compared with the control group, mice exposed to CPZ showed significant $Olig2^+$, $Iba1^+$ and $GFAP^+$ cell activation. However, the activation of $Olig2^+$, $Iba1^+$ and $GFAP^+$ cells was significantly attenuated after cordycepin treatment during remyelination, suggesting a protective effect of cordycepin on neuronal damage (Fig. 4D–F).

3.4. Cordycepin attenuated CPZ-induced activation of microglia and astrocytes in the cerebral cortex

To investigate the cell-specific effect of cordycepin on CPZ-induced animal models, we performed immunostaining in the cerebral cortex brain region with microglial marker (Iba1, Fig. 5A), astrocyte marker (GFAP, Fig. 5B) and oligodendrocyte precursor cell marker (Olig2, Fig. 5C) antibodies. All photos were captured at a constant condition based on exposure time, saturation, gain and gamma value. The images were used to calculate the related number of positive cell and the integrated optical density in a relatively impartial manner. Compared with the control group, mice exposed to CPZ showed significant $Iba1^+$ and $GFAP^+$ cell activation and an increase in $Olig2^+$ cell numbers. However, the activation of $Iba1^+$ and $GFAP^+$ cells was significantly attenuated after cordycepin treatment during remyelination, suggesting a protective effect of cordycepin on neuronal damage (Fig. 5D–E). The increase in $Olig2^+$ cell number was also reduced after cordycepin treatment (Fig. 5F).

3.5. Cordycepin attenuated pro-inflammatory cytokines IL-1β and IL-6 and enhanced anti-inflammatory cytokines IL-4 and neurotrophic factor BDNF expression in the corpus callosum and hippocampus

Demyelination is associated with inflammation and benefits from anti-inflammatory and neurotrophic factor. To investigate the effect of cordycepin on CPZ-induced alterations of pro-inflammatory, anti-inflammatory cytokines and neurotrophic factor, we detected pro-inflammatory cytokine (IL-1β and IL-6), anti-inflammatory cytokine IL-4 and neurotrophic factor BDNF expression by Western blot analysis in the corpus callosum (Fig. 6A–D) and hippocampal brain regions (Fig. 7A–D). Compared with the control group, mice exposed to CPZ exhibited a significant increase in both IL-1β and IL-6 pro-inflammatory cytokine levels in the corpus callosum and hippocampus, and these levels were attenuated by cordycepin treatment in the remyelination phase (Fig. 6A, B, Fig. 7A, B). In contrast, the neurotrophic factor BDNF showed a remarkable decrease, while IL-4 exhibited significant enhancement in the corpus callosum (Fig. 6C). However, compared with CPZ-exposed mice, anti-inflammatory cytokines IL-4 and neurotrophic factor BDNF were remarkably increased after cordycepin treatment in the remyelination phase in the corpus callosum and hippocampus (Fig. 6C, D, Fig. 7C, D). Taken together, Cordycepin reduced IL-1β and IL-6 expression and improved IL-4 and BDNF expression in CPZ-exposed mice during remyelination in corpus callosum and hippocampus tissues.

4. Discussion

We sought to study the effects of cordycepin on remyelination after CPZ-induced demyelination and its underlying mechanisms. We found that 1) cordycepin significantly ameliorated motor dysfunction and partially reversed CPZ-induced body weight loss in the CPZ-treated animal model; 2) cordycepin effectively promoted remyelination and MBP expression; 3) cordycepin inhibited CPZ-induced increases in the number of Iba1-positive microglia, GFAP-positive astrocytes, and Olig2-positive oligodendrocytes precursor cells in the corpus callosum and cerebral cortex; 4) cordycepin prominently inhibited CPZ-induced IL-1β and IL-6 expression and promoted BDNF and IL-4 release in the corpus callosum and hippocampus.

4.1. Cordycepin significantly improved motor dysfunction in the CPZ-treated animal model

Animal models of CPZ-induced demyelination represent certain aspects of MS pathology [27], including motor dysfunction, acute demyelination, glial cell activation, and pro-inflammatory or anti-inflammatory cytokine variation. In this study, mice received a standard diet containing 0.2% CPZ, which caused remarkable body weight loss and motor abnormality, as demonstrated by rotarod test. The result was consistent with a previous study [26,28]. In fact, we tested the mice food intake daily, and we found that the mice fed a CPZ diet ate less than the mice fed a normal standard diet, which could represent one of the reasons for the body weight loss.

While a CPZ diet induced demyelination, the withdrawal of CPZ caused spontaneous remyelination after acute demyelination. In a previous study, investigators observed spontaneous remyelination in the corpus callosum after 5–6 weeks of CPZ administration [8,24]. Treatment with cordycepin significantly improved motor dysfunction during the remyelination period, indicating that cordycepin improves motor dysfunction after CPZ-induced demyelination.

4.2. Cordycepin effectively enhanced MBP expression and remyelination

Myelin sheath loss is a major hallmark of MS [29]. MBP is the major myelin protein that is distributed in the myelin sheath and seems to be integral to the compaction process that generates the closely opposed multilayered structure of mature myelin [30]. LFB and MBP staining represents common technologies used for validating the remyelination process [31]. In this study, demyelination remarkably occurred after CPZ ingestion, which was consistent with a previous study [28]. However, cordycepin treatment during the remyelination phase significantly facilitated remyelination in CPZ-induced demyelination animal models. Consistent with the behavioral data, MBP expression levels and corpus callosum remyelination significantly improved after cordycepin treatment, suggesting that cordycepin indeed enhanced remyelination of the corpus callosum.

4.3. Cordycepin inhibited CPZ-induced increases in $Olig2^+$, $Iba1^+$ and $GFAP^+$ cell numbers in the corpus callosum and cerebral cortex

Glial cells activation is a key characteristic of neuroinflammatory and neurodegenerative disease. Depending on their functional state, glial cells exert either beneficial or harmful effects on the course of neurological disease. During remyelination in the CPZ-induced

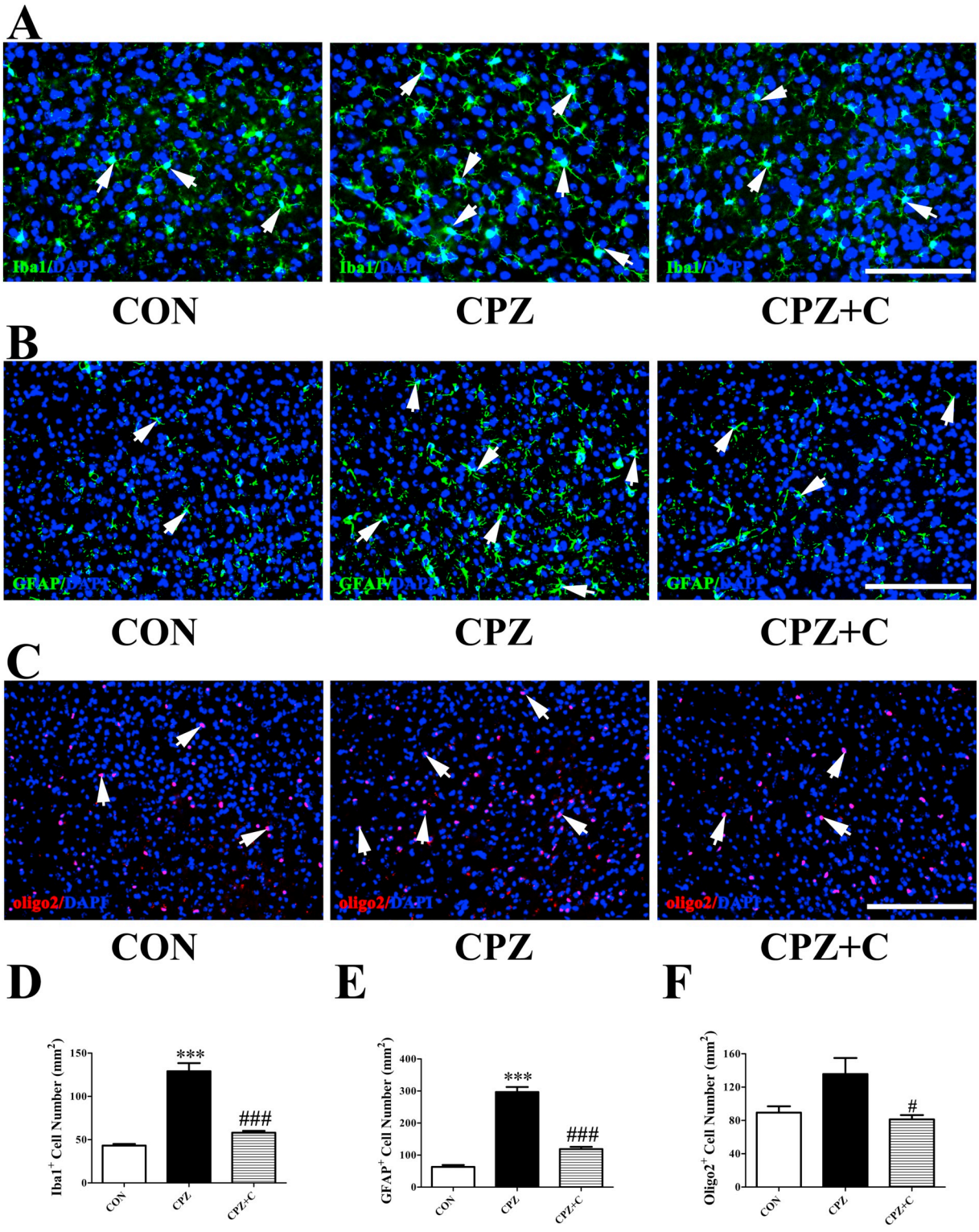

Fig. 5. Immunofluorescence staining of markers for oligodendrocyte precursor cells, microglia, and astrocytes in the cerebral cortex of CPZ-exposed mice with or without cordycepin treatment.

After establishment of demyelination animal models with CPZ and remyelination with cordycepin treatment, immunofluorescence staining with anti-Iba1 (microglial marker, A), anti-GFAP (astrocyte marker, C) and anti-Olig2 (oligodendrocytes precursor cell marker, C) antibodies was performed. The number of cells positive for Iba1 (D), GFAP (E) and Olig2 (F) was determined using the Image Pro Plus analysis program in an average area of 1 mm^2, and the cerebral cortex of both hemispheres was included. Data were presented as Mean ± SEM. Scale bar equals to 200 μM. N = 4–5. ($^{***}P < 0.001$ vs. CON; $^{\#\#\#}P < 0.001$ vs. CPZ; $^{\#}P < 0.05$ vs. CPZ). Statistical analysis was performed using one-way ANOVA followed by post hoc Turkey tests.

demyelination animal model, oligodendrocytes precursor cells become recruited, proliferate, and undergo maturation. On a cellular/molecular level, failure in myelin repair is linked to inadequate activation, inefficient recruitment, and/or lack of differentiation of oligodendrocytes precursor cells [32,33]. A previous study reported that Olig2 expression was greatly upregulated after CPZ treatment at different

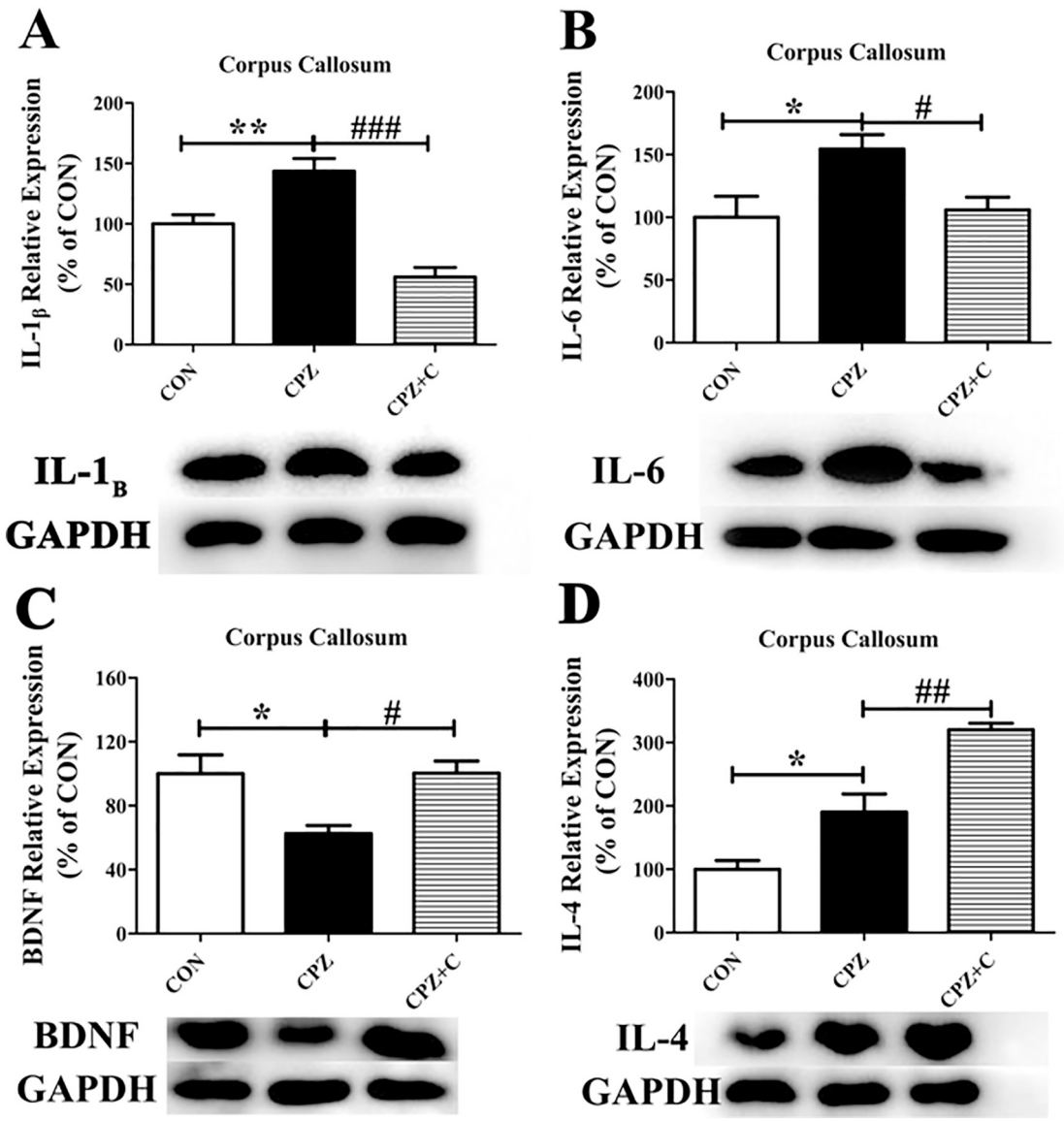

Fig. 6. The effects of cordycepin on IL-1β (A), IL-6 (B), BDNF (C) and IL-4 (D) expression levels in the corpus callosum.
Mice were fed standard rodent chows containing 0.2% CPZ for 5 weeks to induce acute demyelination followed by cordycepin treatment for an additional 7 days. The corpus callosum was isolated, and protein samples were analyzed by Western blot analysis. After normalization to the control, data were analyzed using one-way ANOVA followed by post hoc Turkey tests and were presented as Mean ± SEM. N = 5–6. (**$P < 0.01$ vs. CON; *$P < 0.05$ vs. CON; $^{###}P < 0.001$ vs. CPZ, $^{##}P < 0.01$ vs. CPZ, $^{#}P < 0.05$ vs. CPZ).

time points and in different brain regions [34,35], which is consistent with our findings. Published data have also shown that Olig2 upregulation enhances oligodendrocytes precursor cells differentiation and myelin repair [36,37]. In our study, the increase in Olig2-positive cells by CPZ treatment was significantly attenuated by cordycepin in the remyelination phase, suggesting that cordycepin facilitates oligodendrocytes precursor cells differentiation in the corpus callosum and cerebral cortex, which may be beneficial for myelin functional recovery.

The immune system plays a pivotal role in remyelination. The presence of microglia/macrophages in every step of the evolving gray matter lesion suggests a role in clearing the environment of growth inhibitory molecules and expression of growth beneficial molecules, such as BDNF [37,38]. We found that microglia were remarkably activated after CPZ ingestion for 5 consecutive weeks, which is consistent with previous results [28]. The increase in Iba1-positive microglia cells induced by CPZ treatment was significantly attenuated by cordycepin, suggesting that cordycepin plays a protective role in microglia-mediated demyelination.

Astrocytes become reactive in response to neuroinflammatory stimuli, and GFAP expression was upregulated during the process of astrogliosis [39]. Several studies showed that activated astrocytes promote demyelination, prevent remyelination, exert toxic effects on oligodendrocytes by secreting proinflammatory cytokines [40], and release trophic factors that exert protective effects against axonal and neuronal damage [30]. In the present study, mice treated with CPZ induced prominent astrocyte activation in the corpus callosum and cerebral cortex, which was reversed by cordycepin treatment.

4.4. Cordycepin prominently inhibited pro-inflammatory cytokine release and promoted anti-inflammatory cytokine, neurotrophic factor expression in the corpus callosum and hippocampus

Despite the critical role of inflammation in the pathogenesis of the disease, it is also one of the main determinants for proficient remyelination and enhancement of reparative processes [41]. The timely

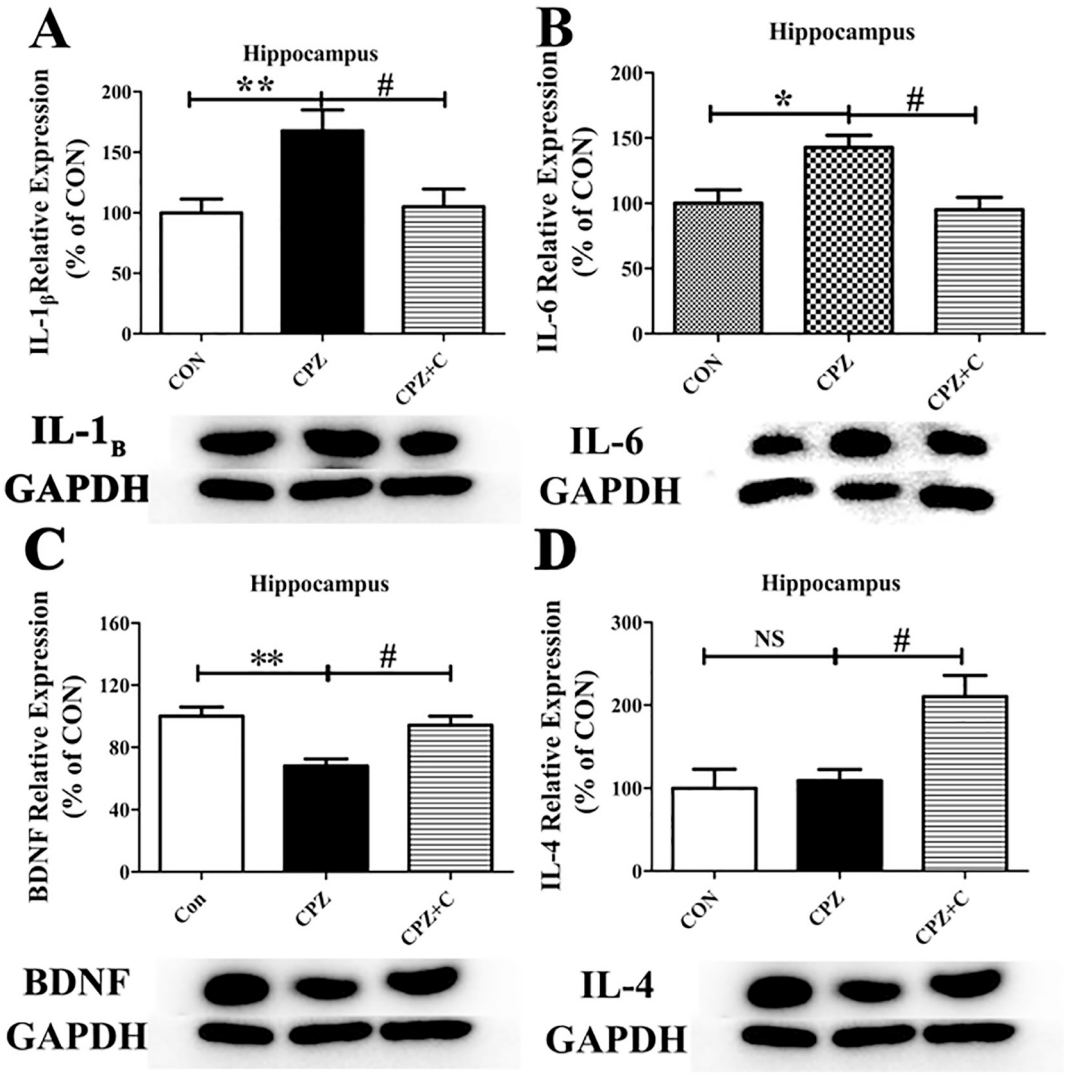

Fig. 7. The effects of cordycepin on IL-1_β (A), IL-6 (B), BDNF (C) and IL-4 (D) expression levels in the hippocampus.
Mice were fed standard rodent chows containing 0.2% CPZ for 5 weeks to induce acute demyelination followed by cordycepin treatment for an additional 7 days. The hippocampus was isolated, and protein samples were analyzed by Western blot analysis. After normalization to the control, data were analyzed using one-way ANOVA followed by post hoc Turkey tests and were presented as Mean ± SEM. N = 5–6. (**$P < 0.01$ vs. CON; *$P < 0.05$ vs. CON; $^{\#}P < 0.05$ vs. CPZ).

transition from the inflammatory state to the repair and regeneration state is critical for rapid and efficient remyelination. Microglia/macrophages can indeed shape the environment by directly secreting local neurotrophic factors (e.g., brain-derived neurotrophic factor, nerve growth factor, neurotrophin-3 and oncomodulin) and indirectly stimulating (via IL-1β) local CNTF production [42,43]. Administration of lipopolysaccharide (LPS) also increased the concentration of pro-inflammatory cytokines TNF-α and IL-1β in the brain and caused cell stress and neuronal cell death [44,45]. Cordycepin downregulated the gene expression of pro-inflammatory factors IL-1β, TNF-α, and IL-2 and upregulated anti-inflammatory factors IL-4, IL-10, and TGF-β in macrophages and mononuclear cells in the peripheral immune system [22,46,47]. We found that CPZ ingestion upregulates the expression of pro-inflammatory cytokines (IL-6, IL-1β) and downregulates the expression of neurotrophic factor BDNF in the corpus callosum and hippocampus, which was consistent with previous studies [48,49].

So far, there are no well-established drugs clinically used for the treatment of MS by promoting remyelination [7,25]. Although there are some molecules, which were proved to be effective for remyelination in some animal models [25,50], it is too early to use them classically as a positive control. Therefore, we did not choose any positive control for this study. The main purpose of this study is to evaluate the efficacy of cordycepin whether it could promote remyelination during remyelination and how it works.

In conclusion, cordycepin improved remyelination in the CPZ-induced mice demyelination model at multiple levels to ameliorate motor dysfunction, myelination of the corpus callosum, MBP expression, decreased numbers of glial cells, expression of inflammatory cytokines, and increased anti-inflammatory cytokine and neurotrophic factor. Therefore, cordycepin may be a valuable anti-inflammatory and neuroprotective candidate for the treatment of demyelination-associated disease.

Declaration of Competing Interest

The authors have no conflicts of interest to disclose, financial or otherwise.

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