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Cordycepin combined with antioxidant effects improves fatigue caused by excessive exercise

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Excessive exercise can lead to physical fatigue and disruption of the antioxidant system, resulting in neurological damage and cognitive decline. Cordycepin, the main component of *Cordyceps militaris*, has anti-inflammatory, antioxidant and neuroprotective effects. In this study, the anti-fatigue effect and potential mechanism of action of cordycepin were investigated using a forced exercise mouse model. The results showed that oral administration of cordycepin enhanced exercise endurance, increased liver and muscle glycogen content, and simultaneously decreased serum levels of lactic acid, lactate dehydrogenase, creatine kinase, and blood urea nitrogen ($p < 0.05$). In addition, cordycepin had antioxidant effects, increasing superoxide dismutase activity and decreasing serum malondialdehyde (MDA) levels ($p < 0.01$). In vitro experiments further demonstrated the antioxidant and anti-fatigue effects of cordycepin. Behavioral tests showed that the learning and memory ability of mice in the excessive exercise model group decreased to 40% compared with the control group. Cordycepin alleviated the learning and memory deficits in the over-exercised mice, significantly reduced the levels of fatigue metabolites and oxidative stress in vivo ($p < 0.05$), and altered the levels of neurotransmitters levels ($p < 0.05$). Furthermore, cordycepin modulated Keap1/Nrf2/HO-1-mediated oxidative stress and enhanced BDNF levels ($p < 0.05$). These findings suggest that cordycepin can alleviate excessive exercise-induced fatigue by modulating the Keap1/Nrf2/HO-1 signaling pathway and BDNF expression, providing strong supporting evidence for the development of cordycepin-functional foods or anti-fatigue drugs.

Keywords Anti-fatigue, Cordycepin, Antioxidant, Learning and memory

Fatigue has become increasingly common and significant with societal progress and faster pace of life. Fatigue is a complex physiological and biochemical process that occurs when brain or physical strength reaches a certain stage¹. Delaying fatigue and promoting recovery are current research priorities in sports medicine. Exercise-induced fatigue can be categorized into central and peripheral fatigue, based on the underlying mechanisms². Research has indicated that prolonged vigorous exercise depletes energy stores, causes excessive metabolite accumulation, disrupts redox balance, and disturbs internal homeostasis, leading to exercise fatigue and central nervous system imbalance³. The brain, however, an organ with a high oxygen consumption rate, is particularly vulnerable to oxidative stress. Hypoxia, hypoglycemia, and oxidative stress caused by excessive exercise can result in damage to the hippocampal neurons, increased cell death, and impaired learning and memory. The hippocampus, in particular, is a key area of the brain for learning and memory, adult neurogenesis and development, and one of the most sensitive tissues in the central nervous system. Prolonged high-intensity exercise may cause oxidative

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stress, leading to an imbalance in central excitatory and inhibitory neurotransmitters, significantly impacting the excitability of hippocampal neurones and the plasticity of synaptic transmission within the hippocampus, which reduces the ability to learn and remember^{4–6}. Oxidative stress results from an imbalance between the antioxidant defense system and ROS production of reactive oxygen species, leading to neuronal death or neurodegeneration. The Nrf2/Keap1 signalling pathway is a crucial regulatory pathway involved in antioxidant responses. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a redox-sensitive transcription factor that induces the expression of several antioxidant proteins. These antioxidant proteins are known to reduce the cellular damage caused by oxidative stress⁷. Overexpression of the Nrf2 downstream gene heme oxygenase 1 (HO-1) also enhances anti-fatigue effects and responses to oxidative stress⁸. Therefore, antioxidant therapy targeting Nrf2 and HO-1 may effectively prevent exercise-induced fatigue and learning and memory impairment.

Cordycepin, also known as 3'-deoxyadenosine, is a key active compound found in *Cordyceps militaris*⁹. Research has shown that cordycepin exhibits antioxidant¹⁰, anti-inflammatory¹¹, and neuroprotective effects¹². Recent studies have suggested that cordycepin can significantly improve hippocampal neural injury induced by hypoxia and ischemia, protect the electrophysiological generation characteristics of hippocampal neurons in a hypoxic environment, enhance the anti-hypoxic ability of pyramidal neurons in the central hippocampal CA1 region, and notably enhance the learning and memory capacity of hypoxia-injured animals¹³. Furthermore, previous research has indicated that cordycepin can enhance contractility of isolated skeletal muscles and delay the onset of exercise fatigue in isolated skeletal muscles¹⁴. Forced treadmill exercise is a common modeling method to induce fatigue by forcing experimental animals to perform excessive exercise, in addition, forced treadmill exercise is widely used in pharmacology, fatigue nutritional supplements, and sports-related fields. Therefore, in order to accurately assess the anti-fatigue effect, we used a mouse model of fatigue induced by forced running exercise to investigate the anti-fatigue effect of cordycepin and its related mechanisms, which provide a scientific foundation for the development of cordycepin nutraceuticals for anti-fatigue.

Materials and methods

Materials and reagents

Cordycepin (HPLC ≥ 98%, CAS77378-04-2, extracted from *Cordyceps militaris*) was procured from Aladdin Biochemical Science and Technology Company Limited (Shanghai, China) and stored at −20 °C. Lactic acid (LA), blood urea nitrogen (BUN), liver glycogen (LG), muscle glycogen (MG), creatine kinase (CK), lactate dehydrogenase (LDH), superoxide dismutase (SOD), and malondialdehyde (MDA) were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The LDH, SOD, and MDA assay kits used for cells were sourced from BioSwamp (Wuhan, Hubei, China). Enzyme-linked immunosorbent assay (ELISA) kits for acetylcholine (ACh, A105-1-1), glutamate (Glu, A074-1-1), 5-Hydroxytryptophan (5-HT, H104-1-1), and gamma-aminobutyric acid (GABA, H168-1-2) were purchased from Bioengineering Institute (Nanjing, Jiangsu, China). A BCA protein quantification kit was purchased from Comwin Biotech Co., Ltd. (Beijing, China). The specific antibody against β-actin (AC038) was purchased from ABclonal (Wuhan, China). Specific antibodies against Brain-Derived Neurotrophic Factor (BDNF; Cat25699-1-AP), Nuclear Factor Erythroid 2-Related Factor 2 (NRF2; Cat16396-1-AP), heme oxygenase 1 (HO-1; Cat10701-1-AP), and Kelch-like ech-associated protein 1 (Keap1; Cat60027-1-Ig) were obtained from Proteintech (Wuhan, Hubei, China). The kits used in this study were evaluated for consistency of performance across different batches of kits by recovery tests with recoveries ranging from 90 to 110% and calibrated by standards. The equipment used had a measurement accuracy of 0.01% and was calibrated before each experiment.

Experimental animals and drug treatment

All animal experiments and methods were performed in accordance with ARRIVE guidelines and regulations. All animal experiments were conducted in accordance with the protocols and regulations approved by the Ethics Committee of Jiangxi University of Science and Technology (the approval number was No. IACUC Y202445). Male Kunming mice (6 weeks old) were purchased from the Center for Laboratory Animal Science and Technology, Jiangxi University of Traditional Chinese Medicine (Jiangxi, China), and housed in group cages with a 12 h light/dark cycle. The ambient temperature was maintained at 22 ± 2 °C and the relative humidity was 40–60%. Mice had free access to food and sterile water throughout the experiment.

Following a one-week acclimatization period to the environment and a standardized diet, the animals were used for the treadmill exercise experiments. During this time, the mice were trained to run and those with poor performance were excluded (10 min). Mice that demonstrated satisfactory performance were randomly allocated to five groups (12 mice per group): silent control (Con + saline) (intragastric gavage of saline and no exercise), excessive exercise (Ex + saline) model group (intragastric gavage of saline and forced excessive exercise), excessive exercise + cordycepin group (Ex + cor) (intragastric gavage of cordycepin at 5 mg/kg/day and forced excessive exercise), excessive exercise + cordycepin group (Ex + cor) (intragastric gavage of 10 mg/kg/day cordycepin and forced excessive exercise), and excessive exercise + cordycepin group (Ex + cor) (intragastric gavage of 25 mg/kg/day cordycepin and forced excessive exercise). The control and excessive exercise groups received the same volume of saline (0.9% sodium chloride solution in a gavage volume of 0.2 mL/10 g) orally every morning, whereas the cordycepin group underwent intragastric administration (5 mg/kg, 10 mg/kg, and 25 mg/kg, gavage volume of 0.2 mL/10 g)⁴. The animals were administered saline and the drug orally once daily for 22 days (Fig. 1A). These mice were subsequently re-evaluated to determine the treatment efficacy. The weight of the mice was recorded daily. The gavage solution was prepared by dissolving and quantifying 0.9 g of sodium chloride to 100 mL in distilled water, and 20 mg of cordycepin was dissolved in different volumes of saline to prepare concentrations of 5 mg/kg, 10 mg/kg, and 25 mg/kg. The solution was agitated using a vortex mixer (MIX-25P; Hangzhou, China) at 2000 rpm for 5 min.

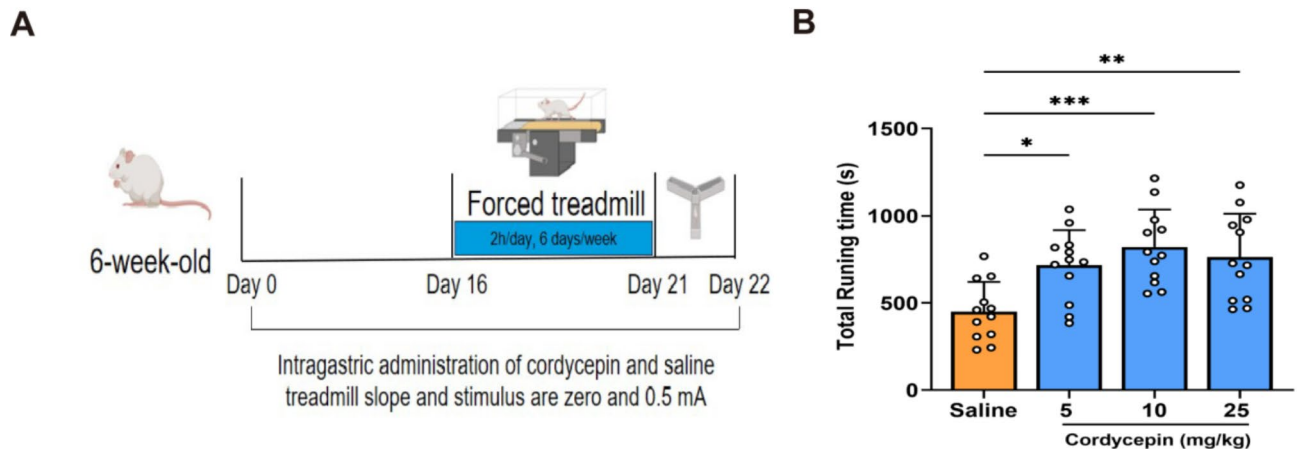


Fig. 1. Cordycepin-enhanced endurance in exercise-fatigued mice. **(A)** Experimental protocol for the forced treadmill exercise. **(B)** Cordycepin enhanced endurance in exercise-fatigue mice. Data are expressed as the mean \pm SEM, $^{\#}p < 0.05$, vs. Con + saline; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Ex + saline, $n = 12$ mice per group.

Forced treadmill exercise build fatigue model

The training protocol was adjusted based on previous studies^{15,16}. The exercise fatigue model followed the classic 3-stage incremental load treadmill training protocol. Treadmill training sessions were conducted every two days for 2 h at varying speeds, starting on day 16 of gavage administration and lasting 6 days. Exercise duration included forced running at different speeds: 18 m/min for 2 h from the first to the second day, 25 m/min for 2 h from the third to the fourth day, and 30 m/min for 2 h from the fifth to the sixth day. All the mice, except those in the quiet control group, ran on a treadmill until exhaustion. Throughout the experiment, the treadmill remained at a zero-degree inclination, and a 0.5 mA electrode stimulus was administered once the mice stopped running. The treadmill used was the ZL-013 animal experimental platform (Anhui Yaokun Biotechnology Co. Ltd.). The exhaustion criterion was defined as the inability of the mouse to maintain a speed of 30 m/min for more than three times during the operation. It is important to note that electricity, sound, and light had no stimulating effect.

Spontaneous alternation behavior test

The Y-maze test was conducted to evaluate working memory and spatial exploration activities, in accordance with previous descriptions¹⁷. The experimental mice were positioned in the center of a Y-maze, featuring three arms at 120° angles (arm length: 60 cm, arm width: 30 cm, wall height: 15 cm; Anhui Yaokun Science and Technology Co., Ltd.), with the three outer arms labelled as “1”, “2”, and “3”. Prior to the experiment, mice underwent a dark adaptation period of approximately 20 min. Throughout the experimental period, the test environment was quiet and dark. Each mouse was placed in any arm of the Y-maze and the number of arm entries and alternations (triads) were recorded. Entry was considered to have occurred when all the four limbs of the mouse were within the arm. An alternation was identified if the mouse's arm entry differed from that of the two arms previously visited, and an error was recorded if the mouse returned to either of the two arms that were just visited. The number of arm entries and changes was automatically documented using the ANY-maze software (Stoelting Company, USA). The percentage of relative alternations was calculated as the ratio of the number of alternations to the total number of entrances; this value was multiplied by 100.

Sample preparation

After the forced running test and the behavioral test, the mice were anesthetized with pentobarbital by intraperitoneal injection (35 mg/kg), and blood was collected from their hearts, the blood was centrifuged at 3500 rpm for 15 min at 4 °C, extract the serum and then stored at -80 °C. Samples of the gastrocnemius muscle, liver, brain, and hippocampus were isolated immediately. The samples were then washed with chilled saline and dried on a filter paper. Tissues were divided into two parts: one part was fixed in 4% phosphate-buffered paraformaldehyde and the other part was immediately frozen in liquid nitrogen.

Determination of fatigue-related biochemical indicators and oxidation-related biochemical indicators

After completing rigorous exercise, we measured LG levels in the liver and MG levels in the gastrocnemius muscle. Additionally, we measured the LA, CK, LDH, BUN, SOD, and MDA levels in the serum using the specified kits, following the manufacturer's instructions. Hippocampal tissue homogenates were rapidly prepared in an ice bath and centrifuged at 12,000 rpm for 10 min at 4 °C to collect the supernatant. The levels of LA, CK, LDH, SOD, and MDA in the hippocampal tissue were measured according to the manufacturer's protocol. The absorbance was measured at 450, 530, 532, 550, 620, 640, and 660 nm using a spectrophotometer or microplate reader. The kits used in this study were evaluated for consistency of performance across different batches of kits.

by recovery tests with recoveries ranging from 90%–110% and calibrated by standards. The equipment used had a measurement accuracy of 0.01% and was calibrated before each experiment.

Cell lines and cell culture

C2C12 myoblasts were procured from the Procell Life Sciences and Technology Co. Ltd. (Wuhan, China), and cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Cell-specific DMEM; Procell, Wuhan, China) in an incubator at 37 °C with 5% CO₂. Upon reaching 80% confluence, the cells were switched to DMEM containing 2% horse serum to initiate differentiation for four days, with the medium being refreshed every other day.

H₂O₂-induced C2C12 myoblast viability assay

H₂O₂ is commonly used to induce cellular oxidative stress¹⁵. To investigate the anti-fatigue mechanism of cordycepin, we initially examined the effect of H₂O₂-induced oxidative stress on C2C12 cells, and then evaluated the antioxidant properties of cordycepin by assessing the viability of C2C12 cells. C2C12 cells were seeded in triplicates in 96-well plates. After 4 days of induced differentiation at 37 °C in a 5% CO₂ incubator, cells were pretreated with various concentrations of cordycepin (0.1, 0.5, and 1 μM) for 24 h. Subsequently, cells were exposed to 500 μM H₂O₂ for 3 h. MTT (120 μL) was added to each well, and the plate was incubated for 4 h. The culture medium was then removed and 150 μL of DMSO was added. Absorbance was measured at 492 nm using a microplate reader.

Analysis of SOD, LDH, and CK activities in C2C12 cells

C2C12 cells were seeded into culture dishes, processed, and harvested according to the aforementioned protocol. Subsequently, LDH, SOD, and MDA activities in cells were assessed according to the manufacturer's guidelines. SOD activity was determined using the WST-8 method and MDA activity was evaluated based on the principle of MDA-TBA adduct formation. NAD acts as a hydrogen acceptor, and the conversion of lactate to pyruvate is catalyzed by lactate dehydrogenase. The resulting pyruvate then reacted with dinitrophenylhydrazine to form dinitrophenylhydrazone. The pyruvate content was measured to determine LDH enzyme activity. Absorbance readings were recorded at 450, 440, and 553 nm using a spectrophotometer and microplate reader (Thermo Fisher, USA).

Determination of central hippocampal neurotransmitters

Hippocampal tissue was collected from the mice and homogenates were rapidly prepared in an ice bath. The samples were then centrifuged at 3000 rpm for 10 min at 4 °C to collect the supernatant. The levels of the neurotransmitters acetylcholine (ACh), glutamate (Glu), gamma-aminobutyric acid (GABA), and serotonin (5-HT), which are related to fatigue, were measured using ELISA following the manufacturer's instructions. The absorbance at 550 nm and 340 nm was measured using a spectrophotometer or microplate reader. The kits used in this study were evaluated for consistency of performance across different batches of kits by recovery tests with recoveries ranging from 90%–110% and calibrated by standards. The equipment used had a measurement accuracy of 0.01% and was calibrated before each experiment.

Western blot analysis

Whole proteins were extracted from hippocampal tissue using a protein lysate. A BCA protein quantification kit was used to establish a standard curve and determine the protein concentration, which was adjusted accordingly. Proteins were separated by 8% SDS-PAGE. Membranes were blocked, washed, and transferred onto polyvinylidene fluoride membranes. Subsequently, primary antibodies against β-actin (1:10,000, ABclonal, Wuhan, China), BDNF (1:1000, Proteintech), Keap1 (1:5000, Proteintech), NRF2 (1:6000, Proteintech), and HO-1 (1:3000, Proteintech) were added and the samples were incubated overnight at 4 °C. After completion of the primary antibody incubation, the membrane was washed with TBST and the secondary antibody working solution (1:5000, Cwbio, Shanghai, China) was added. The samples were incubated at room temperature for 1 h. After three washes with TBST, ECL chemiluminescence reagent was added, and chemiluminescent signals on the western blots were visualized using a Tanon5200 system (Shanghai, China). β-actin antibody was used as a loading control for normalization. The bands were analyzed and quantified using ImageJ software.

Statistical analysis

For statistical analysis of the experimental data, we used GraphPad Prism software (version 9.5.0, CA, USA). Results are presented as the mean ± SEM. To determine statistical differences among multiple groups, one-way analysis of variance (ANOVA) was employed, with post-hoc comparisons conducted using Tukey's or Bonferroni correction to control for multiple testing errors. Data normality was assessed using the Shapiro–Wilk test prior to applying ANOVA. All comparisons were made using $p < 0.05$ as the criterion for statistically significant differences.

Results

Effects of cordycepin on exercise endurance with excessive fatigued mice

This study evaluated the effects of cordycepin on exercise endurance in a mouse fatigue model using forced treadmill exercise, and recorded the total exercise time. As shown in Fig. 1B, the results indicated that cordycepin significantly improved exercise endurance in mice ($p < 0.05$ vs Ex + saline group).

Effects of cordycepin on peripheral fatigued-related biochemical indicators

Compared with the Con + saline group, the Ex + saline group exhibited Lower levels of LG and MG (down to 8.04 mg/g and 0.44 mg/g, respectively) and higher levels of LA, BUN, CK, and LDH (respectively, 12.54 mmol/L, 12.1 mmol/L, 1.35 U/mL, 7259.14 U/L). However, cordycepin treatment significantly increased LG and MG levels, and decreased LA, BUN, CK, and LDH levels (Fig. 2A–F). This suggests that cordycepin effectively relieves exercise fatigue by enhancing LG and MG reserves and by reducing LA levels during excessive exercise ($p < 0.01$ vs Ex + saline group). These results demonstrate that cordycepin accelerated the clearance of lactate and free radicals generated during exercise and effectively slowed down the protein metabolic rate.

Effects of cordycepin on oxidation-related biochemical indicators

In contrast to the Con + saline group, there was a notable reduction in SOD activity (106.662 U/mL) and a significant increase in MDA content in the Ex + saline group (6.482 nmol/mL), whereas the cordycepin group exhibited increased SOD activity and reduced MDA content (Fig. 3A, B). Additionally, we verified these in vitro results and found that MDA and LDH levels decreased in cordycepin-treated myoblasts, whereas SOD activity increased significantly (Fig. 3D–F), suggesting that cordycepin has anti-fatigue effects on C2C12 myoblasts through antioxidant responses. These findings further indicated that cordycepin exerts anti-fatigue effects in combination with antioxidant properties.

Effects of cordycepin on central hippocampus fatigue and oxidation related biochemical indices in excessive fatigued mice

Our study focused on assessing the central fatigue indicators in the hippocampus. The beneficial effects of cordycepin on the learning and memory functions of fatigued mice were consistent with changes in fatigue-related biochemical markers in the hippocampus. Our results showed that the levels of LA, CK, and LDH in the hippocampal tissues of mice in the Ex + saline group were higher than those in the Con + saline group (respectively 0.5826 mmol/g prot, 1.28 U/mg prot, 7482.22 U/g prot). However, the levels of these markers were significantly lower in the cordycepin-treated group ($p < 0.01$ vs Ex + saline group, Fig. 4A–C). Furthermore, we observed a significant decrease in SOD activity and increase in MDA content in the hippocampal tissues of the Ex + saline group, whereas the cordycepin group exhibited increased SOD activity and decreased MDA content (Fig. 4D, E). These results indicate that cordycepin may enhance learning and memory in fatigued mice

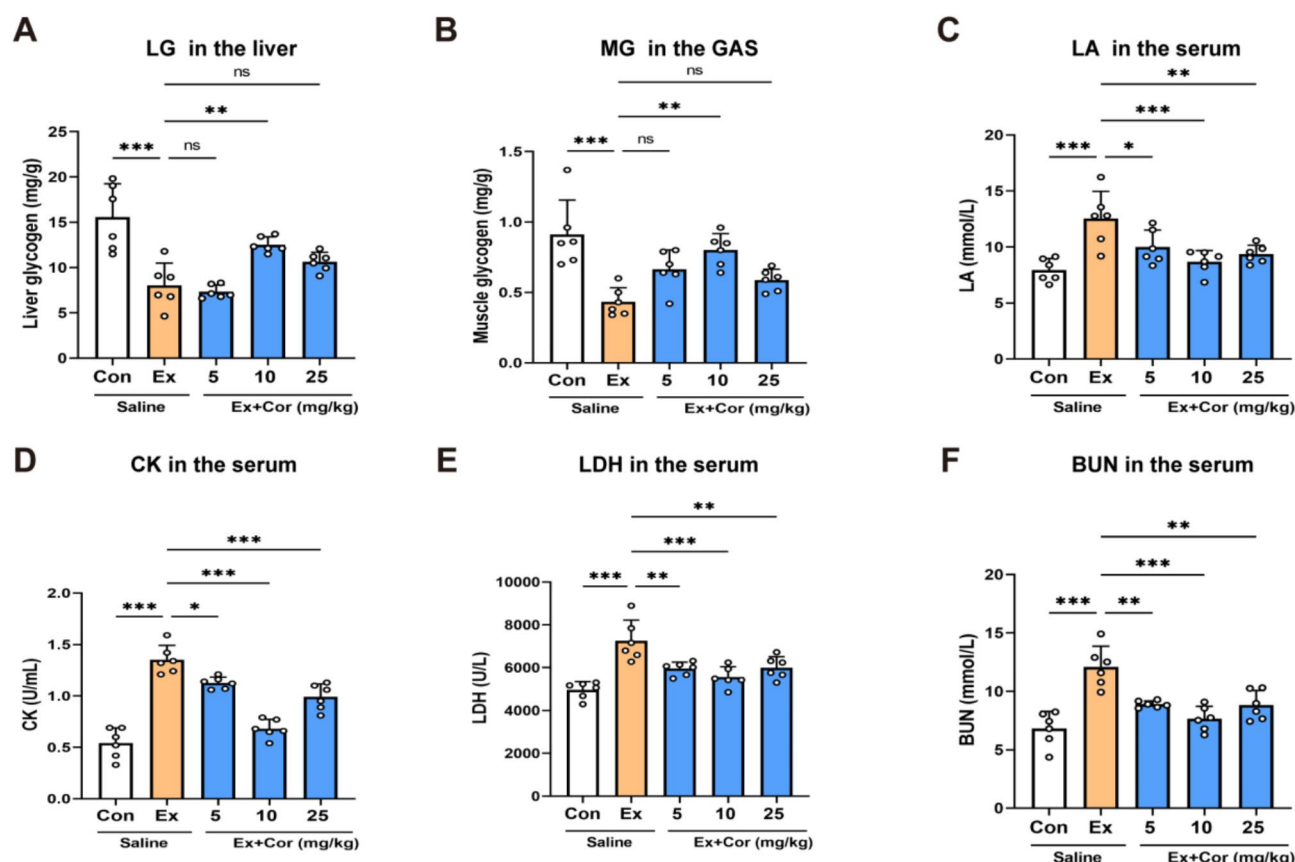


Fig. 2. Cordycepin supplementation restored peripheral biochemical indicators in exercise-fed mice. (A) Liver glycogen (LG), (B) muscle glycogen (MG), (C) lactic acid (LA), (D) creatine kinase (CK), and (E) lactic dehydrogenase (LDH) levels. Data are expressed as the mean \pm SEM. ** $p < 0.01$ and *** $p < 0.001$ Con + saline vs. Ex + saline or Ex + Cor vs. Ex + saline; $n = 6$ mice per group.

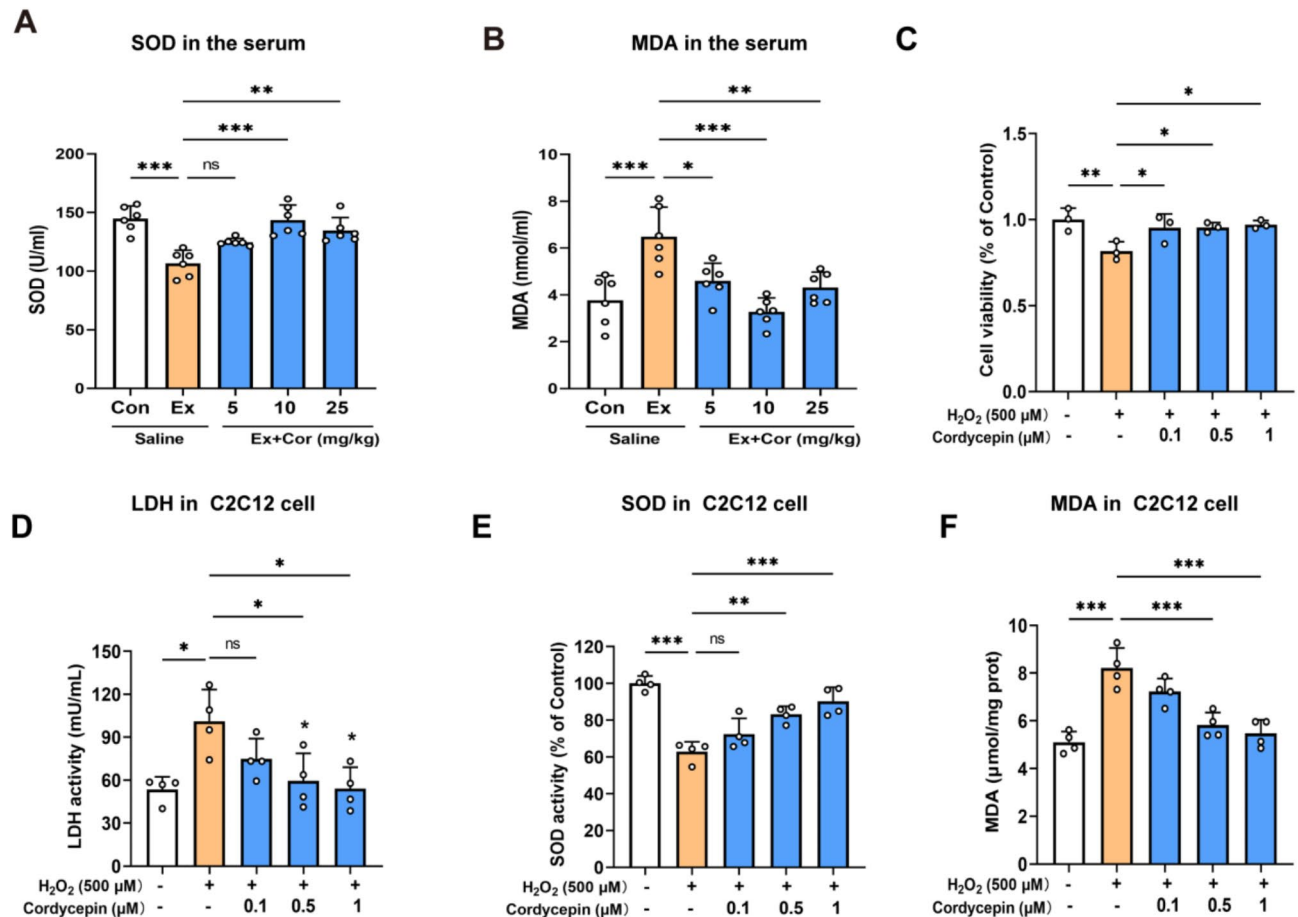


Fig. 3. Cordycepin supplementation restored oxidation-related biochemical indicators in exercise-fatigued mice and C2C12 cells treated with H₂O₂. (A) superoxide dismutase (SOD); (B) malondialdehyde (MDA) (n=6). (C) Effect of cordycepin on oxidative stress-induced C2C12 cell viability (n=3). (D–F) MDA, LDH, and SOD activities were assayed in C2C12 cells (n=4). Data are expressed as the mean ± SEM. ***p* < 0.01 and ****p* < 0.001 Con + saline vs. Ex + saline or Ex + Cor vs. Ex + saline; **p* < 0.05, ***p* < 0.01 vs. Con vs. the H₂O₂ group or Cor group vs. H₂O₂ group.

by mitigating the excessive accumulation of fatigue-related metabolites induced by exercise and by reducing oxidative stress in the brain tissue.

Effects of cordycepin on biochemical indices related to learning and memory in excessive fatigued mice

Further examination of neurotransmitters revealed that, compared to the Con + saline group, the levels of ACh and Glu in the hippocampal tissue of mice in the Ex + saline group were significantly reduced, while the levels of ACh and Glu in the hippocampal tissue of the cordycepin-treated group were notably higher than those in the Ex + saline group (Fig. 4F, G). GABA and 5-HT levels in the hippocampal tissues of mice in the Ex + saline group were higher than those in the Con + saline group, and were further decreased in the cordycepin group (*p* < 0.01 vs Ex + saline group) (Fig. 4H, I). These findings suggest that cordycepin may enhance learning and memory capacity by significantly improving the neurotransmitter levels associated with learning and memory function in mouse hippocampal tissues affected by exercise fatigue.

Effects of cordycepin on the behavioral test of SAB in excessive fatigued mice

In a study conducted by Sun et al.¹⁶, it was found that excessive exercise or training can negatively impact the learning and memory abilities of animals, thereby affecting their cognitive function. The Y-maze test was employed to investigate the effects of exercise fatigue on learning and memory abilities in mice and the potential role of cordycepin in mitigating these deficits. The results depicted in Fig. 5B, C revealed no significant difference in the total number of arms entered between the control and drug groups. However, a notable disparity was observed in the number of correct arms that were entered. Notably, cordycepin administration led to a significant increase in the percentage of relative alterations in mice, particularly at a dose of 10 mg/kg (showing 62.83%, *p* < 0.01 vs Ex + saline group), surpassing the effects observed at doses of 5 mg/kg and 25 mg/kg. These findings suggest that cordycepin effectively ameliorates the learning and memory abilities of mice experiencing exercise fatigue.

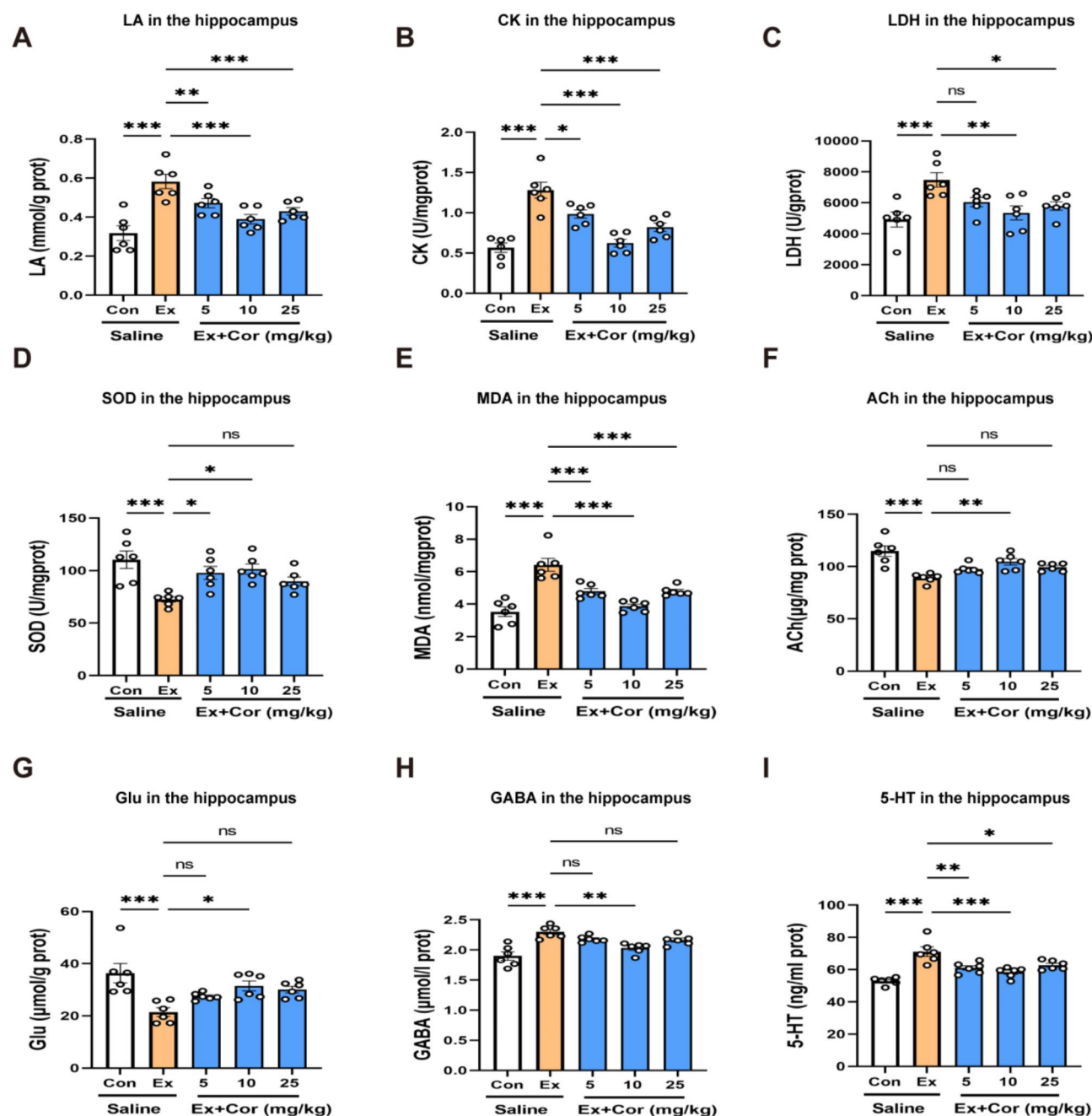


Fig. 4. Cordycepin supplementation restored central fatigue, oxidative biochemical indices, and biochemical indices related to learning and memory in exercise-fatigued mice. (A) Lactic acid (LA) in hippocampal tissue; (B) creatine kinase (CK) in hippocampal tissue; (C) lactic dehydrogenase (LDH) in hippocampal tissue; (D) superoxide dismutase (SOD) in hippocampal tissue; (E) malondialdehyde (MDA) in hippocampal tissue; (F) acetylcholine (ACh) in hippocampal tissue; (G) glutamate (Glu) in hippocampal tissue; (H) γ -aminobutyric acid (GABA) in hippocampal tissue; (I) 5-hydroxy tryptamine (5-HT) in hippocampal tissue. Data are expressed as the mean \pm SEM. ** p < 0.01 and *** p < 0.001 Con + saline vs. Ex + saline or Ex + Cor vs. Ex + saline; n = 6 mice per group.

Effects of cordycepin on the expression of BDNF, Keap1, Nrf2, and HO-1 proteins in excessive fatigued mice

The western blotting results (Fig. 5D, E) indicated that the expression levels of BDNF, Nrf2, and HO-1 proteins in the hippocampus of the Ex + saline group were significantly lower than those in the Con + saline group, whereas the expression level of Keap1 protein was significantly higher than that in the Con + saline group. In contrast, the expression levels of BDNF, Nrf2, and HO-1 proteins in the hippocampus of the cordycepin group were significantly higher than those in the Con + saline group, and the expression level of the Keap1 protein was significantly lower than that of the Ex + saline group (p < 0.05 vs Ex + saline group). These findings suggest

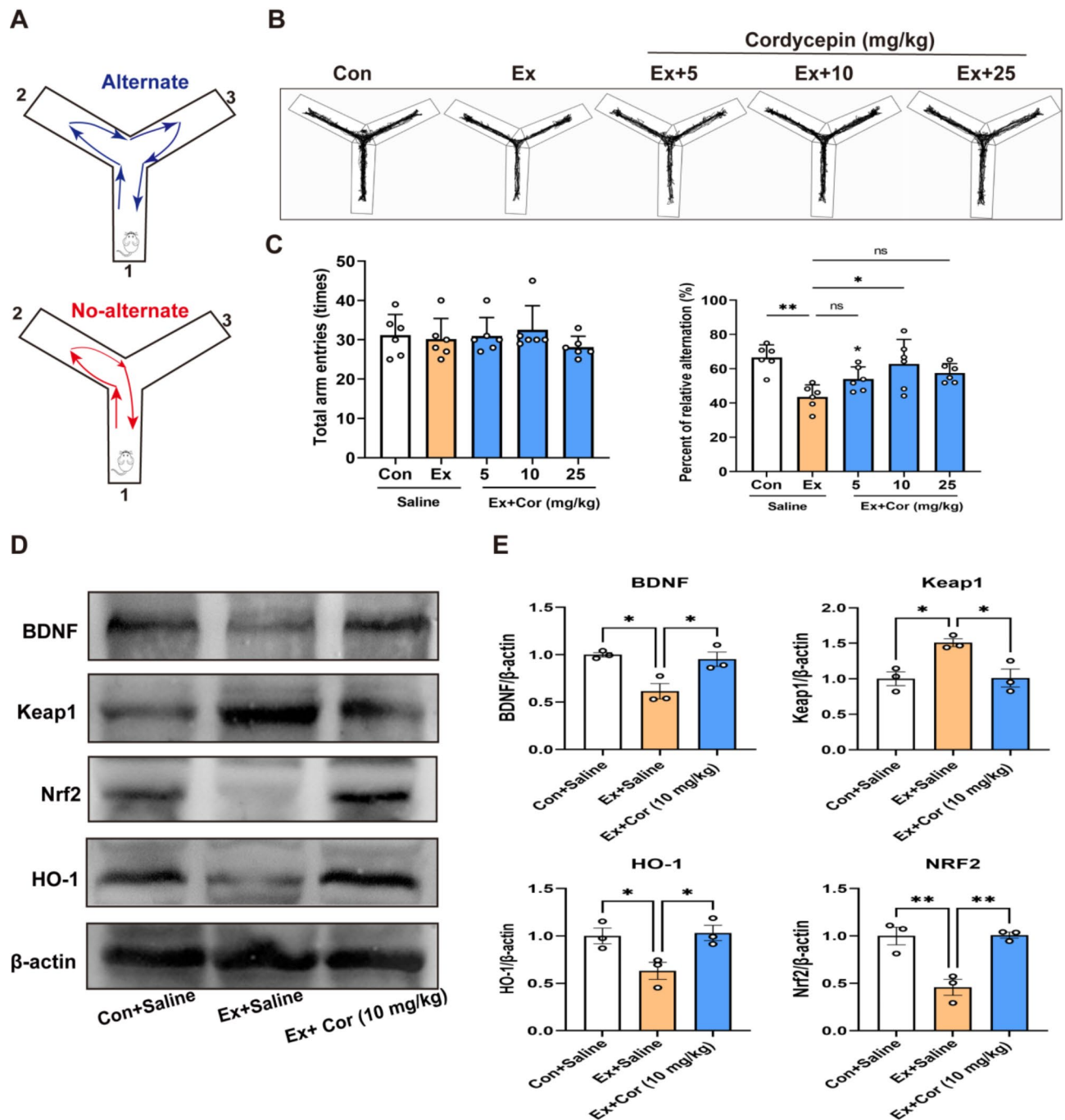


Fig. 5. The effects of cordycepin on cognitive behavior and protein expression in the hippocampus of fatigued mice. **(A, B)** Representative Y-maze test trajectories. **(C)** The percentage of spontaneous rate of change and total number of entries into the arm were measured using the Y-maze test. **(D)** Protein expression of BDNF, Keap1, Nrf2, and HO-1 in the hippocampus of the brain tissue of fatigued mice. Western blot images of BDNF, Keap1, Nrf2, and HO-1. **(E)** Quantitative analysis of BDNF, Keap1, Nrf2, and HO-1 protein expression using ImageJ software ($n = 3$); β -actin was used as a loading control. Data are expressed as the mean \pm SEM. ** $p < 0.01$ and *** $p < 0.001$ Con + saline vs. Ex + saline or Ex + Cor vs. Ex + saline; $n = 6$ mice per group.

that cordycepin may regulate learning and memory impairments caused by excessive exercise-induced fatigue through its influence on BDNF, Keap1, Nrf2, and HO-1 proteins.

Discussion

Cordycepin, also known as 3'-deoxyadenosine, is a key active component found in *Cordyceps militaris*¹⁷. This compound exhibits various pharmacological effects, such as potent antioxidant¹⁰, anti-inflammatory¹¹, and neuroprotective effects¹². A previous study demonstrated that cordycepin reduces the recovery time from

muscle fatigue in isolated skeletal muscles¹³. Based on these results, we propose that cordycepin exerts anti-fatigue effects.

In this study, we utilized classical forced treadmill training to create a mouse model of exercise fatigue, which involved 6 days of forced excessive exercise. We found that cordycepin improved endurance in forced exercise mice (Fig. 1B). We also measured biochemical indicators, including LG, MG, LA, CK, LDH, and BUN levels, to assess fatigue. Previous research has highlighted the importance of glycogen as an energy source during exercise, with sufficient hepatic glycogen and myoglycogen enhancing endurance and sustaining high-intensity exercise^{18–20}. LA is a key indicator for assessing fatigue levels as it is the end product of anaerobic glycolysis during high-intensity exercise. This process can lead to a decrease in the muscle and blood pH, which can cause tissue damage and increased fatigue^{21,22}. Elevated LDH and CK levels reflect skeletal muscle cell necrosis and tissue damage^{23,24}. When fatigue arises from high-intensity exercise, insufficient energy from carbohydrate and fat metabolism occurs, resulting in protein and amino acid depletion and increased urea nitrogen levels²². Our results showed significant changes in the serum levels of LA, LDH, CK, and BUN in fatigued mice, suggesting that cordycepin can mitigate fatigue (Fig. 2A–F). Therefore, the regulation of metabolite accumulation by cordycepin may be a potential mechanism for its anti-fatigue effect.

Oxidative stress is associated with fatigue and fatigue-related diseases²⁵. High energy expenditure during prolonged strenuous exercise leads to an imbalance between oxidative and antioxidant systems. SOD protects cells against oxidative stress damage²⁶. MDA, a product of phospholipid peroxidation, serves as a major biomarker for assessing the extent of oxidative damage²⁷. Our study showed that cordycepin treatment significantly reduced the exercise-induced increase in MDA, a biomarker of oxidative stress, whereas the decrease in SOD activity induced by excessive exercise was markedly enhanced after cordycepin treatment (Fig. 3A–B). In vitro cellular experiments further confirmed that cordycepin improved oxidative stress-induced reductions in MDA and LDH levels, as well as a significant increase in SOD activity in C2C12 cells, thus reducing skeletal muscle fatigue (Fig. 3C–F). These results suggest that prevention of oxidative stress and enhancement of related enzyme activities are involved in the anti-fatigue effect of cordycepin. Peripheral and central fatigue are often simultaneous and interconnected. Peripheral fatigue is linked to muscle activity, whereas changes in the central nervous system owing to muscle signals contribute to central fatigue²⁸. Our study showed that cordycepin significantly increased fatigue-related and oxidative indicators as well as neurotransmitter alterations in the hippocampus of exercise-fatigued mice (Fig. 4A–E, F–I).

The brain, an organ with a high oxygen consumption rate, is particularly vulnerable to oxidative stress. Conditions such as hypoxia, hypoglycemia, and oxidative stress resulting from excessive exercise can damage hippocampal neurons, leading to increased cell death and impaired cognitive functions. Research has also indicated that cordycepin can alleviate learning and memory impairments induced by certain substances and conditions^{4,29}. Our study revealed that cordycepin significantly improved the learning and memory impairments in exercise-fatigued mice, especially, the 10 mg/kg group was superior to the low-dose and high-concentration groups, which may be due to the side effects of the high-concentration group, such as over-activation or saturation of hormones and receptors, which may affect the experimental performance of mice (Fig. 5A–C). Additionally, cordycepin decreased the levels of certain substances associated with cell damage and oxidative stress in the hippocampus while increasing the levels of others that support nerve cell health. When the body experiences oxidative stress owing to prolonged exercise, it activates a series of protective proteins to mitigate damage. The Nrf2/Keap1 signalling pathway is a crucial regulatory pathway involved in antioxidant responses^{30–32}. Studies have shown that activation of the Nrf2/HO-1 signalling pathway prevents oxidative stress-mediated neuronal apoptosis³². Additionally, studies have shown that cognitive dysfunction is linked to decreased BDNF expression, and that increased BDNF levels can ameliorate learning and memory deficits³³. However, it is worth noting that other mechanisms such as AMPK, mitochondrial biogenesis and FOXO3 may also play an important role in fatigue. It has been shown that AMPK activation is associated with enhanced antioxidant response, possibly through interaction with Nrf2²⁷. Gene expression related to mitochondrial function also plays a key role in fatigue and energy metabolism¹⁸. Studies have shown that FOXO3 can synergize with Nrf2 to enhance cellular resistance to oxidative stress³⁴. Although AMPK, mitochondrial biogenesis and FOXO3 play important roles in the cellular stress response, the Nrf2/HO-1 signalling pathway is still considered to be the most critical regulator in this process. To further investigate the role of cordycepin in the Keap1/Nrf2/HO-1 signalling pathway, we detected the relevant proteins of this pathway using western blot methodology. Our results indicated that cordycepin may have neuroprotective effects on excessive exercise induced learning and memory abnormalities in fatigued mice by upregulating the expression levels of Nrf2, HO-1, and BDNF, as well as downregulating the expression of Keap1, indicating that cordycepin exerted an anti-fatigue effect by regulating the Keap1/Nrf2/HO-1 signalling pathway (Fig. 5D, E).

In this study, we demonstrated that cordycepin protects the brain from oxidative damage caused by excessive exercise, alleviates central fatigue, and improves learning and memory in excessive exercise induced-fatigued mice by modulating the activity of Keap1/Nrf2/HO-1 and BDNF in the hippocampus. However, this study has some limitations. The study only involved male mice; therefore, future research should include female animals, considering the gender differences in sports. The treadmill running exercise involved electrical stimulation, which, while essential for inducing complete fatigue, introduced new stresses that may affect brain function. We did not include a sham group in our study, and all groups were subjected to gavage to minimize the effects of this procedure. Although gavage may induce some stress and inflammatory responses, we closely monitored the mice's food and water intake, as well as their activity levels throughout the experiment. It is important to note that inflammation can also play a role in enhancing athletic performance and accelerating recovery. Future studies should incorporate sham groups and investigate the associated inflammatory responses. Furthermore, while we mainly focused on fatigue resulting from excessive exercise, the nuclear translocation of Nrf2 and its

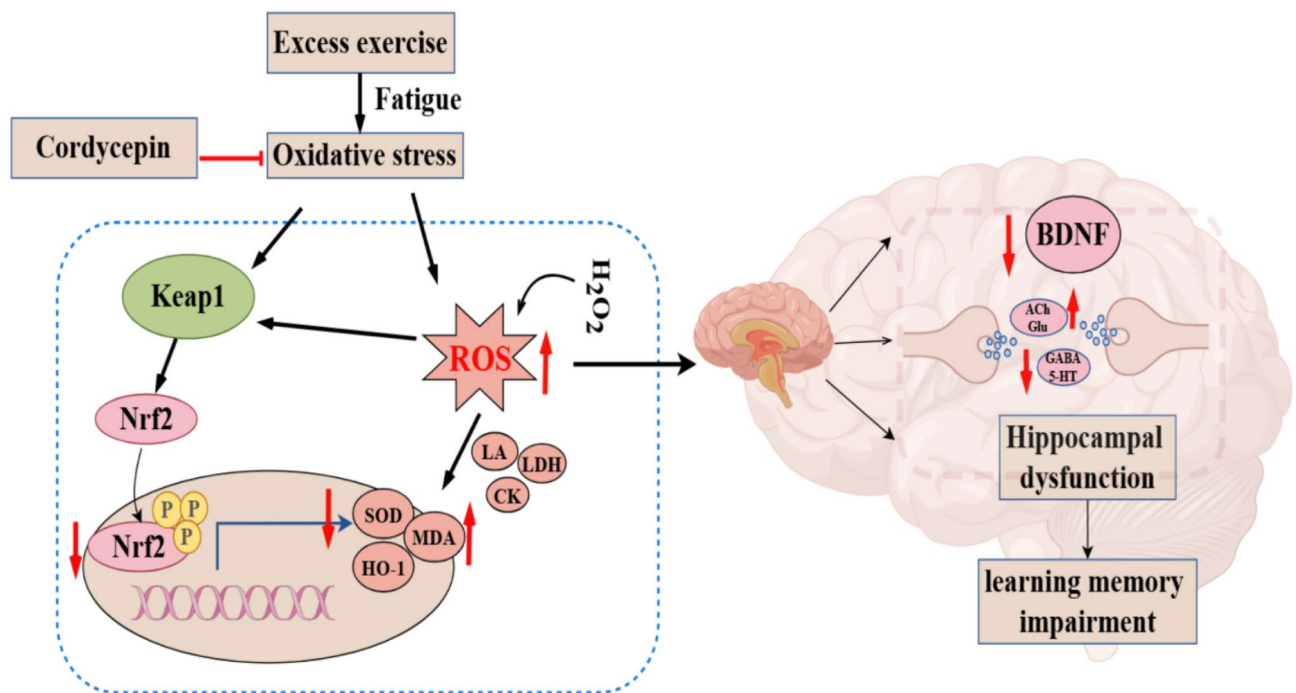


Fig. 6. Schematic diagram of the mechanisms associated with the anti-fatigue effects of cordycepin. The figure summarises that Cordycepin may delay exercise fatigue and ameliorate learning memory deficits due to excessive exercise-induced fatigue through the Keap1/Nrf2/HO-1 signalling pathway and BDNF. Created with Figdraw.com.

specific mechanisms in cellular stress responses warrant further exploration. Future investigations should utilize inhibitors or genetic knockouts to validate the role of Nrf2 in these processes.

Conclusion

In summary, cordycepin combined with its antioxidant action improved the accumulation of oxidative stress and fatigue metabolites, increased glycogen content, and improved exercise endurance to exert anti-fatigue effects. At the same time, our behavioral results show that cordycepin improves learning and memory impairment by reducing the accumulation of metabolites and oxidative stress levels, and improving the imbalance of neurotransmitters in brain tissue caused by excessive exercise. Its potential mechanism may be related to the regulation of the Keap1/NRF2/HO-1 signaling pathway and BDNF expression, thereby enhancing the body's antioxidant capacity (Fig. 6). These results show that cordycepin combined with antioxidant action exerts an anti-fatigue effect and can also improve learning and memory impairment caused by fatigue caused by excessive exercise, indicating that cordycepin may be a promising new antioxidant or anti-fatigue health food suitable for the general population and people engaged in sports and physical exercise.

Data availability

The authors of this article will make the raw data supporting their conclusions available, without any hesitation or reservation. Li-hua Yao (yaoh7905@163.com) should be contacted if someone wants to request the data from this study.

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References

- Davis, J. M. & Bailey, S. P. Possible mechanisms of central nervous system fatigue during exercise. *Med. Sci. Sports Exerc.* **29**, 45–57. <https://doi.org/10.1097/00005768-199701000-00008> (1997).
- O'Leary, T. J., Morris, M. G., Collett, J. & Howells, K. Central and peripheral fatigue following non-exhaustive and exhaustive exercise of disparate metabolic demands. *Scand J. Med. Sci. Sports.* **26**, 1287–1300. <https://doi.org/10.1111/sms.12582> (2016).
- Yang, D. et al. The anti-fatigue and anti-anoxia effects of *Tremella* extract. *Saudi. J. Biol. Sci.* **26**, 2052–2056. <https://doi.org/10.1016/j.sjbs.2019.08.014> (2019).
- Cai, Z. L. et al. Effects of cordycepin on Y-maze learning task in mice. *Eur. J. Pharmacol.* **714**, 249–253. <https://doi.org/10.1016/j.ejphar.2013.05.049> (2013).
- Bai, L. et al. Cordyceps militaris acidic polysaccharides improve learning and memory impairment in mice with exercise fatigue through the PI3K/NRF2/HO-1 signalling pathway. *Int. J. Biol. Macromol.* **227**, 158–172. <https://doi.org/10.1016/j.ijbiomac.2022.12.071> (2023).

6. Lin, H. et al. Schisantherin A improves learning and memory abilities partly through regulating the Nrf2/Keap1/ARE signaling pathway in chronic fatigue mice. *Exp. Ther. Med.* **21**, 385. <https://doi.org/10.3892/etm.2021.9816> (2021).
7. Zhang, M. et al. Effects and mechanism of gastrodin supplementation on exercise-induced fatigue in mice. *Food Funct.* **14**, 787–795. <https://doi.org/10.1039/d2fo03095k> (2023).
8. Peng, Y. et al. Anti-fatigue effects of *Lycium barbarum* polysaccharide and effervescent tablets by regulating oxidative stress and energy metabolism in rats. *Int. J. Mol. Sci.* **23**, 10920. <https://doi.org/10.3390/ijms231810920> (2022).
9. Das, G. et al. Cordyceps spp: A review on its immune-stimulatory and other biological potentials. *Front. Pharmacol.* **11**, 602364. <https://doi.org/10.3389/fphar.2020.602364> (2021).
10. Chen, C. et al. Anti-effects of cordycepin to hypoxia-induced membrane depolarization on hippocampal CA1 pyramidal neuron. *Eur. J. Pharmacol.* **796**, 1–6. <https://doi.org/10.1016/j.ejphar.2016.12.021> (2017).
11. Zhang, X. L. et al. Anti-inflammatory and neuroprotective effects of natural cordycepin in rotenone-induced PD models through inhibiting Drp1-mediated mitochondrial fission. *Neurotoxicology* **84**, 1–13. <https://doi.org/10.1016/j.neuro.2021.02.002> (2021).
12. Song, H. et al. Neuroprotective effects of cordycepin inhibit A β -induced apoptosis in hippocampal neurons. *Neurotoxicology* **68**, 73–80. <https://doi.org/10.1016/j.neuro.2018.07.008> (2018).
13. Liu, Z. B., Liu, C., Zeng, B., Huang, L. P. & Yao, L. H. Modulation effects of cordycepin on voltage-gated sodium channels in rat hippocampal CA1 pyramidal neurons in the presence/absence of oxygen. *Neural. Plast.* **2017**, 2459053. <https://doi.org/10.1155/2017/2459053> (2017).
14. Yao, L. H. et al. Modulation effects of cordycepin on the skeletal muscle contraction of toad gastrocnemius muscle. *Eur. J. Pharmacol.* **726**, 9–15. <https://doi.org/10.1016/j.ejphar.2014.01.016> (2014).
15. Sun, Y. et al. Anti-fatigue effect of hypericin in a chronic forced exercise mouse model. *J. Ethnopharmacol.* **284**, 114767. <https://doi.org/10.1016/j.jep.2021.114767> (2022).
16. Sun, L. N. et al. High-intensity treadmill running impairs cognitive behavior and hippocampal synaptic plasticity of rats via activation of inflammatory response. *J. Neurosci. Res.* **95**, 1611–1620. <https://doi.org/10.1002/jnr.23996> (2017).
17. Kaczka, E. A., Trenner, N. R., Arison, B., Walker, R. W. & Folkers, K. Identification of cordycepin, a metabolite of *Cordyceps militaris*, as 3'-deoxyadenosine. *Biochem. Biophys. Res. Commun.* **14**, 456–457. [https://doi.org/10.1016/0006-291x\(64\)90086-5](https://doi.org/10.1016/0006-291x(64)90086-5) (1964).
18. Zhang, X. et al. Anti-fatigue effect of anwulignan via the NRF2 and PGC-1 α signaling pathway in mice. *Food Funct.* **10**, 7755–7766. <https://doi.org/10.1039/c9fo01182j> (2019).
19. Yang, Q. Effects of macamides on endurance capacity and anti-fatigue property in prolonged swimming mice. *Pharm. Biol.* **54**, 827–834. <https://doi.org/10.3109/13880209.2015.1087036> (2016).
20. Li, D. et al. Anti-fatigue effects of small-molecule oligopeptides isolated from *Panax quinquefolium* L. in mice. *Food Funct.* **9**, 4266–4273. <https://doi.org/10.1039/c7fo01658a> (2018).
21. Chen, X. et al. Anti-fatigue effect of quercetin on enhancing muscle function and antioxidant capacity. *J. Food Biochem.* **45**, e13968. <https://doi.org/10.1111/jfbc.13968> (2021).
22. Tung, Y. T. et al. Antifatigue activity and exercise performance of phenolic-rich extracts from *Calendula officinalis*, *Ribes nigrum*, and *Vaccinium myrtillus*. *Nutrients* **11**, 1715. <https://doi.org/10.3390/nu11081715> (2019).
23. Lin, T. C. et al. Anti-fatigue, antioxidation, and anti-inflammatory effects of eucalyptus oil aromatherapy in swimming-exercised rats. *Chin. J. Physiol.* **61**, 257–265. <https://doi.org/10.4077/CJP.2018.BAG572> (2018).
24. Pal, S., Chaki, B., Chattopadhyay, S. & Bandyopadhyay, A. High-intensity exercise induced oxidative stress and skeletal muscle damage in postpubertal boys and girls: A comparative study. *J. Strength Cond. Res.* **32**, 1045–1052. <https://doi.org/10.1519/JSC.0000000000002167> (2018).
25. Yuan, T. et al. Anti-fatigue activity of aqueous extracts of *Sonchus arvensis* L. in exercise trained mice. *Molecules* **24**, 1168. <https://doi.org/10.3390/molecules24061168> (2019).
26. Shan, X., Zhou, J., Ma, T. & Chai, Q. *Lycium barbarum* polysaccharides reduce exercise-induced oxidative stress. *Int. J. Mol. Sci.* **12**, 1081–1088. <https://doi.org/10.3390/ijms12021081> (2011).
27. Liu, Z. et al. Effects of total soy saponins on free radicals in the quadriceps femoris, serum testosterone, LDH, and BUN of exhausted rats. *J. Sport Health Sci.* **6**, 359–364. <https://doi.org/10.1016/j.jshs.2016.01.016> (2017).
28. Azevedo, R. A., Silva-Cavalcante, M. D., Lima-Silva, A. E. & Bertuzzi, R. Fatigue development and perceived response during self-paced endurance exercise: State-of-the-art review. *Eur. J. Appl. Physiol.* **121**, 687–696. <https://doi.org/10.1007/s00421-020-04549-5> (2021).
29. Cao, Z. P. et al. Effects of cordycepin on spontaneous alternation behavior and adenosine receptors expression in hippocampus. *Physiol. Behav.* **184**, 135–142. <https://doi.org/10.1016/j.physbeh.2017.11.026> (2018).
30. Jangra, A. et al. Edaravone alleviates cisplatin-induced neurobehavioral deficits via modulation of oxidative stress and inflammatory mediators in the rat hippocampus. *Eur. J. Pharmacol.* **791**, 51–61. <https://doi.org/10.1016/j.ejphar.2016.08.003> (2016).
31. Chen, Y. et al. Anti-fatigue and anti-oxidant effects of curcumin supplementation in exhaustive swimming mice via Nrf2/Keap1 signal pathway. *Curr. Res. Food Sci.* **5**, 1148–1157. <https://doi.org/10.1016/j.crfs.2022.07.006> (2022).
32. Wan, T. et al. FA-97, a new synthetic caffeic acid phenethyl ester derivative, protects against oxidative stress-mediated neuronal cell apoptosis and scopolamine-induced cognitive impairment by activating Nrf2/HO-1 signaling. *Oxid. Med. Cell. Longev.* **2019**, 8239642. <https://doi.org/10.1155/2019/8239642> (2019).
33. Lee, H. W. Targeting cathepsin S promotes activation of the OLF1-BDNF/TrkB axis to enhance cognitive function. *J. Biomed. Sci.* **31**, 46. <https://doi.org/10.1186/s12929-024-01037-2> (2024).
34. Deepika, N., Rajendra Prasad, N. & Radhiga, T. Auranofin sensitizes breast cancer cells to paclitaxel mediated cell death via regulating FOXO3/Nrf2/Keap1 signaling pathway. *Cell Biochem. Funct.* **42**, e3903. <https://doi.org/10.1002/cbf.3903> (2024).

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Author contributions

C.F.C: Writing the original draft, formal analysis, and data curation. S.S.Z: Methodology, Data curation. C.C: Methodology, Data curation. Y.C.G: Methodology, Data curation. K.Z.D: Methodology, Data curation. G.Y.L: Methodology, Data curation. W.J: Methodology, Data curation. B.H: Methodology, Data curation. Z.H.H: Methodology, Data curation. Y.H.L: Methodology, Data curation. Z.Y.Z: Methodology, Resource. L.H.Y: Supervision, Project administration, Funding acquisition, Writing—review & editing, Conceptualization.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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