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Effect of Polysaccharide from *Cordyceps militaris* (Ascomycetes) on Physical Fatigue Induced by Forced Swimming

Yan-Feng Xu

Department of Physical Education, Xinxiang Medical College, 601 Jinsui Avenue, Hongqi District, Xinxiang 453000, China; Tel.: +86-18937393878; Fax: +86-0373-3029076; yanfengxuei@126.com

ABSTRACT: *Cordyceps militaris* is the one of the most important medicinal mushrooms, widely used in East Asian countries. Polysaccharide is considered to be the principal active component in *C. militaris* and has a wide range of biological and pharmacological properties. This study was undertaken to investigate the effect of polysaccharide from *C. militaris* (PCM) on physical fatigue induced in animals through a forced swimming test. The mice were divided into 4 groups receiving 28 days' treatment with drinking water (exercise control) or low-, medium-, and high-dose PCM (40, 80, and 160 mg/kg/day, respectively). After 28 days, the mice were subjected to the forced swimming test; the exhaustive swimming time was measured and fatigue-related biochemical parameters, including serum lactic acid, urea nitrogen, creatine kinase, alanine aminotransferase, aspartate aminotransferase, superoxide dismutase, glutathione peroxidase, catalase, malondialdehyde, liver glycogen, and muscle glycogen, were analyzed. The results showed that PCM could significantly prolong the exhaustive swimming time of mice; decrease concentrations of serum lactic acid, urea nitrogen, creatine kinase, aspartate aminotransferase, alanine aminotransferase, and malondialdehyde; and increase liver and muscle glycogen contents and the concentrations of serum superoxide dismutase, glutathione peroxidase, and catalase. The data suggest that PCM has an antifatigue effect, and it might become a new functional food or medicine for fatigue resistance.

KEY WORDS: antifatigue, biochemical parameters, *Cordyceps militaris*, forced swimming test, medicinal mushrooms, mice, polysaccharide

ABBREVIATIONS: ALT, alanine aminotransferase; **AST**, aspartate aminotransferase; **CAT**, catalase; **CK**, creatine kinase; **EC**, exercise control group; **GPx**, glutathione peroxidase; **LA**, lactic acid; **MDA**, malondialdehyde; **PCM**, polysaccharide from *Cordyceps militaris*; **PH**, high-dose PCM group; **PL**, low-dose PCM group; **PM**, medium-dose PCM group; **ROS**, reactive oxygen species; **SOD**, superoxide dismutase; **UN**, urea nitrogen

I. INTRODUCTION

Fatigue is a complex phenomenon that can be defined as difficulty initiating or sustaining voluntary activity.[1] In general, it can be divided into physical fatigue and mental fatigue. Physical fatigue is bodily weakness that can occur because of repetitive muscle activity. By contrast, mental fatigue is observed as a reduced efficiency of mental tasks.[2] Recent studies have revealed that 2 mechanisms, exhaustion and free radical theories, play important roles in physical fatigue.[3] Exhaustion theory suggests that a depleted energy source and excess metabolite accumulation can cause fatigue.[4] Radical theory suggests that intense exercise can produce an imbalance between the body's oxidation system and its antioxidation system. The accumulation of reactive free radicals (reactive oxygen species [ROS]) puts the body in a state of oxidative stress and injures the body by attacking large molecules and cell organs.[5] Since it is difficult to improve the available therapies for fatigue in modern medicine, natural antifatigue compounds with relatively low toxicity, which can reduce metabolite production and/or increase energy potential, are worth investigating.[6] Polysaccharides from herbal medicines and mushrooms are now considered to be a new sort of natural antifatigue compound.[7]

The genus *Cordyceps* belong to the family Clavicipitaceae of the order Hypocreales. More than 350 species worldwide are described as *Cordyceps*,

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of which about 120 species have been reported from China.[8] Some species of *Cordyceps*, such as *C. sinensis* (Berk.) Sacc. (=*Ophiocordyceps sinensis* (Berk.) G.H. Sung et al., Ophiocordycipitaceae, Ascomycetes), *C. militaris* (L.) Link, *C. gracilis* (Grev.) Durieu & Mon, *C. liangshanensis* M. Zang, D. Liu & R. Hu, *C. sobolifera* (Hill ex Watson) Berk. & Broome, and *C. guangdongensis* Li, Lin & Song, among others, have been traditionally used in many countries for health maintenance and the prevention and treatment of various diseases.[9,10] *C. militaris* belongs to the class of Ascomycetes.[11] It is one of the most important traditional Chinese medicines and folk tonic foods, and the second most commercialized medicinal mushroom species in East Asian countries.[12] *C. militaris* has been widely used as a tonic for its effectiveness in improving lung and kidney functions, restoring health after prolonged sickness, and enhanced physical performance.[13] It contains many active components such as polysaccharides, adenosine, and mannitol, among others.[14,15] Polysaccharides are considered the principal active components and found in the largest amounts in *C. militaris*.[13] Modern studies have reported that polysaccharides from *C. militaris* (PCMs) have a wide range of biological and pharmacological properties, such as anti-inflammatory,[12] antioxidant,[13] antihyperlipidemic,[14] antitumor,[16] antiaging,[17] immunomodulatory,[18–20] hypoglycemic,[21] and hepatoprotective[14] effects. However, there is little information on the antifatigue effect of PCMs. This study was designed to investigate the effect of PCM supplementation on physical fatigue using a forced animal swimming test.

II. MATERIALS AND METHODS

A. Materials

Dry cultured fruiting bodies of *C. militaris* were obtained from Hongyu Agricultural Science and Technology Co., Ltd (Xuzhou, China). The material was identified by Professor J.L. Wang, College of Life Science and Technology, Xinxiang Medical College (Xinxiang, China). A voucher specimen was deposited in the herbarium of Xinxiang Medical College.

B. Chemicals and Reagents

Assay kits for serum lactic acid (LA), urea nitrogen (UN), creatine kinase (CK), alanine aminotransferase (ALT), aspartate aminotransferase (AST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and malondialdehyde (MDA) were purchased from the Jiancheng Institute of Biotechnology (Nanjing, China). Assay kits for liver glycogen and muscle glycogen were purchased from Ruiqi Biological Engineering Research Center (Shanghai, China). All other chemicals and reagents, of at least analytical grade purity, were purchased from local suppliers.

C. Preparation of PCM

PCM was prepared using the method proposed by Lee and Hong[19] and Wang et al.,[22] with slight modification. Dried *C. militaris* was ground into powder, which was then defatted with ethanol for 10 hours and extracted twice with hot water (each time for 10 hours at 65 ± 5°C) . The resulting suspension was centrifuged (1509 × *g* for 15 minutes) and filtered through a 0.45-μm membrane. The filtrate was concentrated in a rotary evaporator under reduced pressure and precipitated with 95% (v/v) ethanol at 4°C for 24 hours. The precipitate was collected after centrifugation (1509 × *g* for 10 minutes), redissolved in distilled water, and the solution was again precipitated with 95% ethanol. The resultant precipitate was washed with acetone and ether, respectively, after suction and then lyophilized *in vacuo*. Polysaccharide content was determined using the phenol–sulfuric acid method, with D-glucose as a standard.

D. Animals and Breeding Conditions

Adult male Kunming mice (weighing 20 ± 2 g) were obtained from the Experimental Animal Center of Xinxiang Medical College (Xinxiang, China), and acclimatized for 1 week before being used. The mice had free access to standard rodent chow and tap water, and were housed under normal laboratory conditions (12-hour light/12-hour dark cycle, 25°C, 60% humidity). All experimental protocols were

prepared and performed based on the Guide for Care and Use of Animal Laboratory of Xinxiang Medical College, and were approved by the local ethics committee (approval no. XXMCAE 2013-0107).

E. Experimental Design and Treatment

After 1 week of adaptation, the mice were divided into 4 groups (n = 12): the exercise control group (EC), low-dose PCM group (PL), medium-dose PCM group (PM), and high-dose PCM group (PH). Animals in the PL, PM, and PH groups received various doses of PCM (40, 80, and 160 mg/kg body weight) dissolved in 1.0 mL of drinking water, while the EC groups received same volume of drinking water. The doses were intragastrically administered once daily for 28 days, and the body weights of mice were measured weekly. After 28 days, the mice in all the groups were subjected to the forced swimming test 30 minutes after the last administration. The test was carried out as previously described, with some modifications.[4,23,24] In brief, the mice were placed in an acrylic plastic pool (50 × 40 × 50 cm) filled with fresh water to a depth of 30 cm and kept at 25 ± 1°C. Every mouse was forced to swim with a lead block weighting approximately 5% of its body weight attached to the tail. point of exhaustion was defined as the animal dropping its head into the water within 7 seconds and being unable to break the water surface,[23] and the exhaustive swimming time was measured.

F. Biochemical Analysis Related to Fatigue

After the forced swimming test, the mice were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight), and blood samples were collected by quickly removing the eyeball from the socket. The serum was separated by centrifugation at room temperature (1006 × *g* for 10 minutes) to measure the serum concentrations of LA, UN, CK, ALT, AST, SOD, CAT, GPx, and MDA. Upon completion of blood collection, the mice were sacrificed by cervical dislocation, and the liver and skeletal muscle from both hind limbs were immediately removed, washed with physiological saline, and frozen in liquid nitrogen for storage at −80°C until required for tissue glycogen. All the biochemical parameters were determined according to the procedures provided by the assay kits.

G. Acute Toxicity Test

The acute toxicity test was carried out according to the guidelines of the Organization for Economic Co-operation and Development. Two groups of mice of both sexes (8 mice per group, 4 females and 4 males) weighing 20 ± 2 g were orally administered PCM at increasing dose levels of 175, 550, 2000, and 5000 mg/kg body weight. The mice were monitored for 14 days, and the general behavior and neurological profiles were observed.

H. Statistical Analysis

Results were expressed as the mean ± standard error of the mean. Statistical analysis was performed by 1-way analysis of variance followed by the Student *t* test. *P* values <0.05 were considered significant.

III. RESULTS

A. Effect of PCM on the Body Weights of Mice

As shown in Fig. 1, body weights were not significantly different between groups during the experiment. Therefore, PCM had no significant effect on the body weights of the mice.

B. Effect of PCM on the Exhaustive Swimming Time of Mice

As shown in Fig. 2, the exhaustive swimming time in the PL, PM, and PH groups was significantly longer ($P < 0.05$) than that in the EC group: 29.81%, 45.22%, and 70.39% higher, respectively.

C. Effect of PCM on the Serum LA and UN Concentrations in Mice

As shown in Fig. 3, the serum LA concentrations in the PL, PM, and PH groups were significantly lower ($P < 0.05$) than those in the EC group: 24.49%, 32.31%, and 41.69% lower, respectively. The serum

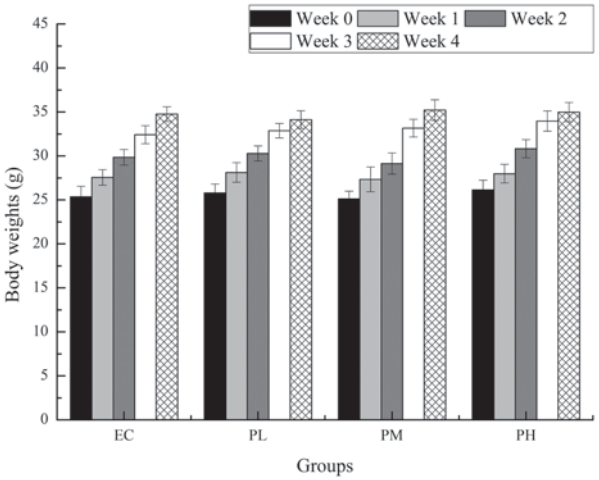

FIG. 1: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the body weights of mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg).

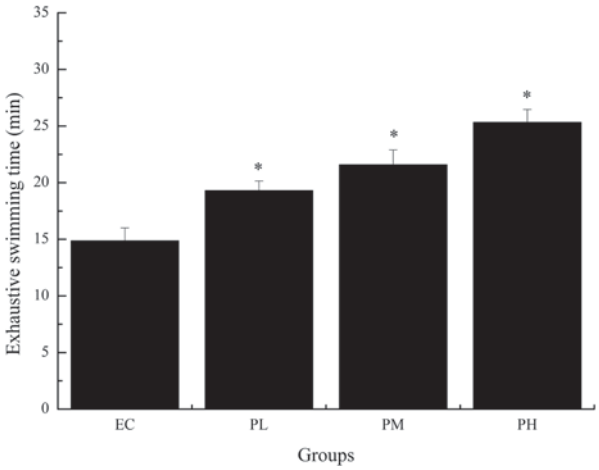

FIG. 2: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the exhaustive swimming time of mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg). *P < 0.05 compared to the EC group.

UN concentrations in the PM and PH groups were significantly lower (P < 0.05) than those in the EC group: 14.44% and 22.05% lower, respectively. Although the serum UN concentrations in the PL group also decreased, no significant difference was observed (P > 0.05).

D. Effect of PCM on the Tissue Glycogen Content in Mice

As shown in Fig. 4, the liver glycogen contents in the PL, PM, and PH groups were significantly higher (P < 0.05) than those in the EC group: 37.52%, 47.53% and 74.61% higher, respectively. The muscle glycogen contents in the PH group was significantly higher (by 24.13%; P < 0.05) than those in the EC group. Although the muscle glycogen contents in the PM and PL groups also increased, no significant difference was observed (P >0.05).

E. Effect of PCM on the Serum Cytosolic Enzyme Concentrations in Mice

As shown in Fig. 5, the serum CK concentrations in the PL, PM, and PH groups were significantly lower (P <0.05) than those in the EC group: 15.68%, 24.4%, and 28.94% lower, respectively. The serum AST concentrations in the PH group were significantly lower (by 19.65%; P <0.05) than those in the EC group. Although the serum AST concentrations in the PL and PM groups also decreased, no significant difference was observed (P >0.05). The serum ALT concentrations in the PM and PH groups were significantly lower (P <0.05) than those in the EC group: 18.12% and 24.51% lower, respectively. Although the serum ALT concentrations in the PL group also decreased, no significant difference was observed (P >0.05).

F. Effect of PCM on the Serum Antioxidant Enzyme Concentrations in Mice

As shown in Fig. 6, the serum SOD concentrations in the PL, PM, and PH groups were significantly higher (P <0.05) than those in the EC group: 27.67%, 38.83%, and 53.33% higher, respectively. The serum GPx concentrations in the PM and PH groups were significantly higher (P <0.05) than those in the EC group: 14.1% and 44.05% higher, respectively. Although the serum GPx concentrations in the PL group also increased, no significant difference was

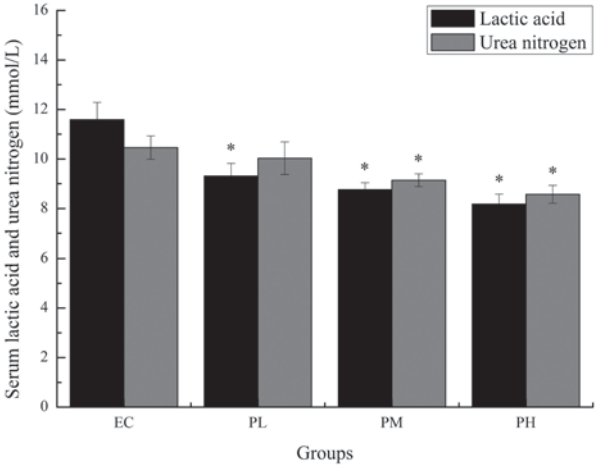

FIG. 3: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the serum lactic acid and urea nitrogen concentrations in mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg). **P* < 0.05 compared to the EC group.

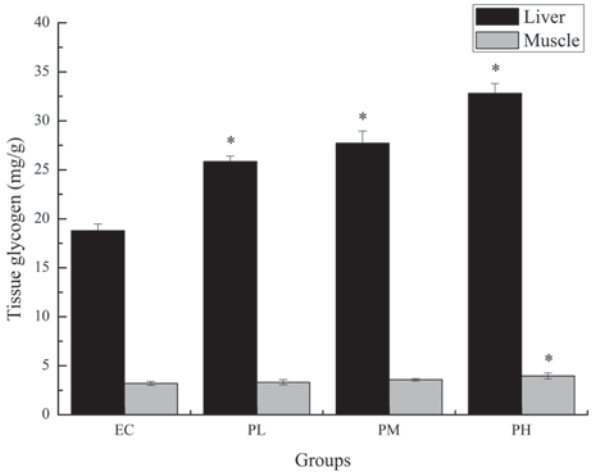

FIG. 4: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the tissue glycogen contents in mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg). **P* < 0.05 compared to the EC group.

observed (*P* >0.05). The CAT concentrations in the PL, PM, and PH groups were significantly higher (*P* <0.05) than those in the EC group: 29.93%, 57.75%, and 73.01% higher, respectively.

G. Effect of PCM on the Serum MDA Concentrations in Mice

As shown in Fig. 7, the serum MDA concentrations in the PL, PM, and PH groups were significantly lower (*P* <0.05) than those in the EC group: 29.36%, 37.1%, and 79.52% lower, respectively.

H. Acute Toxicity Studies

The general behavior of the treated mice appeared normal. No obvious symptoms of toxicity nor any significant changes were observed, and there were no deaths in any of the groups. Therefore, PCMs were considered practically nontoxic substances.

IV. DISCUSSION

During the past 2 decades, researchers have been looking for natural active products that not only can improve athletic ability, postpone fatigue, and accelerate the elimination of fatigue in human beings, but that also have few side effects.[7] Various natural compounds including peptides, polyphenols, flavonoids, alkaloids, triterpenoids, and polysaccharides from the animals, plants, and fungi sources have been found to possess an antifatigue effect. You et al.[5] found that loach peptide could increase endurance capacity and facilitate recovery from fatigue. Sun et al.[25] indicated that an egg white peptide fraction with a molecular weight 2–5 kDa possessed strong antioxidant activity and exhibited an antifatigue effect. Ma et al.[26] showed that salidroside had a significant antifatigue effect that was dose-dependent, and the strongest effect on most biomarkers was seen with an intermediate dose (180 mg/kg). Sheng et al.[27] indicated that polysaccharides of *Radix pseudostellariae* were beneficial to chronic fatigue syndrome, and the underlying mechanisms of action involved neuroendocrine and immune systems. Qi et al.[23] found that ginsenoside-Rb_1 exhibited a protective effect on exercise (swimming)–induced oxidative stress in mice. Tang et al.[28] reported that intranasal administration of ginsenoside-Rg_3 showed an

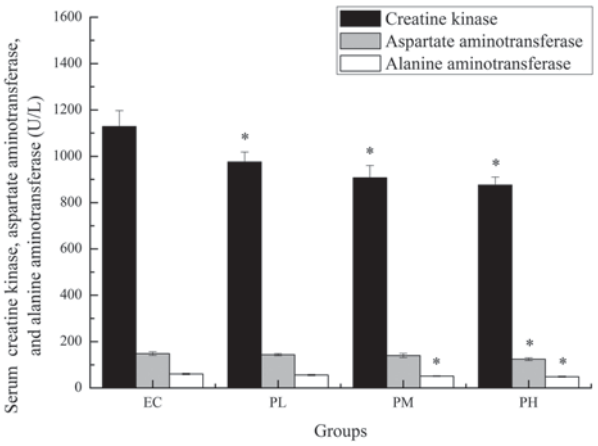

FIG. 5: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the serum creatine kinase, aspartate aminotransferase, and alanine aminotransferase concentrations in mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg). **P* < 0.05 compared to the EC group.

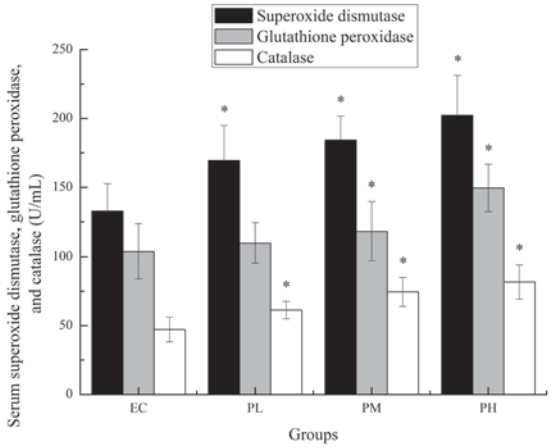

FIG. 6: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the serum superoxide dismutase, glutathione peroxidase, and catalase levels of mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg). **P* < 0.05 compared to the EC group.

antifatigue effect. The mechanism was related to an increase in the storage of hepatic glycogen and a decrease in the accumulation of metabolites such as lactic acid and serum urea nitrogen. Yu et al.[29] indicated that flavonoids from *Cynomorium songaricum* could not only reduce free radical formation and scavenge free radicals but also enhance endurance exercise performance by reducing muscle fatigue. Song et al.[30] indicated that a *C. militaris* extract had an antifatigue effect that attenuated the production of lactate, increased ATP levels, and reduced oxidative stress in animals. Yan et al.[31] showed that a *C. guangdongensis* extract could potently alleviate fatigue through reducing the accumulation of blood lactic acid; the refined polysaccharide was the functional constituent. Other recent studies have also shown that polysaccharides from *C. sinensis* have an antifatigue effect and could ameliorate exhaustive exercise–induced oxidative stress. The antifatigue effect of polysaccharides from *C. sinensis* was related to their antioxidant properties.[32,33] It could be hypothesized that PCMs might attenuate fatigue during exercise, because of the similar functions of polysaccharides from other *Cordyceps* species. Therefore this study was designed to investigate the effect of PCM supplementation on physical fatigue in mice, and the findings indicated that PCM could prolong the exhaustive swimming time of mice and had an antifatigue effect.

The forced swimming test is one of the most valid models for evaluating antifatigue effects on animals.[6] Other forced exercise tests, such as with a motor-driven treadmill or wheel, can injure the animals and may not be routinely acceptable.[34] Prolonged exhaustive swimming time in a test means a lessening of fatigue. To standardize the workload and reduce the swimming time, weights at specific body weight percentages were added to the chest or tail of the animal.[34] This study showed that the exhaustive swimming time in the PL, PM, and PH groups was significantly longer than that in the EC group. The results indicate that PCM had an antifatigue effect, the strongest of which was observed at a dose of 160 mg/kg.

LA is the product of carbohydrates under anaerobic conditions during glycolysis, and glycolysis is the main energy source for intense exercise in a short time.[35] The accumulation of serum LA

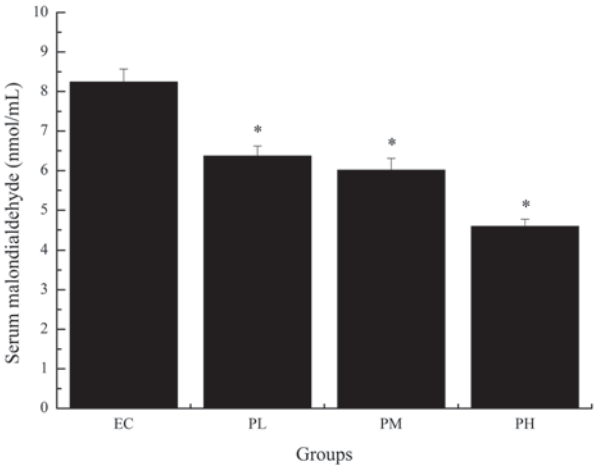

FIG. 7: Effect of polysaccharide from *Cordyceps militaris* (PCM) treatment for 28 days on the serum malondialdehyde levels of mice. Data are mean ± SD (n = 12 mice/group). EC, exercise control group; PL, PCM low-dose group (40 mg/kg); PM, PCM medium-dose group (80 mg/kg); PH, PCM high-dose group (160 mg/kg). *$P < 0.05$ compared to the EC group

during intense exercise might change the internal pH value, which could result in various biochemical and physiological side effects on glycolysis, phosphofructokinase, and muscular contractions caused by calcium ion release.[36] Thus the accumulation of LA could illustrate the speed and degree of fatigue development.[37] This study showed that the serum LA concentrations in the PL, PM, and PH groups were significantly lower than those in the EC group. The results indicate that PCMs could effectively retard and lower the serum LA produced, which might be another pathway through which PCM alleviates physical fatigue.

Serum UN is the metabolic outcome of proteins and amino acids and is a sensitive index to evaluate the bearing capability when a body is weighed down with a physical load.[33] After intense exercise, proteins and amino acids strengthen catabolic metabolism to cover the shortage of energy through sugar and fat catabolic metabolism, resulting in an additional increase of UN.[33,37] This study showed that the serum UN concentrations in the PM and PH groups were significantly lower than those in the EC group. The results indicate that PCMs could reduce the catabolic decomposition of protein for energy and thereby delay physical fatigue.

Glycogen, an important resource of energy, is used to complement the consumption of blood glucose during exercise and maintain blood glucose in a physiological range.[38] After intense exercise, muscle glycogen is exhausted, and later, energy forms circulating glucose released by the liver.[39] Fatigue happens when liver glycogen is consumed. Increasing stored glycogen or reducing glycogen consumption in the liver and muscle can enhance exercise endurance, delaying the onset of fatigue.[40] This study showed that the liver glycogen contents in the PL, PM, and PH groups, as well as the muscle glycogen contents in the PH group, were significantly higher than those in the EC group. The results indicate that PCMs made mice resistant to physical fatigue by improving the glycogen reserve, reducing glycogen consumption, or both. The reason for this might be related to the activation of energy metabolism.[39]

It is well known that intense exercise increases levels of various cytosolic enzymes, such as CK, AST, and ALT, in blood and causes significant tissue oxidative damage.[41] AST and ALT are not muscle-specific, but marked elevations are usually only associated with muscle damage, whereas CK is more muscle-specific, and increased levels reflect muscle damage.[42] Therefore, CK, AST, and ALT were used as important markers of tissue damage. This study showed that serum CK concentrations in the PL, PM, and PH groups, serum AST concentrations in the PH group, and serum ALT concentrations in the PM and PH groups were significantly lower than those in the EC group. The results indicate that PCMs attenuated exercise-induced oxidative damage by modifying cytosolic enzyme activities.

Intense exercise is associated with the accelerated generation of ROS, leading to oxidative stress, which can lead to damage or destroy cellular macromolecules such as lipids, proteins, and nucleic acids.[43] Therefore, oxidative stress plays a very important role in the occurrence of fatigue, oxidative damage, and physical performance.[44] To prevent exercise-induced oxidative stress, organisms are well equipped with antioxidant defense systems including enzymes and nonenzymatic substances

that act in synergy.[45] Principal antioxidant enzymes include SOD, GPx, and CAT. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. GPx is a selenoenzyme that catalyzes the reduction of hydroperoxides at the expense of reduced glutathione. CAT is a primary antioxidant defense component that works to catalyze the decomposition of hydrogen peroxide to water, sharing this function with GPx.[44] This study showed that the serum SOD and CAT concentrations in the PL, PM, and PH groups, and the serum GPx concentrations in the PM and PH groups, were significantly higher than those in the EC group. The results indicate that the antifatigue effect of PCMs probably occurred by increasing antioxidant enzymes activities.

Previous studies have shown that intense exercise-induced ROS could attack polyunsaturated fatty acids, which leads to lipid peroxidation, directly reflects the oxidative damage to a cell membrane, and also contributes to the pathophysiology of fatigue.[3,23,24] MDA, a secondary product generated during the oxidation of polyunsaturated fatty acids, has frequently been used as an indicator of the degree of lipid peroxidation.[3] This study showed that the serum MDA concentrations in the PL, PM, and PH groups were significantly lower than those in the EC group. The results indicate that PCMs were able to reduce lipid peroxidation and ameliorate physical fatigue

V. CONCLUSIONS

These findings indicate that PCMs could prolong the exhaustive swimming time of mice and had an antifatigue effect. The antifatigue mechanisms of PCMs might be attributable to various factors: (1) PCMs could eliminate the accumulation of detrimental metabolites and activate energy metabolism by dose-dependently decreasing serum LA and UN concentrations and dose-dependently increasing liver and muscle glycogen contents. (2) PCMs could attenuate exercise-induced oxidative damage by dose-dependently decreasing serum CK, AST, and ALT concentrations. (3) PCMs could protect exercise-induced oxidative stress by dose-dependently increasing serum SOD, GPx, and CAT concentrations and dose-dependently decreasing serum MDA concentrations. However, recent studies have shown that the biological activities of polysaccharides are highly related to their chemical structure, especially their molecular weight.[46] In this study, the relationships between PCMs with different molecular weight fractions and their antifatigue effects were not investigated. In addition, previous studies also confirmed that *C. militaris* extract had an antifatigue effect, and its molecular mechanism might be mainly through activating 5′-AMP-activated protein kinase and protein kinase B (AKT)/mammalian target of rapamycin pathways and regulating serum hormone levels.[30] Therefore, further study is warranted to elucidate the antifatigue effect of various molecular weight fractions of PCMs and their molecular mechanisms and antifatigue-related gene regulation through the above pathways. Taken together, the results of this study provide an important basis for developing PCMs as an antifatigue functional food or medicine for wide use.

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