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Protective Effects on Mitochondria and Anti-Aging Activity of Polysaccharides from Cultivated Fruiting Bodies of *Cordyceps militaris*

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Abstract: *Cordyceps militaris* (L.) Link is an entomopathogenic fungus parasitic to *Lepidoptera* larvae, and is widely used as a folk tonic or invigorant for longevity in China. Although *C. militaris* has been used in traditional Chinese medicine for millennia, there is still a lack convincing evidence for its anti-aging activities. This study was performed to investigate the effects of polysaccharides from cultivated fruiting bodies of *C. militaris* (CMP) on mitochondrial injury, antioxidation and anti-aging activity. Fruiting bodies of *C. militaris* were cultivated artificially under optimized conditions. The spectrophotometric method was used to measure thiobarbituric acid reactive substances (TBARS), mitochondrial swelling, and activities of scavenging superoxide anions *in vitro*. D-galactose (100 mg/kg/day) was injected subcutaneously into back of the neck of mice for 7 weeks to induce an aging model. The effects of CMP on the activities of catalase (CAT), surperoxide dismutase (SOD), glutathione peroxidase (GPx) and anti-hydroxyl radicals were assayed *in vivo* using commercial monitoring kits. The results showed that CMP could inhibit mitochondrial injury and swelling induced by Fe^{2+}-L-Cysteine in a concentration- dependent manner and it also had a significant superoxide anion scavenging effect. Moreover, the activities of CAT, SOD, GPx and anti-hydroxyl radicals in mice liver were increased significantly by CMP. These results indicate that CMP protects mitochondria by scavenging reactive oxygen species (ROS),

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inhibiting mitochondrial swelling, and increasing the activities of antioxidases. Therefore, CMP may have pharmaceutical values for mitochondrial protection and anti-aging. CMP was the major bioactive component in *C. militaris*.

Keywords: *Cordyceps militaris* Polysaccharides; Mitochondria; Anti-Aging; Reactive Oxygen Species; Antioxidase.

Introduction

In the Orient, some Cordyceps species are among the most potent herbs in traditional medicine and are widely used as a tonic for longevity, endurance and vitality (Park *et al.*, 2005). *Cordyceps militaris* (L.) Link, an edible and medicinal mushroom, is an entomopathogenic fungus parasitic to *Lepidoptera* larvae, which belongs to the class Ascomycetes and the DongChongXiaCao group in Chinese herbs. Besides its usage as a crude drug, it has been extensively used as folk tonic food or invigorant since ancient times (Wu *et al.*, 2000). Various constituents, such as nucleosides and polysaccharides, are associated with the pharmacological activities of the Cordyceps species (Shin *et al.*, 2001; Zhou *et al.*, 2008). The metabolites extracted from *C. militaris*, for example, cordycepin, cordycepic acid, superoxide dismutase (SOD) and polysaccharides, have been proven to have pronounced biological effects on the human body (Zhan *et al.*, 2006). Previous studies on polysaccharides from various Cordyceps species have demonstrated many interesting biological activities, including antitumour (Chen *et al.*, 1997; Xiao *et al.*, 2002), anti-influenza virus (Ohta *et al.*, 2007), immunopotentiation (Nakamura *et al.*, 1999), hypoglycemic activity (Kiho *et al.*, 1999) and hypocholesterolemic effect (Koh *et al.*, 2003). The medical merits of Cordyceps polysaccharides have drawn a great amount of research interest. The exopolysaccharides from cultured liquid of *C. militaris* were reported to have moderate anti-oxidative effects for lard (Li and Xu, 1997). The antioxidant activity of aqueous extract from cultivated *C. militaris* has also been found to scavenge hydroxyl and superoxide anion radicals (Zhan *et al.*, 2006). Scavenging of free radicals and anti- cancer activity were correlated closely with the quantities of polysaccharides (Wang *et al.*, 2005). The free radical theory of aging is based in the works of Gerschman and Harman, and, when focused around mitochondria, emerged as the mitochondrial hypothesis of aging (Vina *et al.*, 2003; Harman, 2006). It holds that during aging, an increase in ROS in mitochondria causes mutations in the mtDNA and damages mitochondrial components, resulting in senescence (Manczak *et al.*, 2005). Mitochondria are considered the pacemakers of tissue aging due to the continuous production of free radicals, oxygen, nitrogen free radicals and related reactive species, and the selective oxidative damage that leads to mitochondrial dysfunction (Navarro and Boveris, 2007). There have been no reports on the protective effect of the polysaccharides from cultivated fruiting bodies of *C. militaris* (CMP) on mitochondrial injury so far. Total polysaccharides in *C. militaris* thus may relate to these biological activities. In the present study, the mitochondrial protection and anti-aging activities of CMP were investigated and evaluated by its scavenging abilities on superoxide anion ($O_2^{\bullet -}$) and its effects on the activities of CAT, SOD, GPx and anti-hydroxyl radicals.

Materials and Methods

Animals and Materials

Male Kunming mice (Grade II, Certificate No. 2002-5), weighing 22 ± 2.0 g, were purchased from the Experimental Animal Center, Dalian University. All mice were cared for according to the Guiding Principles in the Care and Use of Animals. The experiment was approved by the Medical College Council on Animal Care Committee of Dalian University (China) in accordance with NIH guidelines (NIH, 2002). Rodent laboratory chow and tap water were available *ad libitum* during the period. Coomassie brilliant blue G-250 (CBBG-250) and N-methylphenazonium methyl sulfate (PMS) were purchased from Fluka (Bushs SG, Switzerland). Bovine serum albumin (BSA), 4-nitrobluetetrazolium chloride (NBT) and reduced nicotinamide adenine dinucleotide (NADH) were from the Boehringer Mannheim Corp. (Indianapolis, IN, USA). 2-Thiobarbituric acid (TBA) and 1, 1, 3, 3-tetraethoxypropane (TEP) were from Sigma Chemical (St Louis, MO, USA). N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (HEPES) was from Merck (Darmstadt, Germany). Tris (hydroxymethyl) aminomethane (Tris) was from Gibco BRL (Grand Island, NY, USA). Commercial catalase (CAT), surperoxide dismutase (SOD), glutathione peroxidase (GPx) and anti-hydroxyl radical monitoring kits were from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Vitamin E (Vit E) was from the Shanghai Xinyi Pharmaceutical Factory (Shanghai, China). D-galactose was from the Shanghai Second Reagent Factory (Shanghai, China). All other chemicals and solvents used in the study were of analytical grade made in China. The strain of *Cordyceps militaris* (registration no. 5.700) was purchased from the China General Microbiological Culture Collection Center (CGMCC) (Beijing, China).

Culture and Constituents Assay of Fruiting Bodies of Cordyceps militaris

The stock culture was maintained on potato-dextrose-agar slants. The slants were inoculated with mycelia and incubated at 25°C for 7 days and then used for seed culture inoculation. The seed culture medium (1 L) consisted of the following components: 20 g glucose, 1 g yeast extract, 3 g KH_2PO_4, 1.5 g $MgSO_4 7H_2O$ and 20 mg vitamin B_1. The mycelia of *C. militaris* were inoculated to the seed culture medium slant and cultivated at 25°C for 4 days under the light intensity of 600 Lux. The fine mycelia were selected to subculture three times. A mixture of millet and sorghum (1:1) was used as an artificial culture medium, which also contains 5 mg/l vitamin B_1, 0.1 g/l 2,4-dichlorophenoxyacetic acid (2,4-D), K^+, Mg^{2+} and Ca^{2+}. Soluble starch and peptone were used as a carbon and nitrogen source respectively. The relative humidity was 80% and the light intensity was 600 Lux. The cultivation temperature was changed to 25°C after the mycelia were filled the container at 15°C. After the fruit bodies of *C. militaris* were dried to a consistent weight at 45°C, they were weighed and ground into a fine powder. The crude fat, water-soluble proteins and carbohydrates in the dry fruit bodies are 2.98%, 23.01% and 14.20% respectively.

Animal Groups for in Vivo Experiments

Sixty mice were randomly assigned into 6 groups (Normal, Model, Vit E and CMP1, 2, 3. n = 10 for each group). CMP (40, 80, 160 mg/kg/day) was administered by oral gavage to mice in CMP 1, 2, 3 groups respectively. Vitamin E (100 mg/kg/day) was administered to mice in the Vit E group and an equivalent volume of normal saline to mice in the Normal and Model groups. All mice except those in the Normal group were administered D-galactose (100 mg/kg/day) subcutaneously, while the Normal group mice were given an equivalent volume of normal saline for 7 weeks. Each index was determined later.

Preparation of Cordyceps militaris Polysaccharides (CMP)

Powdered dry fruit bodies (200 mesh) of *C. militaris* were degreased with water-free ethanol in a Soxhlet extractor at 70°C for 7 hours in a water bath, the residue was extracted three times with 10 times volume of distilled water for 0.5 hour each in a boiling water bath, the aqueous extracts were mixed and centrifuged at 10,000 g for 15 min and the supernatant was condensed to 1 g crude drug/ml under decompression. Sevage reagents (ratio of chloroform and n-butanol was 4:1) were used to remove protein constituents in the concentrated liquid. Activated charcoal was added to a final concentration of 0.2% and boiled for 5 min. The decolored liquor was centrifuged at 10,000 g for 15 min and was sucked. 95% ethanol was added to the filtrate to a final concentration of 80%, and the solution was then refrigerated at 4°C for 24 hours. The precipitate was dissolved in distilled water after centrifugation at 10,000 g for 15 min. The solution was then precipitated with ethanol for a second time. The resultant precipitate was washed with acetone and ether respectively after suction and then lyophilized *in vacuo*. The polysaccharide content (89.86%) of the extracts was determined using the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Preparation of Liver Homogenate and Mitochondria

Mice were dislocated and their livers were excised immediately, weighed, placed in pre-cooled normal saline and made into 10% homogenate with a motor-driven glass homogenizer at 0°C and then stored on ice. Mitochondria were isolated by differential centrifugation using a modified protocol adapted from Fink *et al.* (2005). Mice livers were placed in precooled normal saline to wash off the blood on the surface, then they were placed in an ice-cold isolation medium (containing 0.25 M sucrose, 0.5 mM EDTA and 3 mM HEPES, pH 7.4) and homogenized with a motor-driven Teflon pestle on ice. After homogenization, samples were centrifuged at 1000 g for 10 min. A Beckman JA-25.50 rotor and Beckman Coulter Avanti J-E centrifuge were used in this and all other centrifugation steps at 4°C. Supernatants were removed and centrifuged at 12,000 g for 10 min. The pellets were washed twice in the isolation medium, and respun at 12,000 g each. Following the final wash, mitochondria were resuspended in the same medium and stored on ice until use. Protein determinations were carried out via the Bradford (1976) method, using BSA as a standard.

Assay of Thiobarbituric Acid Reactive Substances (TBARS)

The contents of TBARS in the liver mitochondria and homogenate were measured according to the method of Chen *et al.* (2007), with slight modifications. Mitochondrial protein (0.5 mg) or 0.20 ml of 10% liver homogenate in each tube was incubated with graded concentrations (1.5–24 mg/l) of CMP (the normal and model group were excluded) at 37°C for 5 min. Then, $FeSO_4$ (50 μM, not for the normal group) and L-cysteine (L-Cys) (0.2 mM) were added. 0.1 M PBS with a pH of 7.4 was added to a final volume of 2 ml (homogenate, mitochondria and CMP were not added to the blank reference tube). After incubating the mixture in a vibratory incubator at 37°C for 30 min, 0.5 ml of 20% trichloroacetic acid (TCA) was added to end the reaction. Two milliliters of supernatant were transferred to another tube after centrifugation (9000 g) for 10 min, 1.0 ml of 0.67% TBA was added and the tube was then heated in a boiling water bath for 10 min. After cooling with tap water, the absorbance at 532 nm was determined on an UV-visible spectrophotometer and the blank reference tube was used as zero. TBARS was measured by linear regression analysis of an aliquot using TEP as an external standard and the action of CMP was expressed by the inhibitory rate (IR%).

$$\mathrm{IR}\% = (\mathrm{TBARS}_{\mathrm{model}} - \mathrm{TBARS}_{\mathrm{CMP}})/(\mathrm{TBARS}_{\mathrm{model}} - \mathrm{TBARS}_{\mathrm{normal}}) \times 100\%.$$

Determination of Mitochondrial Swelling

The reactive system contains 0.5 mg mitochondrial protein, 0.5 mM $FeSO_4$ (excluding the normal group), 0.2 mM L-Cys and various concentrations of CMP (3 and 6 mg/l) (excluding the normal and model groups). PBS (0.1 M, pH 7.4) was used as a buffer system and the reactive system totaled a volume of 3 ml and was incubated at 37°C for 0, 5, 10 and 15 min. The absorbance (A) value of the system was measured at 520 nm (Hunter *et al.*, 1963).

Assay of Superoxide Anion Scavenging Activity

Superoxide anions generated in the NADH-NBT-PMS system was measured by the nitrobluetetrazolium (NBT) reduction method (Liu *et al.*, 2006), with slight modification. Tris-HCl buffer (16 mM, pH 8.0) containing NADH (73 μM) and NBT (50 μM) was mixed with various concentrations of CMP (1.5–24 mg/l) except the control group. The final volume was 3 ml. After adding PMS solution (15 μM, the blank was excluded) to the mixture, the reaction mixture was incubated at 25°C for 2 min, and the absorbance (A) at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The inhibitory rate (IR%) of superoxide anion production was expressed by $[1-(\mathrm{A}_{\mathrm{CMP}}/\mathrm{A}_{\mathrm{Control}})] \times 100\%$.

Determination on the Activities of CAT, SOD, GPx and Anti-Hydroxyl Radicals

Mice were sacrificed via dislocation and their livers were rapidly removed, weighed and made into 1% homogenates with a normal saline solution at 0°C. Two milliliters of homogenate was centrifuged at 2000 g for 5 min, 100 μl supernatant was mixed with 900 μl normal saline,

and then 10 μl of the solution was used to determine the CAT, SOD, GPx and anti-hydroxyl radical activities using commercial monitoring kits and following the manufacturer's protocol.

Statistical Analysis

Data were expressed as means $\pm$ SD and statistical differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) *post hoc* multiple comparisons test using the statistical software package SPSS 16.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Results were considered statistically significant at the probability (p) values < 0.05 level.

Results

Effect of CMP on the Formation of TBARS in Mice Liver Mitochondria and Homogenate in Vitro

Oxidative damage was studied by measuring lipid peroxidation (LPO) products, such as TBARS. The TBARS level, used as an auto-oxidation index of LPO in tissues, is used to screen the antioxidants. TBARS is a main marker of LPO. A measurement of TBARS content may directly reflect the production condition of free radicals (Suji and Sivakami, 2004). Pro-oxidants and reactive oxygen species (ROS) result in LPO in mitochondria (Armstrong, 2006). It has been shown that the amount of lipid peroxides in mitochondria increases with age (Hruszkewycz, 1992). Peroxidation of membrane lipids has been suggested to be one of the major causes of decreased mitochondrial membrane function (Paradies *et al.*, 1997). In fact, peroxidation alters the structure of membrane lipids, which can disrupt the structural organization of the lipid double layer, altering membrane fluidity and permeability (Valls *et al.*, 2005). LPO reactions are generally chain reactions driven by free radicals in which one radical can induce the oxidation of a comparatively large number of substrate molecules, particularly the highly vulnerable polyunsaturated fatty acids (PUFAs) (Abuja and Albertini, 2001). Unsaturated lipids in liver tissue are very susceptible to peroxidation when they are exposed to pro-oxidative metal ions such as Fe^{2+}. It has long been thought that Fe^{2+} is the most likely active species, producing oxidants through the interaction of Fe^{2+} with oxygen. In the current investigation, we have incubated the mouse liver mitochondria and liver homogenate with or without the presence of Fe^{2+}, and examined the effects on liver mitochondria and liver homogenate by measuring the absorbance at 532 nm. The results showed that formation of TBARS in liver mitochondria and liver homogenate, which was enhanced significantly following treatment with Fe^{2+}-L-Cys, was inhibited in a concentration-dependent manner in the presence of CMP (Table 1), indicating that CMP possess the antioxidant activity.

Effect of CMP on Liver Mitochondrial Swelling

Permeability and fluidity of the mitochondrial membrane are prerequisites for maintaining mitochondrial functions. The status of the mitochondrial membrane potential, which is an

Table 1. The Effects of CMP on Formation of TBARS in Mice Liver Mitochondria and Homogenate

Group	Concentration (mg/L)	TBARS (Mitochondria) (nmol/mg Protein)	IR%	TBARS (Homogenate) (nmol/g· Liver)	IR%
Normal	—	$0.26 \pm 0.11^{***}$	—	$50 \pm 16^{***}$	—
Model	—	2.59 ± 0.60	—	176 ± 42	—
CMP	1.5	$1.92 \pm 0.44^{**}$	28.76	163 ± 30	10.32
	3.0	$1.51 \pm 0.30^{***}$	46.35	148 ± 25	22.22
	6.0	$1.06 \pm 0.35^{***}$	65.67	$124 \pm 37^{**}$	41.27
	12.0	$0.73 \pm 0.30^{***}$	79.83	$105 \pm 28^{***}$	56.35
	24.0	$0.50 \pm 0.26^{***}$	89.70	$88 \pm 23^{***}$	69.84

Notes: All values are mean ± SD ($n = 6$). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the model group. CMP: *Cordyceps militaris* polysaccharides; TBARS: thiobarbituric acid reactive substances; IR: inhibitory rate.

estimate of the electrochemical gradient across the inner mitochondrial membrane, and thus the mitochondrial permeability transition pore (MPTP) opening. ROS can stimulate the opening of MPTP (Halestrap *et al.*, 2004). Mitochondrial swelling is a sensitive index for surveying the permeability of the membrane and assessing mitochondrial functions. Mitochondria are abundant in PUFA, which react easily with free radicals to form lipid peroxides. Mitochondrial oxidative stress has been implicated in cell death (Orrenius *et al.*, 2007). High levels of pro-oxidants produced by mitochondria can induce apoptosis by changing cellular redox status, which depletes reduced glutathione (GSH) (Kroemer *et al.*, 1998). Pro-oxidants and ROS result in the opening of MPTP in mitochondria (Armstrong, 2006). LPO can lead to an increase in membrane permeability, thus initiating mitochondrial swelling. The generation of oxygen free radicals was induced by Fe^{2+}-L-Cys and can result in the swelling of mitochondria. The decrease of absorbance (A) value at 520 nm indicates the swelling of mitochondria. CMP inhibited Fe^{2+}-L-Cys induced mitochondrial swelling significantly, and the inhibitory potency was stronger when the concentration of CMP was higher (Table 2).

Table 2. The Effect of CMP on Mice Liver Mitochondrial Swelling Induced by Fe^{2+} -L-Cys

Group	Concentration (mg/L)	A_{520} 0 min	A_{520} 5 min	A_{520} 10 min	A_{520} 15 min
Normal	—	$0.432 \pm 0.045^{***}$	$0.355 \pm 0.036^{***}$	$0.284 \pm 0.030^{***}$	$0.224 \pm 0.038^{***}$
Model	—	0.237 ± 0.032	0.187 ± 0.049	0.155 ± 0.023	0.136 ± 0.029
CMP	3.0	0.276 ± 0.036	$0.254 \pm 0.031^{**}$	$0.205 \pm 0.029^{**}$	$0.179 \pm 0.026^{*}$
	6.0	$0.302 \pm 0.029^{**}$	$0.296 \pm 0.038^{***}$	$0.251 \pm 0.035^{***}$	$0.217 \pm 0.027^{***}$

Notes: The decrease of absorbance value at 520 nm indicates the swelling of mitochondria. All values are mean ± SD ($n = 6$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the model group. CMP: *Cordyceps militaris* polysaccharides; A_{520}: absorbance value at 520 nm.

Table 3. The Scavenging Effect of CMP on $O_2^{\bullet -}$ Produced by NADH-NBT-PMS System

Group	Concentration (mg/L)	$A_{560\,nm}$	IR%
Control	—	0.255 ± 0.034	
CMP	1.5	$0.209 \pm 0.027^{*}$	18.04
	3.0	$0.184 \pm 0.026^{***}$	27.84
	6.0	$0.159 \pm 0.042^{***}$	37.65
	12.0	$0.120 \pm 0.034^{***}$	52.94
	24.0	$0.086 \pm 0.029^{***}$	66.27

Notes: All values are mean ± SD (n = 6). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. the control group. CMP: *Cordyceps militaris* polysaccharides; A_{560}: absorbance value at 560 nm; IR: inhibitory rate.

Effect of CMP on the Generation of Superoxide Anion

The mitochondrial electron transport chain (ETC) consumes more than 90% of the oxygen taken up by the cells and up to 5% of that is converted into superoxide anions ($O_2^{\bullet -}$), even during a normal physiological state (Leeuwenburgh and Heinecke, 2001). The primary ROS generated in the mitochondria is $O_2^{\bullet -}$, which are then converted to hydrogen peroxide (H_2O_2) by spontaneous dismutation or superoxide dismutase (SOD) (Brookes, 2005). The superoxide anions were generated by the NADH-NBT-PMS system *in vitro* in the current study. The absorbance (A) value at 560 nm of the CMP group decreased significantly compared to the control group. The present studies show that CMP scavenged superoxide anions concentration-dependently (Table 3).

Effect of CMP on the CAT, SOD, GPx and Anti-Hydroxyl Radical Activities

GPx, which is probably the best studied mitochondrial antioxidant enzyme, plays an important role in the decomposition of H_2O_2 produced in mitochondria. GPx catalyzes H_2O_2 and ROOH reduction by GSH [$H_2O_2(ROOH) + 2GSH => GSSG + 2H_2O(ROH + H_2O)$] and is an unique enzyme that uses H_2O_2 in the mitochondria of most mammalian organs (Chance *et al.*, 1979). In fact, GPx activity seems to exceed that of any competing H_2O_2 scavenger in mitochondria (Cadenas and Davies, 2000). Although it is mostly present in the peroxisomes, CAT might also play a role in the decomposition of mitochondrial H_2O_2 to H_2O (Cadenas and Davies, 2000). The recent work by Schriner *et al.* (2005) clearly demonstrates that mitochondrially targeted CAT decreases free radicals (mainly H_2O_2), leads to reduced mitochondrial oxidative damage, and increases the lifespan of CAT transgenic mice, suggesting that overexpressed CAT in mitochondria decreases ROS and boosts the functioning of mitochondria.

The mitochondrial matrix contains a specific form of SOD, manganese superoxide dismutase (MnSOD), which eliminates the superoxides produced in the matrix (Turrens, 2003) by facilitating the dismutation of the superoxide radical to H_2O_2. It thereby protects the mitochondrial iron-sulfur cluster-containing enzymes from superoxide attack

Table 4. The Effects of CMP on the CAT, SOD, GPx and Anti-Hydroxyl Radical Activities in Mice Liver *in Vivo*

Group	Dose (g/kg/day)	CAT (U/mg Protein)	SOD (U/mg Protein)	GPx (U/mg Protein)	Anti-•OH (U/mg Protein)
Normal	0	$13.8 \pm 2.6^{***}$	$279 \pm 37^{***}$	$54.3 \pm 6.8^{***}$	$96.9 \pm 13.1^{***}$
Model	0	8.6 ± 2.3	215 ± 31	41.5 ± 7.3	72.5 ± 12.7
Vit E	0.1	$11.3 \pm 1.6^{**}$	$262 \pm 23^{***}$	$50.3 \pm 5.7^{**}$	$87.4 \pm 6.8^{**}$
CMP1	0.04	$10.5 \pm 1.2^{*}$	238 ± 25	46.3 ± 6.8	78.8 ± 6.0
CMP2	0.08	$13.2 \pm 2.2^{***}$	$266 \pm 28^{***}$	$50.7 \pm 5.7^{**}$	$88.7 \pm 8.5^{***}$
CMP3	0.16	$14.6 \pm 2.4^{***}$	$287 \pm 36^{***}$	$58.1 \pm 8.3^{***}$	$97.6 \pm 13.4^{***}$

Notes: All values are mean ± SD ($n = 10$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the model group. CMP: *Cordyceps militaris* polysaccharides; CAT: catalase; SOD: surperoxide dismutase; GPx: glutathione peroxidase; Anti-•OH: anti-hydroxyl radical; Vit E: vitamin E.

(Gardner *et al.*, 1995). The lipophilic radical scavenger alpha-tocopherol, which is present in mitochondrial membranes, also has a role in interfering with the propagation of free radical-mediated chain reactions, thereby protecting membrane lipids from peroxidation (Andreyev *et al.*, 2005). CAT, SOD, GPx and anti-hydroxyl radical activities in mice liver homogenate of the model group mice decreased significantly *versus* the normal ones, and CMP increased CAT, SOD, GPx and anti-hydroxyl radical activities in a dose-dependent manner (Table 4).

Discussion

Mitochondria are a major source of ROS, which are a byproduct of mitochondrial electron transfer activity. As much as 0.2–2% of the molecular oxygen consumed by mitochondria during respiration is converted to superoxides, primarily by Complexes I and III (Turrens, 2003; Chen *et al.*, 2003). Normally, superoxides are detoxified by the combined activities of the mitochondrial antioxidant enzymes MnSOD, CAT and GPx (Nordberg and Arner, 2001; Giordano, 2005). $O_2^{\bullet -}$ is the precursor of most ROS and it is a mediator in oxidative chain reactions. Dismutation of $O_2^{\bullet -}$, either spontaneously or through a catalytic reaction by SOD, produces hydrogen peroxide (H_2O_2), which in turn may be fully reduced to water by CAT and GPx, or partially reduced to a hydroxyl radical (•OH), one of the strongest oxidants in nature (Turrens, 2003). •OH may be re-reduced by $O_2^{\bullet -}$ and may propagate the entire ROS process (Reddy, 2006). As a consequence of their biological functions, mitochondria are always exposed to ROS production and have a complex antioxidant defense system to counteract it. Oxidative stress occurs when the homeostatic balance between oxidant and antioxidant capacities in a determined biological system is disturbed and the redox state becomes more prooxidizing (Droge, 2002). Thus, under conditions of oxidative stress where mitochondrial ROS production exceeds the antioxidant capacity, mitochondria may suffer oxidative damage to their biomolecules.

The present study shows that CMP could inhibit mitochondrial injury in a concentration-dependent manner and also have a significant $O_2^{\bullet -}$ scavenging effect. Oxidative

damage was determined by the content of TBARS and mitochondrial swelling. CMP could obviously inhibit the generation of TBARS in liver tissue and mitochondria, indicating that it can protect the body from LPO significantly. Moreover, the activities of CAT, SOD, GPx and anti-hydroxyl radical in mice liver homogenate were increased significantly by CMP. CMP protected mitochondria by scavenging reactive oxygen species, and increasing the activities of the antioxidases. CMP ameliorated mitochondrial dysfunction. Therefore, CMP has antioxidant and anti-aging pharmaceutical values. CMP was the major bioactive component of *C. militaris*.

Cellular antioxidant systems have been traditionally divided into two categories: enzymatic and nonenzymatic. Primary antioxidant enzymes include SOD, GPx and CAT (Beckman and Ames, 1998). Non-enzymatic antioxidants, such as vitamin E (α-tocopherol), directly scavenge superoxides and •OH, as well as singlet oxygen (Yu, 1994). The increase in mitochondrial oxidative damage in brain and liver mitochondria of aged and senescent mice was ameliorated by vitamin E supplementation (Navarro *et al.*, 2005). Vitamin E is the major lipid-soluble chain-breaking antioxidant in mammals, and it plays an important role in normal development and physiology (Cuddihy *et al.*, 2008). Alpha-tocopherol has a potency to increase the activities of free radical-scavenging enzymes and it is a potent antioxidant that significantly reduced the LPO levels (Gulcin *et al.*, 2005; Chitra and Shyamala Devi, 2008). The oral supplementation of vitamin E was found to increase the activities of CAT, SOD, GPx and anti-hydroxyl radical in the current study. The ability of CMP to increase CAT, SOD, GPx and anti-hydroxyl radical activities is comparable to vitamin E.

Our work also clearly demonstrates that a low concentration of CMP was effective in scavenging free radicals.. This result suggests that polysaccharides are important in free radical scavenging. The antioxidant mechanism of polysaccharides is not clear. Tsiapali *et al.* (2001) speculated that the abstraction of anomeric hydrogen from monosaccharides was the reason for the free radical scavenging ability. Polysaccharides have enhanced antioxidant activity over monosaccharides because polysaccharides exhibit the greater ease of abstraction of the anomeric hydrogen from one of the internal monosaccharide units. CMP may be a potent hydrogen donator. The data presented here further suggests the biological function of polysaccharides. An acidic polysaccharide (APS) was isolated from the extract of *C. militaris*. Analyses of sugar composition indicated that APS consisted of D-galactose, L-arabinose, D-xylose, L-rhamnose, and D-galacturonic acid (Ohta *et al.*, 2007). However, the sugar components of CMP need further studies.

As for the difference and variation between cultivated and wild type *C. militaris*, the total amino acid and 8 essential amino acid content in *C. militaris* cultivated in natural *Bombyx mori* medium is higher than that of wild and otherwise cultivated *C. militaris* (Liu *et al.*, 1999). All 19 kinds of amino acids in fruiting bodies of wild *C. militaris* exist in cultivated one. The ratio of total amino acid, cordycepic acid, cordycepin and cordyceps polysaccharide content in wild and cultivated *C. militaris* is 1.5, 1.06, 1.54 and 0.42 respectively (Shun *et al.*, 1999). The total content of nucleosides is much higher in cultured cordyceps ($3722 \pm 1446\,\mu g/g$ for *C. militaris*) than in natural ones ($2167 \pm 412\,\mu g/g$). Cordycepin, abundant in cultured *C. militaris* ($2276.5 \pm 842.6\,\mu g/g$), is found very low in natural *C. sinensis* (Yang *et al.*, 2009). Feng *et al*. (2009) investigated genetic and chemical

variations of two natural *C. sinensis*, 16 fungal strains isolated from *C. sinensis*, and two fungal strains of *C. militaris*. Natural and cultured Cordyceps were in two individual subgroups according to their nucleoside content, which suggested that the chemical characteristics among cultured mycelia of different fungal strains isolated from natural *C. sinensis* were similar, but they were different from the natural one. The scavenging ability on hydroxyl radicals from the stroma of cultivated *C. militaris* was not inferior to the wild *C. militaris*, however, that of the fermented mycelium from cultivated *C. militaris* was stronger than the wild one (Wang, 2002). The SOD enzyme vitalities in the mycelium and stroma of wild *C. militaris* were all higher than those in corresponding parts in cultivated *C. militaris*, but their difference was little (Wang and Xue, 2008). These results demonstrate that wild *C. militaris* can be replaced by the artificially cultivated one concerning its functions for health and treatment.

C. militaris may be potentially useful for the prevention and treatment of various pathologies in humans including cancers, inflammation, fibrosis, diabetes, and hypercholesterolemia (Shimada *et al.*, 2008). Our present findings raise the possibility that *C. militaris* may be useful for the treatment of mitochondria-related metabolic disorders and delaying the aging process. A clinical feedback to human medication from this animal study results awaits further studies.

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