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Anti-Oxidation of Cultured *Cordyceps militaris* Growing on Silkworm Pupa

Cheng-Liang Gong*, Zhong-Hua Pan, Xiao-Jian Zheng, Ren-Yu Xue and Guang-Li Cao
Life Science College, Suzhou University, Suzhou, 215123, P. R. China.

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Cordyceps, one of the well-known traditional Chinese medicines, consists of the dried fungus *C. sinensis* growing on the larva of caterpillar. It is commonly used as a tonic of restoring vital body functions. In order to evaluate the pharmacological effects of *C. militaris*, the anti-oxidation and antiaging effect of cultured *C. militaris* growing on pupa of silkworm *Bombyx mori*, were investigated. The results showed that the contents of lipofusin in myocardium were reduced and the SOD activity in the liver were elevated in a dose-dependent manner while no effect was observed for blood GSH-Px activity and liver MDA content in mice perfused with the cultured *C. militaris* compared to those untreated mice. Furthermore, the average life span, maximum life span and 50% death time of the fruit flies treated with cultured *C. militaris* were markedly prolonged. The results suggest that the cultured *C. militaris* growing on pupa of silkworm possesses anti-oxidation and delay senescence activity.

Key words: *Cordyceps militaris*, Senescence delay, Antioxidation

Introduction

Cordyceps, one of the most valuable traditional Chinese medicines, consists of the dried fungus *C. sinensis* growing on the larva of caterpillar. It is also known as "summer-grass and winter worm" because of its appearance in different seasons. The parasitic complex of the fungus and the caterpillar is found in the soil of a prairie at an elevation of 3,500 to 5,000 meters. It is commonly used in China for over two thousands years to improve the function of kidney and lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthenia after severe illness, respiratory disease, renal dysfunction and renal failure, arrhythmias and other heart disease, as well as liver disease (Zhu *et al.*, 1998). Recent studies have demonstrated its multiple pharmacological actions in potentiating the immune system (Xu *et al.*, 1992) and the antitumor activity (Chen *et al.*, 1997). The anti-oxidation activity of Cordycepes was also reported and it was indeed used commonly in China for preventing aging and improving physical performance (Yu and He, 1998; Zhu *et al.*, 1998).

However, the natural Cordyceps (wild *C. sinensis*) is rare and very expensive. In China, the life cycle of Cordyceps has been extensively examined with the aim of developing techniques for isolating fermentable strains of *C. sinensis*. To date, several mycelial strains have been isolated from natural Cordyceps, and they could be manufactured at a large scale by fermentation technology (Yin and Tang, 1995). The mycelial fermentation products have been demonstrated to have a stronger pharmacological efficacy than the natural Cordyceps (Yu and He, 1998).

In addition, *C. militaris*, alias North aweto, is a typical species of Cordyceps family. *C. militaris* has been considered as a substitute of *C. sinensis* because there is no significant difference in the chemical composition and physiological function between *C. sinensis* and *C. militaris* (Pang *et al.*, 1996; Gong *et al.*, 1997). In order to evaluate the pharmacological effects of *C. militaris*, the antiaging effect of *C. militaris* growing on the pupa of silkworm *Bombyx mori*, was investigated. The current study examined its effects of *C. militaris* on the content of lipofuscin, lipid peroxidation, the activity of SOD (Superoxide dismutase) and GSH-Px (Glutathione peroxidase) in senile mice and the life span of *Drosophila* melanogaster.

*To whom correspondence should be addressed.
Life Science College, Suzhou University, Suzhou, 215006, P. R. China. Tel: 86-512-65880183; Fax: 86-512-65880183; E-mail: chlgong@tom.com

Materials and Methods

Cultured *C. militaris*

The *C. militaris* growing on silkworm pupa were obtained via artificial cultivation as described previously (Gong *et al*., 1994). In brief, the silkworm pupa (Breed Su5 × Su6) inoculated with fungi *C. militaris* were incubated for 40 days under imitating naturally ecological environment of the aweto (20 – 22°C, relative humidity ≥ 95%, natural light). The cultured *C. militaris* was dried at 60°C and ground into powder.

Mice preparation

Male Kunming mice at 10 months old were purchased from Animal Center of Chinese Medical Science Academy. The significant diversity of the body weights of mice was not showed among all groups with T test ($P < 0.05$). The mice were divided into three experimental groups randomly and were perfused with cultured *C. militaris* at doses of 0.05, 0.1, 0.3 g/kg/day for 50 days, respectively. Control group was treated with distilled water only.

Determination of lipofuscin

The content of myocardium lipofuscin was measured according to the method of Chen (1996) after mice were sacrificed by decapitation. Myocardium lipofusin was extracted with chloroform and methanol mixture (2 : 1) and measured using spectrophotometer at 360 nm of excitated wavelength and 450 nm of emission intensity. A solution of 0.1 μg/ml of quina in 0.1 M sulfuric acid and the mixture of chloroform and methanol (2 : 1) were used as the standard and blank control, respectively. The content of lipofuscin was expressed as μg/g of tissue and calculated as follows: myocardium lipofuscin content (μg/g) $= SFI - BFI/QFI \times CQ \times VW$; where SFI, BFI and QFI were the fluorescence intensity of sample, blank and standard control, respectively. CQ was the concentration of quina; V was the volume of homogenate; W was the weight of sample.

Determination of superoxide dismutase (SOD) activity

Ten percent of liver tissue homogenate was prepared with Tris-HCl buffer (0.1 M, pH 8.2) and separated by centrifugation at 13,000 rpm for 10 min at 4°C. The supernatant was used for the measurement of SOD using the enzymatic method as described previously (Marklund and Marklund, 1974). In brief, the inhibition of auto-oxidation of pyrogallol in alkaline (pH 8.2) was measured as a function of time. The rate of inhibition was determined and 50% inhibitory concentration was calculated. The amount of enzyme required to obtain 50% inhibition was considered as one unit of SOD activity.

The stock pyrogallol solution (45 mM) was stored at 25°C. The assay mixture contained 4.5 ml of Tris-HCl buffer (0.1 M Tris-HCl, 1 mM EDTA-Na_2, pH 8.2) and 0.5 ml of the liver supernatant. The blank contained 4.5 ml of assay buffer and 0.5 ml of water. The absorbance of each sample was measured at 325 nm before addition of pyrogallol. The increase in absorbance was measured every 30 sec for 4 min. SOD activity was expressed as U/mg liver.

Lipid peroxidation

Malondialdehyde (MDA) production was used as an index of lipid peroxidation. It was determined by the thiobarbituric acid (TBA) assay of Mihara and Uchiyama (1978). Ten percent of liver homogenate was prepared with the solution (pH 7.4) containing 0.15 M KCl and 5 mM Tris-maleate. One ml of homogenate was mixed with 2.0 ml TCA-TBA (0.335% TBA in 50% trichloroacetic acid) and then was separated by centrifugation at 1,500 rpm for 10 min. The supernatant was heated in a boiling water bath for 8 min, and cooled down to room temperature. The absorbance of the sample was measured at 535 nm. All values were expressed as pmoles MDA/mg liver.

Determination of glutathione peroxidase (GSH-Px) activity

The activity of glutathione peroxidase (GSH-Px) was determined by quantifying the rate of oxidizing reduced glutathione (GSH) to oxidized glutathione (GSSG) by hydrogen peroxide (Mills, 1959). The reaction mixture contained 0.4 ml of 1 mM GSH in 0.4 M phosphate buffer (pH 7.0), 0.4 mM EDTA, 0.5% of 0.01 M sodium azide and 0.4 ml of sample (A mixture of 10 μl blood and 1 ml distilled water). After 10 min of incubation at 37°C, 0.2 ml of prewarmed hydrogen peroxide (1.5 mM) was added and incubated for another 3 min. It was then mixed with 4.0 ml of meta phosphoric acid precipitating solution (1000 ml of solution contains 16.7 g of HPO_3, 0.5 g of EDTA and 280 g of NaCl) and centrifuged at 3,000 rpm for 10 min to obtain a protein free filtrate. The GSH in the protein free filtrate was then determined by mixing 2.0 ml of filtrate with 2.5 ml of 0.4 M Na_2HPO_4 and 0.5 ml of 5,5' dithiobis-(2-nitrobenzoic acid) (DTNB) reagent (DTNB: 0.4 mg/ml, sodium citrate: 1%). Absorbance of this solution was measured at 412 nm. A blank was carried through the incubation simultaneously with the samples since non-enzymatic GSH oxidation by hydrogen peroxide occurs during incubation. An enzyme unit of activity was defined as a decrease in 1 mM GSH per min after the decrease in 1 mM GSH per min of non-enzymatic reaction was subtracted. GSH-Px activity was then expressed as U/mg liver.

Test on *Drosophila* melanogaster life

One control and three treatment groups were carried out. The control diet contained 10% corn flour, 10% brown sugar, 1.5% agar, 0.3% benzoic acid, 1% dried yeast powder, and 77% water; pH 7.0). The treatment diets contained 0.2%, 1.0% and 5.0% of the cultured *C. militaris*, respectively. *D. melanogaster* were purchased from Life Science College of Peking University. Eight hr after hatching, the *D. melanogaster* adults were collected and narcotized with ether. Each group had 100 male fruit flies and 100 female fruit flies and they were divided into test tubes of 25 each, respectively. The flies were kept at 25 ± 1°C and the survived and died fruit flies were counted every day. The average life span, maximum life span and half death time were calculated.

Results and Discussion

Fatty acids are important components of biological membranes, in which they serve as building blocks. The unsaturated fatty acids, in particular, impart desirable properties upon the fluidity of membranes. However, these fatty acids are sensitive to oxygen induced damage mediated by lipid peroxidation, and thus may lead to a decrease in the membrane fluidity and disrupt membrane structure and function (Machlin and Bendich, 1987; Slater *et al.*, 1987). The amounts of malondialdehyde (MDA) and lipofusin produced in tissues were used as indexes of lipid peroxidation. To evaluate the anti-oxidation effect of the *C. militaris* growing on the pupa of silkworm *Bombyx mori*, lipid peroxidation in the liver and lipofusin content in myocardium were assessed in senile mice treated with cultured *C. militaris* at different doses for 50 days. Table 1 shows the results of the effects of cultured *C. militaris* on MDA level in the senile mice liver, there was no difference observed in the content of liver MDA among the senile mice treated with the cultured *C. militaris* at the different dosage and control. However, the contents of myocardium lipofusin were reduced by 22%, 13%, and 12%, respectively, in the senile mice treated with the cultured *C. militaris* at doses of 0.05, 0.1, and 0.3 g/kg/day, respectively, as compared with control mice.

Table 1. Effects of *Cordyceps militaris* growing on silkworm pupa on the content of liver MDA in senile mice

Group	Dose (g/kg/day)	mice	MDA (pmol/mg)	P value
Control	0	12	34.6 ± 7.1	-
Cordyceps militaris	0.05	12	40.4 ± 12.9	0.1890
	0.1	12	40.0 ± 11.3	0.1730
	0.3	12	37.8 ± 8.8	0.3485

Table 2. Effects of *Cordyceps militaris* growing on silkworm pupa on the lipofuscin content in senile mice myocardium

Group	Dose (g/kg/day)	mice	Lipofuscin (μg/g)	P value
Control	0	12	33.3 ± 4.6	-
Cordyceps militaris	0.05	12	26.0 ± 4.9***	0.0001
	0.1	12	28.9 ± 5.2*	0.0350
	0.3	12	29.2 ± 4.6*	0.0375

*: vs control $P < 0.05$, ***: vs control $P < 0.001$.

Generation of free radical molecules can lead to damage or destruction of a variety of tissues. Consequences of excessive reactive oxygen species are lipid peroxidation, oxidation of proteins and damage to DNA (Gotz *et al.*, 1994). Generally, Cells are equipped with anti-oxidants for the prevention of free radical damage. For example, GSH-Px and SOD, along with other non-enzymatic anti-oxidants, serve as detoxifying system to prevent cell damage. Anti-oxidant enzymes play a pivotal role in preventing oxidant-induced cell damage. Therefore, the effects of *C. militaris* on liver SOD and blood GSH-Px were investigated in senile mice which were perfused with cultured *C. militaris* at the different doses for 50 days. In comparing with the untreated group, SOD activity was increased by 16% ($P < 0.01$), 19% ($P < 0.01$), and 28% ($P < 0.001$), respectively, in mice treated 0.05, 0.1, 0.3 g/kg/day (Table 3). However, no significant difference was observed in GSH-Px activity (Table 4). There was no significant difference in body weight among the different treatment groups and control group (Table 5).

It has been suggested that free radical damage to cell leads to the pathological changes associated with aging, and the improved antioxidant status may have antiaging and postponing senescence effects (Horwitt, 1986; Packer, 1995; Halliwell, 1996). Cordycepe has been commonly used an agent for anti-aging and improving physical per-

Table 3. Effects of *Cordyceps militaris* growing on silkworm pupa on liver SOD activity in senile mice

Group	Dose (g/kg/day)	mice	SOD (U/mg)	P value
Control	0	12	26.7 ± 2.7	-
Cordyceps militaris	0.05	12	30.9 ± 3.4**	0.0067
	0.1	12	31.8 ± 5.2**	0.000007
	0.3	12	34.2 ± 3.5***	0.0028

: vs control, $P < 0.01$, *: vs control, $P < 0.001$.

Table 4. Effects of *Cordyceps militaris* growing on silkworm pupa on blood GSH-Px activity in senile mice

Group	Dose (g/kg/day)	mice	GSH-Px (U/mg)	P value
Control	0	12	24.4 ± 3.9	-
Cordyceps militaris	0.05	12	24.4 ± 2.4	0.3999
	0.1	12	23.2 ± 2.7	0.8957
	0.3	12	24.2 ± 3.0	0.9899

Table 5. Effects of *Cordyceps militaris* growing on silkworm pupa on the body weights of mice

Group	Dose (g/kg/day)	mice	Body weight (g)	P value
Control	0	12	46.8 ± 2.9	-
Cordyceps militaris	0.05	12	46.6 ± 2.6	0.7217
	0.1	12	47.2 ± 2.3	0.7001
	0.3	12	46.4 ± 2.8	0.8542

formance (Yu and He, 1998; Zhu *et al.*, 1998), which are thought to be a result of anti-oxidation activity of Cordycepes (Yamaguchi *et al.*, 2000; Li *et al.*, 2003; Wang *et al.*, 2004). In the present study, we investigated the antiaging function of the cultured C. militairs growing on the silkworm pupa by assessing the life span of *D. melanogaster* treated with C. militairs. The average life span, maximum life span and 50% death days of both the male and female fruit flies were markedly prolonged in comparison with the untreated fruit flies (Tables 6 and 7). The average life span, maximum life span and 50% death days were prolonged 12% (P < 0.05), 8% (P < 0.05), and 8 days for the male fruit flies and 8% (P < 0.05), 6% (P < 0.01), and 3 days for the female flies, respectively, when the flies were raised with diet containing 1.0% of the cultured *C. militaris*. The results of this study imply that the cultured *C. militaris* has a function of postponing senescence.

Table 7. Effects of *Cordyceps militaris* growing on silkworm pupa on the life-span of *Drosophila*

Group	Sex	n	Average body weight (mg)	50% death time (days)
0%	♂	100	0.83	54
	♀	100	1.00	56
0.2%	♂	100	0.84	58
	♀	100	1.01	58
1.0%	♂	100	0.85	62
	♀	100	1.00	59
5.0%	♂	100	0.85	61
	♀	100	1.01	58

In summary, results of the present study showed that the content of lipofusin of myocardium were significantly reduced, and the SOD activity of liver were obviously elevated in the senile mice treated with the cultured *C. militaris* compared to those of the untreated mice, respectively. The average life span, maximum life span, and 50% death days of the fruit flies were significantly prolonged by feeding flies with diets containing the cultured *C. militaris*. Therefore, we can conclude that the *C. militaris* growing on the silkworm pupa possesses anti-oxidation and delay senescence activity.

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Table 6. Effects of *Cordyceps militaris* growing on silkworm pupa on the life-span of *Drosophila*

Dosage (%)	Sex	n	Average life span (days)	P value	Maximum life span (days)	P value
0%	♂	100	56 ± 17	-	87 ± 3	-
	♀	100	60 ± 16	-	90 ±	-
0.2%	♂	100	62 ± 18*	0.0211	91 ± 4*	0.0101
	♀	100	63 ± 18	0.1169	94 ± 3*	0.0150
1.0%	♂	100	63 ± 16**	0.0037	90 ± 4	0.1565
	♀	100	65 ± 19*	0.0442	95 ± 2**	0.0011
5.0%	♂	100	64 ± 16***	0.0008	91 ± 3**	0.0046
	♀	100	66 ± 19*	0.0140	99 ± 3***	3.2×10^{-6}

*: vs control P < 0.05, **: vs cotrol P < 0.01, ***: vs control P < 0.001.

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