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A 90-day subchronic toxicity study of submerged mycelial culture of *Cordyceps militaris* in rats

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Cordyceps militaris (*C. militaris*) is a parasitic fungus that grows on the larvae of *Lepidoptera*. It is a well-known fungus with immunomodulatory activity. The study was conducted to clarify the edible safety of *C. militaris* mycelium for long term use. Eighty Sprague-Dawley (SD) rats were divided into four groups (10 males and 10 females in each group). Rats were orally administrated with reverse osmosis water or 2000, 3000 and 4000 mg per kg BW per day freeze dried *C. militaris* mycelium powder for 90 consecutive days. Clinical observation was carried out daily. The body weight and feed intake of the rats were recorded weekly. At the end of the study, all rats were sacrificed and the blood and organs were collected for hematology, clinical biochemistry and histopathological examination. All animals survived until the end of the study. During the study period, no abnormality occurred in clinical signs, body weight, feed intake, ophthalmological examination and urinalysis. There were no significant differences upon gross necropsy between the treatment and control group. Hematology, clinical biochemistry parameters and histopathological examination showed no treatment-related change. According to the results, the no-observed-adverse-effect level of *C. militaris* mycelium is 4000 mg per kg BW per day for male and female SD rats.

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Introduction

Cordyceps militaris (*C. militaris*) (L.) Link is an entomopathogenic fungus belonging to the family *Cordycipitaceae* and the genus *Cordyceps*. *C. militaris* is the type species of *Cordyceps*, which internally parasitizes larva or pupa of lepidopteran insects and forms fruiting bodies on their insect hosts.[1–3] It is one of the most important fungi used in traditional Chinese medicine for the treatment of asthma, and bronchial and lung inflammation.[4] *C. militaris* possesses extensive bioactive compounds including polysaccharides, cordycepin, adenosine, amino acids, organic selenium, ergosterol, sterols, cordycepic acid, superoxide dismutase (SOD), and multivitamins with significant pharmacological effects.[5–7] *C. militaris* has been reported to display various biological activities such as anticancer,[8] immunomodulatory, antioxidant,[9] renal-protective,[10] antifibrotic,[11] antiangiogenetic,[12,13] anti-inflammatory,[13] and anti-diabetic[14] activities.

C. militaris has long been recognized as a desirable alternative to *Ophiocordyceps sinensis* (*O. sinensis*) based on the similar compositions and bioactive effects of *C. militaris* and *O. sinensis*[2,9,15–18] as it has been given the Chinese Licence number Z20030034/35. The demand for *O. sinensis* is continuously increasing because of its medicinal uses, while the wild resource is decreasing rapidly due to non-sustainable collection.[19] Hence, *O. sinensis* is gradually being replaced by large amounts of cultivated *C. militaris* manufactured by fermentation technology in the marketplace.

For many years, *C. militaris* mycelium has beensold as a dietary supplement in many countries, including USA, Canada, Japan, Korea and China. The Taiwan Food and Drug Administration (TFDA) issued a notice of the amendment to "Daily consumption limit and Security Marked Warning of the ingredient '*Cordyceps militaris* fruiting body" draft on Jan 12, 2017. In addition to the *C. militaris* fruiting body, the manufacturing method and usage amount of *C. militaris* mycelium are also regulated. Our previous studies demonstrated that no toxic effect was observed in a sub-acute oral toxicity assay,[20] 3 different test systems of genotoxicity test[21] and teratogenicity study.[22] The results from these studies suggested that daily treatment with *C. militaris* mycelium at 3 g per kg BW per day did not induce observable toxicopathologic lesions in male and female rats. However, a 90-day subchronic toxicity study has not yet been conducted for a comprehensive safety profile of this potential mushroom. In the present study, we conducted a 90-day subchronic toxicological assessment of

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C. militaris mycelium in Sprague-Dawley (SD) rats to confirm the edible safety and evaluate the no-observed-adverse-effect level (NOAEL) of *C. militaris* mycelium for long term use.

Materials and methods

Preparation of freeze dried *C. militaris* mycelium powder

For 90-day subchronic toxicological assessment, *C. militaris* cultured on potato dextrose agar was transferred to a 2.0 L flask containing 1.0 L of PDB. The whole medium was cultivated at 25 °C for 5 days in a rotary shaker (120 rpm) for seed culturing prior to its scale-up production step. The fermented broth (1.0 L) was inoculated into a 500 L fermenter with 60% working volume (2% glucose, 1% yeast extract, 1% soybean powder; pH 6.0), and agitated at 60 rpm with an aeration rate of 0.5 vvm at 25 °C for 3 days. The submerged mycelial culture was heated at 100 °C for 1 h, freeze dried, and ground to powder.

Animals

Eighty 6-week-old SD rats were obtained from BioLASCO Taiwan Co., Ltd (Yilan, Taiwan). After quarantine and accommodation for one week, the rats were randomized based on their body weight, and then entered into the study. The rats had free access to a commercial rodent diet and sterile reverse osmosis water (R.O. water), and were maintained at controlled temperature (22 ± 3 °C), relative humidity (60 ± 10%) and light cycle (12 h light/12 h dark).

Study design

This study was performed based on the safety assessment guideline of Health Food announced by the Ministry of Health and Welfare (Taiwan). The protocol was approved by the Institutional Animal Care and Use Committee (IACUC No. MG103086) before the beginning of the study. The rats were randomly divided into four groups (n = 10 per sex per group) and orally administrated with *C. militaris* mycelium (2000, 3000 and 4000 mg per kg BW) for 90 days (daily). The body weights of the rats were measured prior to the study and once a week during the study. Measurement of feed intake for all rats was conducted weekly during the study. *C. militaris* mycelium was prepared freshly by dissolving freeze dried *C. militaris* mycelium powder into R.O. water to a concentration of 100 mg ml^{-1}, 150 mg ml^{-1} and 200 mg ml^{-1}, respectively. Clinical observation for all rats was conducted every day by recording abnormal clinical signs or death. At the end of the experiment, the rats were euthanized with CO_2 asphyxiation and blood samples were collected.

Urinalysis

One day before scarification, all rats were placed in metabolic cages for 16 h in order to collect urine samples. We used a compact urine analyzer (Urisys 2400; Roche, Germany) to analyze color, glucose, bilirubin, ketone bodies, specific gravity, pH, protein, urobilinogen and occult blood. The sediments of the urine were observed for white blood cells (WBC), red blood cells (RBC), epithelial cells (EP), crystals, microbes *etc*. by microscopic examination.

Ophthalmology

Ophthalmology for all rats was conducted prior to the oral administration and at the end of the study. The external appearance and internal structure of the eyes were evaluated with the naked eye and an ophthalmoscope (Optotechnik K-180, Heine, Germany).

Hematology and serum biochemistry

After overnight fasting, all rats were euthanized with CO_2 asphyxiation and blood samples were collected from the abdominal vein. We used an automated hematology analyzer (Sysmex Corporation XT-2000i, Japan) to detect red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), hemoglobin, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets (PLT), mean platelet volume (MPV), white blood cells (WBC), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EOS) and basophiles (BASO). Plasma samples were analyzed using an automated coagulated analyzer (Sysmex Corporation CA-1500, Japan) for prothrombin time (PT) and activated partial thromboplastin time (APTT). The following serum biochemistry parameters were analyzed by using an automated biochemistry analyzer (Johnson & Johnson Vitros 5.1 FS, USA): alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (BIT), γ-glutamyl transpeptidase (GGT), total protein (TP), albumin (ALB), globulin (GLO), amylase (AMY), blood urea nitrogen (BUN), creatinine (CRE), creatine phosphokinase (CPK), glucose (GLU), total cholesterol (TC), triglyceride (TG), sodium (Na), potassium (K), calcium (Cl), calcium (Ca) and inorganic phosphorus (P).

Pathology

Necropsy examination for all rats was conducted at the end of the study. The outer appearance, oral cavity, cranial cavity and all tissues and organs in the thoracic and abdominal cavity were examined visually and recorded. Organ weights including the brain, heart, kidneys, liver, spleen, adrenal, testes or ovaries were measured after the removal of peripheral fat tissue. A histopathological test was performed for the control group and the high dose group to examine the adrenals, aorta, bone, bone marrow, brain, caecum, colon, duodenum, epididymis, esophagus, eyes, Harderian gland, heart, ileum, jejunum, kidneys, liver, lung, lymph nodes, mammary gland, optic nerve, ovaries, oviduct, pancreas, pituitary, prostate gland, rectum, salivary gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid gland, parathyroid gland, trachea, urinary bladder, uterus and vagina. The collected organs were fixed in 10% neutral formalin buffer. Preserved organs and tissues were dehydrated, clarified, infiltrated with paraffin and embedded after trimming, forming paraffin tissue blocks, and cut into

2 μm thickness of a tissue slice using a paraffin tissue slicing machine (Thermo Shandon Ltd Fitness 325, Cheshire, UK), and stained with Hematoxylin & Eosin (H&E). The histopathological changes were evaluated using an optical microscope (Olympus BX51, Tokyo, Japan). If treatment-related changes occurred in a particular organ or tissue in the high dose group, extended examination for the organ or tissue of medium dose and low dose groups was included.

Statistical analysis

Values are expressed as mean ± standard deviation (SD) and analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's test for comparison of group means. A p value <0.05 is considered statistically significant.

Results

Body weight and feed intake

All animals survived during the study (Table 1). No abnormal clinical sign was shown after oral administration of R.O. water and *C. militaris* mycelium. The body weight of rats receiving *C. militaris* mycelium was similar to that of the control groups and was not statistically significant (Fig. 1). There is no significant difference in feed intake of both sexes between the treatment and control groups (Fig. 2).

Urinalysis

There was no significant difference in the examination of urine sediments and the routine test of the urinalysis between the treatment and control groups of both sexes (Tables 2 and 3).

Ophthalmology

The results revealed that no abnormal findings were observed in ophthalmological examination with the naked eye and by ophthalmoscopic diagnosis for the treated and control groups of both sexes prior to the oral administration and at the end of the study (Table 4).

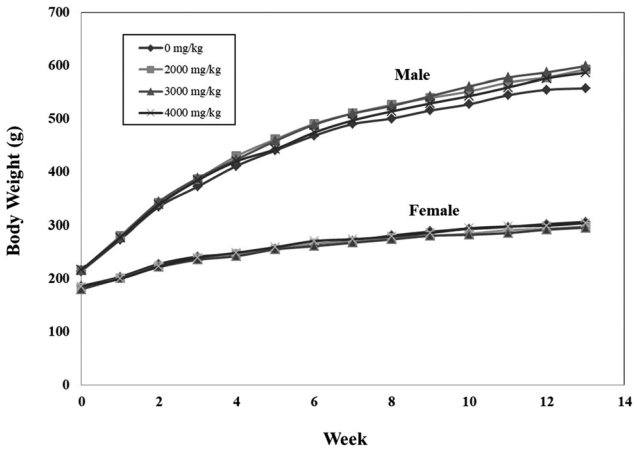

Fig. 1 Effects of *C. militaris* mycelium on body weight in male and female SD rats during the 90-day safety assessment. Data expressed as mean ± S.D., n = 10. Error bars have been omitted for simple presentation.

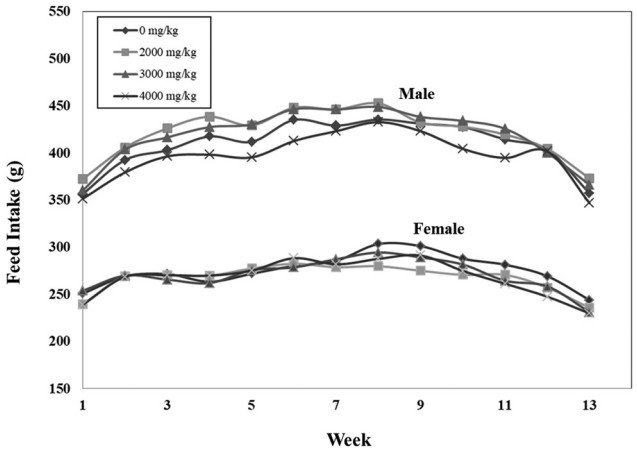

Fig. 2 Effects of *C. militaris* mycelium on feed intake in male and female SD rats during the 90-day safety assessment. Data expressed as mean ± S.D., n = 5 (2 rats in one cage). Error bars have been omitted for simple presentation.

Table 1 Mortality and incidence of abnormal clinical signs of the rats after 90 days of *C. militaris* mycelium administration

	Control		Low dose		Medium dose		High dose	
	0 mg kg^{-1}		2000 mg kg^{-1}		3000 mg kg^{-1}		4000 mg kg^{-1}	
Mortality of the rats								
Sex	M	F	M	F	M	F	M	F
Number of rats in each group	10	10	10	10	10	10	10	10
Number of rats died during the study	0	0	0	0	0	0	0	0
Incidence of abnormal clinical sign								
Sex	M	F	M	F	M	F	M	F
Number of rats in each group	10	10	10	10	10	10	10	10
Number of rats exhibiting abnormal clinical sign	0	0	0	0	0	0	0	0

M: male; F: female.

Table 2 Microscopic examination of urinary sediments of rats after 90 days of *C. militaris* mycelium administration

			Dosage (mg per kg BW)							
			Male				Female			
	Items		0	2000	3000	4000	0	2000	3000	4000
Cell number (HPF)	EP	0–1	10/10	10/10	10/10	10/10	10/10	10/10	10/10	9/10
		0–2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		1–2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		1–3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		2–3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	1/10
	WBC	0–1	0/10	0/10	0/10	0/10	10/10	10/10	10/10	10/10
		0–2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		0–3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		1–2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		1–3	10/10	9/10	9/10	10/10	0/10	0/10	0/10	0/10
		2–3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		2–4	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
	RBC	0–1	10/10	9/10	9/10	10/10	10/10	10/10	10/10	10/10
		0–2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		0–3	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		0–4	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		1–2	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
		1–3	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
Crystals	None	0/10	0/10	0/10	0/10					
	Triple phosphate	10/10	10/10	10/10	10/10					

n/n: affected rats/total examined rats. EP: epithelial cell; WBC: white blood cell; RBC: red blood cell; HPF: high power field.

Table 3 Urinalysis of rats after 90 days of *C. militaris* mycelium administration

		Dosage (mg per kg b.w.)							
		Male				Female			
Items		0	2000	3000	4000	0	2000	3000	4000
Appearance	Yellow	0/10	0/10	0/10	0/10	1/10	3/10	0/10	4/10
	Brown	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	Amber	10/10	10/10	10/10	10/10	9/10	7/10	10/10	6/10
Glucose	N	10/10	10/10	10/10	10/10	10/10	10/10	10/10	10/10
Bilirubin	N	8/10	9/10	9/10	8/10	10/10	9/10	9/10	9/10
	P	2/10	1/10	1/10	2/10	0/10	1/10	1/10	1/10
Ketone bodies	N	0/10	0/10	0/10	0/10	2/10	2/10	1/10	1/10
	1+	0/10	0/10	1/10	1/10	5/10	3/10	2/10	7/10
	2+	9/10	10/10	9/10	9/10	3/10	5/10	7/10	2/10
	3+	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Specific gravity	≦1.030	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	1.031–1.040	3/10	0/10	3/10	2/10	5/10	4/10	0/10	7/10
	1.041–1.050	7/10	10/10	7/10	8/10	5/10	6/10	10/10	3/10
	>1.050	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
pH	≦6.5	1/10	0/10	2/10	2/10	7/10	7/10	4/10	8/10
	7.0	9/10	10/10	5/10	8/10	3/10	3/10	5/10	2/10
	7.5	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	8.0	0/10	0/10	3/10	0/10	0/10	0/10	0/10	0/10
	8.5	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	≧9.0	0/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10
Protein	N	8/10	10/10	10/10	10/10	8/10	10/10	10/10	10/10
	1+	2/10	0/10	0/10	0/10	2/10	0/10	0/10	0/10
Urobilinogen	Normal	0/10	1/10	3/10	1/10	2/10	3/10	0/10	2/10
	1+	8/10	9/10	7/10	9/10	8/10	7/10	9/10	8/10
	2+	2/10	0/10	0/10	0/10	0/10	0/10	1/10	0/10
Occult blood	N	9/10	9/10	9/10	10/10	10/10	10/10	10/10	9/10
	±	1/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	1+	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
	2+	0/10	0/10	1/10	0/10	0/10	0/10	0/10	0/10
	3+	0/10	1/10	0/10	0/10	0/10	0/10	0/10	1/10

N: negative; P: positive; ±: trace. n/n: affected rats/total examined rats.

Table 4 Ophthalmological examination before and at the end of the study

	Group			
	Control		High dose	
	Male	Female	Male	Female
Before the study				
Number of rats in each group	10	10	10	10
Number of rats exhibiting ophthalmic abnormality	0	0	0	0
At the end of the study				
Number of rats in each group	10	10	10	10
Number of rats exhibiting ophthalmic abnormality	0	0	0	0

n/n: affected rats/total examined rats.

Hematology

Five hematological parameters in male rats were noted to be statistically significant (Table 5). In male rats, MPV of the low dose group, WBC of the low dose and high dose groups and LYMPH of the low dose and medium dose groups were significantly lower than the control group ($p < 0.05$). NEUT in the low dose and medium dose groups of *C. militaris*-treated males and EOS in the high dose group of *C. militaris*-treated males were significantly higher than in the control ($p < 0.05$). Four hematological parameters in female rats were noted to be statistically significant (Table 5). In female rats, MCHC of the low dose and medium dose groups and NEUT of the medium dose and high dose groups were significantly higher than in the control ($p < 0.05$). MCV in the medium dose group of *C. militaris*-treated females and LYMPH in the

Table 5 Hematology of rats after 90 days of *C. militaris* mycelium administration

	Hematology[a]			
Items	Control 0 mg kg^{-1}	Low dose 2000 mg kg^{-1}	Medium dose 3000 mg kg^{-1}	High dose 4000 mg kg^{-1}
Male				
RBC (10^6 μl^{-1})	9.143 ± 0.473	9.158 ± 0.419	8.969 ± 0.37	8.966 ± 0.303
HGB (g dL^{-1})	15.86 ± 0.57	15.94 ± 0.57	15.82 ± 0.61	15.71 ± 0.53
HCT (%)	50.11 ± 2.21	49.77 ± 1.88	49.65 ± 2.24	50.13 ± 1.96
MCV (fl)	54.83 ± 1.82	54.39 ± 1.38	55.46 ± 3.69	55.94 ± 2.21
MCH (pg)	17.37 ± 0.47	17.42 ± 0.33	17.67 ± 0.94	17.54 ± 0.73
MCHC (%)	31.67 ± 0.51	32.04 ± 0.30	31.86 ± 0.59	31.36 ± 0.76
PLT (10^3 μl^{-1})	1097.0 ± 90.6	1213.4 ± 124.0	1100.8 ± 119.8	1008.5 ± 312.5
MPV (fl)	8.05 ± 0.34	7.63 ± 0.22*	7.77 ± 0.30	8.04 ± 0.50
WBC (μl)	12 120.0 ± 2802.8	9341.0 ± 1670.9*	11 411.0 ± 2436.7	9512.0 ± 1129.0*
NEUT (%)	14.81 ± 2.66	24.86 ± 5.30*	21.84 ± 9.27*	18.46 ± 5.89
LYMPH (%)	78.35 ± 2.60	67.00 ± 6.34*	69.67 ± 9.83*	73.51 ± 7.66
MONO (%)	5.89 ± 1.28	6.82 ± 1.95	7.24 ± 2.41	6.49 ± 2.32
EOS (%)	0.81 ± 0.32	1.21 ± 0.36	1.11 ± 0.36	1.43 ± 0.58*
BASO (%)	0.14 ± 0.05	0.11 ± 0.06	0.14 ± 0.07	0.11 ± 0.07
PT (s)	11.42 ± 1.13	12.13 ± 0.89	11.88 ± 1.11	12.01 ± 0.80
APTT (s)	23.37 ± 3.77	22.80 ± 1.28	25.75 ± 3.99	25.70 ± 2.53
Female				
RBC (10^6 μl^{-1})	8.215 ± 0.243	8.212 ± 0.389	8.214 ± 0.50	8.197 ± 0.442
HGB (g dL^{-1})	15.26 ± 0.36	15.14 ± 0.63	15.25 ± 0.93	15.11 ± 0.74
HCT (%)	50.65 ± 1.57	48.25 ± 2.30	47.94 ± 2.98	49.15 ± 2.30
MCV (fl)	61.69 ± 2.54	58.82 ± 3.21	58.39 ± 1.91*	60.04 ± 2.88
MCH (pg)	18.59 ± 0.60	18.47 ± 0.77	18.58 ± 0.51	18.45 ± 0.58
MCHC (%)	30.14 ± 0.64	31.41 ± 0.63*	31.80 ± 0.36*	30.74 ± 0.69
PLT (10^3 μl^{-1})	941.2 ± 136.8	991.4 ± 178.8	1008.5 ± 88.2	953.9 ± 72.8
MPV (fl)	7.71 ± 0.25	7.63 ± 0.20	7.56 ± 0.26	7.58 ± 0.28
WBC (μl)	7896.0 ± 2049.5	7810.0 ± 1941.0	7464.0 ± 1649.9	6599.0 ± 1358.6
NEUT (%)	12.22 ± 2.70	15.45 ± 2.32	16.46 ± 4.64*	16.14 ± 2.97*
LYMPH (%)	80.90 ± 4.42	77.36 ± 3.34	76.30 ± 4.66*	76.19 ± 3.62*
MONO (%)	5.67 ± 2.49	5.83 ± 1.94	5.93 ± 1.64	6.05 ± 1.94
EOS (%)	1.08 ± 0.40	1.22 ± 0.45	1.18 ± 0.36	1.49 ± 0.54
BASO (%)	0.13 ± 0.08	0.14 ± 0.05	0.13 ± 0.05	0.13 ± 0.08
PT (s)	9.42 ± 0.85	9.16 ± 0.24	9.22 ± 0.36	9.16 ± 0.28
APTT (s)	18.78 ± 3.86	19.62 ± 3.60	19.35 ± 2.94	20.79 ± 2.78

[a] Data expressed as mean ± S.D., $n = 10$. RBC: red blood cell; HGB: hemoglobin; HCT: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; PLT: platelet count; MPV: mean platelet volume; WBC: white blood cell; NEUT: neutrophil; LYMPH: lymphocyte; MONO: monocyte; EOS: eosinophil; BASO: basophil; PT: prothrombin time; APTT: activated partial thromboplastin time. *Significantly different from the control group ($p < 0.05$). Normal range of MPV of male rats: 7.36–8.84 fL.[30] Normal range of WBC of male rats: 4569.3–15 456.6 μl^{-1}.[30] Normal range of NEUT of male rats: 6.42–28.53%.[30] Normal range of LYMPH of male rats: 64.1–88.68%.[30] Normal range of EOS of male rats: 0.25–1.65%.[30] Normal range of MCHC of female rats: 28.51–32.3%.[30] Normal range of MCV of female rats: 50.60–59.33 fL.[30] Normal range of NEUT of female rats: 4.66–20.16%.[30] Normal range of LYMPH of female rats: 71.08–91.35%.[30]

medium dose and high dose groups of *C. militaris*-treated females were significantly lower than in the control ($p < 0.05$).

Serum biochemistry

Serum biochemistry analysis revealed a lower ($p < 0.05$) AST, CPK and P of three treatment groups. Besides, TC in the medium dose and high dose groups of *C. militaris*-treated males were significantly lower than in the control group ($p < 0.05$). In female rats, GGT of the medium dose and high dose groups, TG of the low dose group and P of the high dose group were significantly lower than the control group ($p < 0.05$). Cl in the low dose, medium dose and high dose groups of *C. militaris*-treated females and K in the medium dose group of *C. militaris*-treated females were significantly higher than in the control group ($p < 0.05$, Table 6).

Pathology

No gross lesions were observed at necropsy (Table 7). In male rats, except for the adrenals and adrenals-to-brain weight ratio

Table 6 Clinical chemistry of rats after 90 days of *C. militaris* mycelium administration

	Clinical chemistry[a]			
Items	Control 0 mg kg^{-1}	Low dose 2000 mg kg^{-1}	Medium dose 3000 mg kg^{-1}	High dose 4000 mg kg^{-1}
Male				
ALT (U L^{-1})	34.7 ± 5.3	35.3 ± 5.2	34.6 ± 4.8	34.3 ± 2.5
AST (U L^{-1})	114.2 ± 16.6	97.3 ± 13.7*	90.5 ± 9.2*	95.1 ± 9.8*
ALP (U L^{-1})	92.4 ± 16.7	91.2 ± 13.3	92.6 ± 16.8	89.2 ± 8.4
TBIL (mg dL^{-1})	0.014 ± 0.007	0.015 ± 0.007	0.013 ± 0.005	0.011 ± 0.0
GGT (U L^{-1})	1.7 ± 0.5	1.3 ± 0.5	1.5 ± 0.7	1.4 ± 0.5
Total protein (g dL^{-1})	7.1 ± 0.4	7.1 ± 0.4	7.0 ± 0.2	6.8 ± 0.3
Albumin (g dL^{-1})	4.4 ± 0.2	4.4 ± 0.3	4.4 ± 0.1	4.3 ± 0.2
Globulin (g dL^{-1})	2.7 ± 0.2	2.7 ± 0.2	2.7 ± 0.1	2.5 ± 0.2
Amylase (U L^{-1})	1979.4 ± 254.2	1858.4 ± 247	1761.6 ± 200.6	1801.4 ± 215.5
BUN (mg dL^{-1})	16.3 ± 1.1	15.4 ± 1	16.2 ± 1.8	15.9 ± 2.2
Creatinine (mg dL^{-1})	0.65 ± 0.05	0.60 ± 0.05	0.61 ± 0.0	0.64 ± 0.1
CPK (U L^{-1})	200.9 ± 69.1	103.5 ± 24.2*	92.0 ± 38.4*	122.8 ± 42.3*
Glucose (mg dL^{-1})	178.6 ± 21.7	200.7 ± 33.7	202.6 ± 23.4	175.7 ± 28.4
TC (mg dL^{-1})	84.9 ± 16.7	70.5 ± 10.1	67.6 ± 17.7*	62.6 ± 14.1*
Triglyceride (mg dL^{-1})	68.2 ± 25.1	51.3 ± 17	54.6 ± 25.6	59.6 ± 16.3
Sodium (mmol L^{-1})	147.1 ± 1.7	147.7 ± 1.5	147.8 ± 1.4	147.5 ± 1.4
Potassium (mmol L^{-1})	7.1 ± 1.2	6.8 ± 0.8	6.7 ± 0.9	6.5 ± 0.9
Chloride (mmol L^{-1})	97.7 ± 2.0	99.3 ± 1.6	99.7 ± 1.9	99.0 ± 0.9
Calcium (mg dL^{-1})	12.5 ± 1	12.3 ± 0.4	13.0 ± 0.7	13.0 ± 0.8
Phosphorus (mg dL^{-1})	11.6 ± 1.1	10.3 ± 0.5*	10.2 ± 0.6*	10.5 ± 0.7*
Female				
ALT (U L^{-1})	35.1 ± 11.0	35.6 ± 8.0	34.8 ± 9.4	35.5 ± 17.4
AST (U L^{-1})	107.4 ± 14.7	96.5 ± 15.8	90.3 ± 12.1	97.6 ± 17.5
ALP (U L^{-1})	40.9 ± 8.9	47.9 ± 7.5	43.7 ± 19.0	47.5 ± 10.4
TBIL (mg dL^{-1})	0.043 ± 0.032	0.020 ± 0.012	0.024 ± 0.022	0.020 ± 0.0
GGT (U L^{-1})	1.8 ± 0.6	1.3 ± 0.5	1.1 ± 0.3*	1.2 ± 0.4*
Total protein (g dL^{-1})	7.9 ± 0.6	7.8 ± 0.5	7.9 ± 0.6	7.6 ± 0.4
Albumin (g dL^{-1})	5.2 ± 0.5	4.9 ± 0.2	5.0 ± 0.4	4.9 ± 0.4
Globulin (g dL^{-1})	2.8 ± 0.2	2.8 ± 0.2	2.9 ± 0.2	2.7 ± 0.2
Amylase (U L^{-1})	1443 ± 380.7	1382.3 ± 242.1	1266.4 ± 342.4	1350.9 ± 328.0
BUN (mg dL^{-1})	18.2 ± 2.7	17.5 ± 1.8	19.3 ± 4.7	19.2 ± 3.0
Creatinine (mg dL^{-1})	0.71 ± 0.06	0.68 ± 0.04	0.67 ± 0.1	0.72 ± 0.1
CPK (U L^{-1})	141.2 ± 39.1	112.0 ± 42.2	95.3 ± 22.7	122.7 ± 57.4
Glucose (mg dL^{-1})	167.4 ± 28.6	160.9 ± 22.6	150.2 ± 16.5	158.6 ± 25.1
TC (mg dL^{-1})	100.7 ± 25.4	95.4 ± 28.0	96.9 ± 23.2	87.9 ± 10.3
Triglyceride (mg dL^{-1})	62.2 ± 19.0	43.5 ± 9.7*	47.6 ± 10.9	51.7 ± 12.1
Sodium (mmol L^{-1})	145.4 ± 0.7	144.9 ± 1.3	144.4 ± 0.8	145.2 ± 1.2
Potassium (mmol L^{-1})	6.2 ± 0.7	6.6 ± 0.7	7.3 ± 0.8*	6.3 ± 0.5
Chloride (mmol L^{-1})	97.6 ± 1.7	99.6 ± 2.0*	99.5 ± 1.2*	99.9 ± 0.7*
Calcium (mg dL^{-1})	12.2 ± 0.6	12.0 ± 0.3	12.5 ± 0.5	12.0 ± 0.3
Phosphorus (mg dL^{-1})	8.9 ± 0.8	8.4 ± 0.7	8.8 ± 0.8	7.4 ± 0.7*

[a] Data expressed as mean ± S.D., $n = 10$. ALT: alanine aminotransferase; AST: aspartate aminotransferase; ALP: alkaline phosphatase; TBIL: total bilirubin; GGT: γ-glutamyl transpeptidase; BUN: blood urea nitrogen; CPK: creatine phosphokinase; TC: total cholesterol. *Significantly different from the control group ($p < 0.05$). Normal range of AST of male rats: 67.24–149.90 U L^{-1}.[30] Normal range of CPK of male rats: 70.1–344.7 U L^{-1}.[30] Normal range of TC of male rats: 20.83–119.10 mg dL^{-1}.[30] Normal range of P of male rats: 7.65–14.47 mg dL^{-1}.[30] Normal range of GGT of female rats: 0–2.37 U L^{-1}.[30] Normal range of TG of female rats: 31.0–90.6 mg dL^{-1}.[30] Normal range of Cl of female rats: 95.0–106.6 mg dL^{-1}.[30] Normal range of P of female rats: 7.33–15.29 mg dL^{-1}.[30]

Table 7 Incidence of gross lesion after 90 days of *C. militaris* mycelium administration

	Control 0 mg kg^{-1}	Low dose 2000 mg kg^{-1}	Medium dose 3000 mg kg^{-1}	High dose 4000 mg kg^{-1}
Male				
Number of rats in each group	10	10	10	10
Number of rats exhibiting gross lesion	0	0	0	0
Female				
Number of rats in each group	10	10	10	10
Number of rats exhibiting gross lesion	0	0	0	0

of the low dose and medium dose groups and kidneys of the medium dose group, no significant difference in organ weights was observed between the treatment and control groups. In female rats, there were no significant differences in organ weight between the treatment and control groups (Table 8).

No significant treatment-related changes for all rats were observed in the adrenals, brain, kidney, liver, spleen, testis (male) and ovary (female) of the treatment and control groups (Table 9). In male rats, focal, slight to moderate severe cortical lipidosis was found in the adrenal gland of the control and high dose groups (incidence rate of 3/10 and 2/10). Only one

Table 8 Organ weight of rats after 90 days of *C. militaris* mycelium administration

		Absolute organ weight[a]			
		Control 0 mg kg^{-1}	Low dose 2000 mg kg^{-1}	Medium dose 3000 mg kg^{-1}	High dose 4000 mg kg^{-1}
Male					
Organ	Weight				
Brain	Weight (g)	2.164 ± 0.081	2.188 ± 0.086	2.204 ± 0.103	2.193 ± 0.089*
Adrenals	Weight (g)	0.0423 ± 0.0065	0.0532 ± 0.0094*	0.0583 ± 0.0075*	0.0479 ± 0.0102
	Ratio (%)	1.9589 ± 0.3192	2.4287 ± 0.4018*	2.6459 ± 0.3296*	2.1793 ± 0.4339
Heart	Weight (g)	1.578 ± 0.132	1.661 ± 0.139	1.677 ± 0.141	1.655 ± 0.151
	Ratio	0.730 ± 0.066	0.758 ± 0.070	0.760 ± 0.052	0.754 ± 0.055
Kidneys	Weight (g)	3.724 ± 0.272	4.136 ± 0.519	4.349 ± 0.525*	4.098 ± 0.288
	Ratio	1.724 ± 0.154	1.892 ± 0.243	1.974 ± 0.224	1.873 ± 0.151
Liver	Weight (g)	15.627 ± 1.842	16.110 ± 2.485	16.473 ± 1.684	16.046 ± 1.008
	Ratio	7.230 ± 0.887	7.382 ± 1.260	7.486 ± 0.802	7.327 ± 0.547
Spleen	Weight (g)	0.781 ± 0.094	0.755 ± 0.077	0.777 ± 0.153	0.803 ± 0.121
	Ratio	0.359 ± 0.041	0.343 ± 0.035	0.352 ± 0.059	0.367 ± 0.050
Testes	Weight (g)	3.800 ± 1.196	3.490 ± 0.345	3.673 ± 0.221	3.492 ± 0.353
	Ratio	1.752 ± 0.510	1.596 ± 0.140	1.669 ± 0.122	1.593 ± 0.142
Thymus	Weight (g)	0.306 ± 0.062	0.281 ± 0.061	0.328 ± 0.066	0.340 ± 0.080
	Ratio	0.142 ± 0.028	0.128 ± 0.030	0.149 ± 0.030	0.156 ± 0.041
Female					
Organ	Weight				
Brain	Weight (g)	1.943 ± 0.097	1.971 ± 0.082	1.991 ± 0.058	1.979 ± 0.077
Adrenals	Weight (g)	0.0554 ± 0.0094	0.0615 ± 0.0095	0.0681 ± 0.0161	0.0592 ± 0.0070
	Ratio (%)	2.8459 ± 0.4254	3.1299 ± 0.5245	3.4153 ± 0.7895	3.0008 ± 0.4290
Heart	Weight (g)	0.941 ± 0.096	0.911 ± 0.080	0.923 ± 0.093	0.959 ± 0.100
	Ratio	0.486 ± 0.059	0.463 ± 0.051	0.464 ± 0.049	0.485 ± 0.051
Kidneys	Weight (g)	1.994 ± 0.180	2.039 ± 0.160	2.124 ± 0.265	2.063 ± 0.167
	Ratio	1.025 ± 0.065	1.038 ± 0.102	1.068 ± 0.133	1.044 ± 0.105
Liver	Weight (g)	8.541 ± 0.710	7.953 ± 0.982	8.239 ± 1.247	8.202 ± 0.725
	Ratio	4.403 ± 0.379	4.048 ± 0.581	4.136 ± 0.610	4.151 ± 0.385
Spleen	Weight (g)	0.413 ± 0.069	0.472 ± 0.165	0.455 ± 0.045	0.435 ± 0.052
	Ratio	0.215 ± 0.040	0.240 ± 0.085	0.228 ± 0.025	0.220 ± 0.026
Testes	Weight (g)	0.0670 ± 0.0176	0.0686 ± 0.0100	0.0767 ± 0.0162	0.0774 ± 0.0281
	Ratio	0.0342 ± 0.0080	0.0348 ± 0.0054	0.0386 ± 0.0071	0.0392 ± 0.0140
Thymus	Weight (g)	0.254 ± 0.060	0.233 ± 0.067	0.202 ± 0.040	0.247 ± 0.059
	Ratio	0.131 ± 0.032	0.117 ± 0.035	0.101 ± 0.021	0.124 ± 0.031

[a] Data expressed as mean ± S.D., n = 10. Ratio: organ weight/brain weight; ratio (%): (organ weight/brain weight) × 100%. *Significantly different from the control group ($p < 0.05$). Normal range of the adrenals weight of male rats: 0.027–0.060 g.[30] Normal range of the adrenals-to-brain weight ratio of male rats: 1.259–2.818%.[30] Normal range of the kidney weight of male rats: 2.970–4.373 g.[30]

Table 9 Histopathological examination of rats after 90 days of *C. militaris* mycelium administration

		Group			
		Control		High dose	
Organ	Lesions	Male	Female	Male	Female
Adrenal gland					
	Lipidosis, cortex, focal, slight to moderate severe	3/10	—	2/10	—
Aorta		—	—	—	—
Bain					
Fore		—	—	—	—
Middle		—	—	—	—
		—	—	—	—
Cerebellum					
Bone		—	—	—	—
Bone marrow		—	—	—	—
Epididymis			N		N
	Degeneration/ necrosis, sperm, focal, slight	1/10		—	
Esophagus		—	—	—	—
Eyes		—	—	—	—
Harderian gland					
	Infiltration, mononuclear cell, focal, slight	1/10	—	—	—
Heart					
	Infiltration, mononuclear cell, focal, minimal to slight	1/10	1/10	2/10	—
Intestine, small duodenum		—	—	—	—
Jejunum		—	—	—	—
Ileum		—	—	—	—
Intestine, large					
Caecum		—	—	—	—
Colon		—	—	—	—
Rectum		—	—	—	—
Kidney					
	Fibrosis, interstitial, focal, minimal to slight	1/10	—	1/10	—
	Cast, tubular, focal, minimal to slight	1/10	—	1/10	—
	Mineralization, tubular, focal, minimal to slight	—	4/10	—	5/10
Liver		—	—	—	—
Lung		—	—	—	—
Lymph node					
Cervical		—	—	—	—
Mesenteric		—	—	—	—

—: no effect. N: tissue was not available. n/n: affected rats/total examined rats. Degree of lesion was graded from one to five depending on severity: minimal (<1%); slight (1–25%); moderate (26–50%); moderate/severe (51–75%); severe/high (76–100%). Incidence: affected rats/total examined rats (n = 10).

male rat in the control group exhibited focal and slight mononuclear cell infiltration in Harderian gland. Focal, minimal to slight mononuclear cell infiltration was found in the heart of the control and high dose male rats (incidence rate of 1/10 and 2/10) and control female rats (incidence rate of 1/10). Focal, minimal to slight interstitial fibrosis and focal, minimal to slight tubular cast were observed in the kidney of the control and high dose male rats (incidence rate of 1/10 and 1/10). Focal, minimal to slight tubular mineralization was also observed in the kidney of the control and high dose female rats (incidence rate of 4/10 and 5/10). There was one male rat in the control group that exhibited focal and slight sperm degeneration and necrosis in the epididymis.

Discussion

Because of long-term excessive collection of wild *C. militaris*, supply of wild *C. militaris* is failing to meet the market demands. Artificially cultivated *C. militaris* is a better alternative for healthcare product development. Liu *et al.* (2015) yielded mass production of *C. militaris* mycelium using a variety of carbon sources, nitrogen sources and inorganic salts combined with a liquid fermentation technique in a 50 ton fermenter, offering the obvious advantages of less pollution and a short production cycle.[21] Furthermore, the functional component contents and physiological activity of the produced *C. militaris* mycelium were not inferior to those of wild *C. militaris* mycelium. Previous studies reported the proximate composition of natural and cultivated *Cordyceps* mycelium. *Cordyceps* contains a high amount of polysaccharide with a wide range.[23,24] Protein levels in *Cordyceps* mycelium have been reported to be approximately 5.6–31.6 g per 100 g.[24–26] Fat contents reported previously were above 4.5 g per 100 g.[24,27–29] We have performed an analysis of bioactive compounds and found that the adenosine, cordycepin, N^6-(2-hydroxyethyl)-adenosine and ergosterol contents in *C. militaris* mycelium were 1.7, 0.3, 0.1 and 2.2 mg g^{-1}, respectively.[21] The nutritional values of *C. militaris* detected indicate its potential use in well-balanced diets and as a source of bioactive compounds.

During the study period, no abnormality occurred in clinical signs, body weight, feed intake and ophthalmological examination. There were no significant differences in urinalysis between the treatment and control group. A few parameters in hematology and clinical biochemistry analysis showed significant differences between the treatment and control group, including MPV, WBC, NEUT, LYMPH, EOS, AST, CPK, P, and TC in male rats, and MCHC, MCV, NEUT, LYMPH, GGT, TG, Cl, K and P in female rats. The above findings are changes within the range observed in normal SD rats,[30] and the changes were not found to be dose-dependent.

Necropsy and histopathological examination found no treatment-related change. Although the organ weights and organ-to-brain weight ratio of the adrenal gland of the low and medium dose groups and the organ weights of the kidneys of the medium dose group in male rats were significantly higher than the control group, no differences ($p > 0.05$) were found between treatments regarding the serum biochemical parameters and histopathological effects of these organs. Non-

specific histopathological changes were observed, including focal cortical lipidosis in the adrenal gland, focal mononuclear cell infiltration in the Harderian gland and heart, focal interstitial fibrosis, focal tubular cast and focal tubular mineralization in the kidney, and focal sperm degeneration and necrosis in the epididymis. According to the results, the incidence of histopathological change was not directly correlated with the oral administration of *C. militaris* mycelium. These histopathological changes were non-specific, and not induced by oral administration of *C. militaris* mycelium.

Conclusion

The present study demonstrates that the 90-day subchronic toxicological assessment showed no systemic toxicity attributable to dietary exposure to the powder of *C. militaris* submerged mycelial culture, and no significant toxicity occurred even at the highest dose of 4000 mg per kg BW per day in SD rats. The results from the 90-day subchronic toxicity study of *C. militaris* mycelium do not raise concern with respect to its possible use as a functional food ingredient. According to the results, the no-observed-adverse-effect level (NOAEL) of *C. militaris* mycelium was 4000 mg per kg BW per day in SD rats. This information provides evidence supporting the use of the *C. militaris* fermentation product as a safe agent for functional foods.

Conflicts of interest

There are no conflicts of interest to declare.

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