International Journal of Medicinal Mushrooms, 17(8): 771–781 (2015)

A 90-Day Subchronic Toxicity Study of Submerged Mycelial Culture of *Cordyceps cicadae* (Ascomycetes) in Rats

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ABSTRACT: *Cordyceps cicadae* is a parasitic fungus that hibernates inside a host (*Cicada flammata* Dist.) and then grows its fruiting body on the surface of the insect. The complete insect/fungus combination of *C. cicadae* has been widely applied in Chinese traditional medicine. Recent studies have demonstrated that the medicinal benefits of cultured mycelia are as effective as those found in the wild. However, toxicological information regarding the chronic consumption of *C. cicadae* mycelia culture is not available. This study was conducted to evaluate the possible toxicity arising from repeated exposure to freeze-dried submerged mycelial culture of *C. cicadae* for 90 days. A total of eighty 8-week-old Sprague-Dawley rats were divided into 4 groups (10 males and 10 females in each group). *C. cicadae* was administered daily to animals by gavage at doses of 0, 500, 1000, and 2000 mg/kg body weight for 90 days. No animal deaths occurred and no treatment-related clinical signs were observed during the study period. No statistical differences in body weight gain, relative organ weight, hematology, serum chemistry, and urinalysis were observed. Gross necropsy and histopathological findings indicated that there was no treatment-related abnormality. Based on the results, the no observed adverse effect level of *C. cicadae* whole broth is determined to be >2000 mg/kg for male and female Sprague-Dawley rats. The results of this study provides support for the use of *C. cicadae* fermentation product as a safe agent in functional food.

KEY WORDS: medicinal fungi and mushrooms, *Cordyceps cicadae*, Sprague-Dawley rats, 90-day subchronic toxicity

ABBREVIATIONS: A:G, albumin-to-globulin ratio; **BUN**, blood urea nitrogen; **HEA**, N^6-(2-hydroxyethyl)adenosine; **MCV**, mean corpuscular volume; **RBC**, red blood cell; **SD**, Sprague-Dawley.

I. INTRODUCTION

Cordyceps cicadae (Miq.) Massee (Cordycepitaceae, Hypocreales, Ascomycetes) is a parasitic fungus that hibernates inside a host (*Cicada flammata* Dist.) and then forms a fruiting body on the surface of the insect. It has been considered as a substitute for *Ophiocordyceps sinensis* because its biological components are similar, and it has been recorded in Chinese medicinal prescriptions for 1580 years for sedation and for the treatment of childhood palpitation, epilepsy, and convulsion.[1–3] In recent years, *C. cicadae* has been reported to exhibit other pharmacological properties, including renoprotective,[4–7] hypoglycemic,[8] and antitumor properties.[9] Moreover, some effective chemical constituents such as N^6-(2-hydroxyethyl) adenosine (HEA) isolated from cultured mycelia of *C. cicadae* were reported to have sedative hypnotic activity.[10,11]

Previous studies have demonstrated that the medicinal benefits of cultured mycelia are similarly effective as those found in the wild.[12,13] As the interest in *C. cicadae* is growing because of its rarity and beneficial curative effects, it seems highly desirable

1045-4403/15/$35.00

to produce *C. cicadae* by submerged fermentation in a batch stirred tank bioreactor for higher mycelial production in a shorter incubation time with a less chance of contamination. We previously performed a 28-day feeding study and teratology of HEA-enriched submerged mycelial culture of *C. cicadae* in Sprague-Dawley (SD) rats and found no treatment-related abnormality at a dose of 1680 mg/kg body weight (data not shown). Based on these results, the aim of this study was to investigate further the possible health hazards arising from repeated exposure to submerged mycelial culture of *C. cicadae* over 90 days.

II. MATERIALS AND METHODS

A. Fungal Material Preparation

C. cicadae (MU30106) procured from the Bioresource Collection and Research Center at the Food Industry Research and Development Institute (Hsinchu, Taiwan) was grown on potato dextrose agar at 25°C for 5 days, transferred to a 2.0-L flask containing 1.0 L of PDB, and incubated at 25°C on a rotary shaker at 120 rpm for 5 days. The fermented broth (1.0 L) was inoculated into a 200-L fermentor (BioTop, Taichung, Taiwan) 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. Analysis of crude protein, crude fat, carbohydrate, crude fiber, ash, and moister of the freeze-dried powder was performed according to the Association of Official Analytical Chemists official procedure.[14] Similarly, heavy metal contents were determined using standard procedures.[14] HEA, the bioactive ingredient of *C. cicadae*, was measured using a high-performance liquid chromatograph equipped with an ultraviolet detector and a reverse phase column (Luna 5µ C18(2), 250 × 4.6 mm; Phenomenex, Torrance, CA). The mobile phase consisted of 10 mmol/L KH_2PO_4 and acetonitrile (94:6). The flow rate was 1.0 mL/min and the column was kept at 40°C.

B. Animals

Eighty 8-week old male and female SD rats (BioLASCO, Taipei, Taiwan) were randomly assigned to either the control or treatment groups (10 rats per sex in each group) after the animals were quarantined for 2 weeks and acclimated for 1 week. The animals were housed in pairs with the same sex and maintained under the following conditions: 22 ± 4°C, 40–70% relatively humidity, and a 12-h light/12-h dark light cycle. Standard rodent diet (Laboratory Autoclavable Rodent Diet 5010; PMI Nutrition International, St. Louis, MO) and reverse osmosis water were provided *ad libitum*.

C. Study Design

The study was designed on the basis of Organization for Economic Co-operation and Development Guideline 408 and carried out in accordance with Good Laboratory Practice. Animals were administered freeze-dried powder of *C. cicadae* submerged mycelial culture daily by gavage at doses of 0, 500, 1000, and 2000 mg/kg (acceptable daily intake was up to 100 mg/kg body weight/day, with a default assessment factor of 100). The freeze-dried powder was dissolved in reverse osmosis water and given at 20 mL/kg. Body weight was measured before the first dosing on day 0 and weekly thereafter for the duration of the study period. Averaged feed and water intakes were calculated every week. Clinical observation for possible signs of toxicity, morbidity, and mortality was carried out daily. At the end of the 90-day period, all rats were anesthetized with carbon dioxide and then killed after blood collection.

D. Ophthalmology

Ophthalmologic examinations were performed for all rats on the day before the first dosing and on the last day of study period. The lens, cornea, conjunctiva, anterior chamber, and iris were examined using an ophthalmoscope.

E. Urinalysis

One day before being killed, animals were placed in metabolic cages individually for 16 hours to collect urine. Color, pH, specific gravity, urobilinogen, bilirubin, ketone, protein, glucose, nitrite, and occult blood of urine were analyzed using a semiquantitative urinalysis system (Urisys 2400; Roche, Basel, Switzerland).

F. Hematology and Serum Biochemistry

At the end of 90 days, all animals were killed after overnight fasting. Blood was obtained by heart puncture and collected in EDTA-coated tubes. The following parameters were evaluated using an automatic blood analyzer (Gen. S; Beckman Coulter, Pasadena, CA): white blood cells, red blood cells (RBCs), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and reticulocytes. Another automatic analyzer (LX-20; Beckman Coulter) was applied to measure aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, total protein, albumin, globulin, blood urea nitrogen (BUN), creatinine, glucose, triglyceride, cholesterol, sodium, potassium, calcium, chloride, and phosphorus.

G. Gross Necropsy and Histopathology

On the day of terminal sacrifice, all rats were anesthetized with carbon dioxide, followed by blood collections and necropsy. The wet weights of major organs/tissues were measured and the relative weight was calculated as follows: organ weight (g)/body weight (g) × 100. The gross necropsies included the external surface of the body, thoracic and abdominal cavities, and visceral and intestinal organs. Following gross necropsy, histopathological examinations of the adrenal gland, aorta, brain, bone, cervix, epididymis, esophagus, eyes, Harderian gland, heart, intestines, kidneys, liver, lung, lymph nodes, mammary gland, ovaries, pancreas, parathyroid gland, pituitary gland, prostate gland, salivary gland, skin, seminal vesicle, spinal cord, spleen, stomach, testes, thymus, thyroid gland, tongue, trachea, urinary bladder, uterus, and vagina were carried out. The sampled tissues listed above were fixed in 10% neutral buffered formalin. The selective tissues from the control group and the high-dose group were trimmed, dehydrated, clarified, infiltrated with paraffin, embedded, sectioned (5 μm), and stained with hematoxylin and eosin. Microscopic examination (Opticphot-2; Nikon, Tokyo, Japan) was then performed.

H. Statistical Analysis

SAS software (SAS Institute, Cary, NC) was applied for one-way analysis of variance and Duncan's test to determine significant differences among means ($\alpha = 0.05$). All data were expressed as mean ± standard deviation.

III. RESULTS AND DISCUSSION

A. Proximate Composition of *C. cicadae*

Because of the complexity of *Cordyceps* spp., the fungal strain used for this study was confirmed by the Food Industry Research and Development Institute (Hsinchu City, Taiwan) based on morphological characters and analysis of nuclear ribosomal DNA, internal transcribed spacer 1–5.8S and small subunit region, and partial β-tubulin sequences. Composition analysis showed that freeze-dried powder of *C. cicadae* submerged mycelial culture contains approximately 5.05% moisture, 25.67% crude protein, 5.48% crude fat, 5.44% crude fiber, and 3.28% ash. The results obtained were comparable to those of the natural fruit body of *C. cicadae* (9.81%, 19.65%, 8.41%, 3.12%, and 7.84%, respectively).[15] Furthermore, HEA, a Ca^{2+} antagonist and inotropic agent, is the bioactive ingredient of *Cordyceps* spp. and is used as a marker for quality control of *Cordyceps*.[16–19] High-performance liquid chromatographic analysis indicated that the peak of HEA occurred at a retention time of 10.180 min (Fig. 1). HEA was found to be 1.5 mg/g in the test material. Such yield was

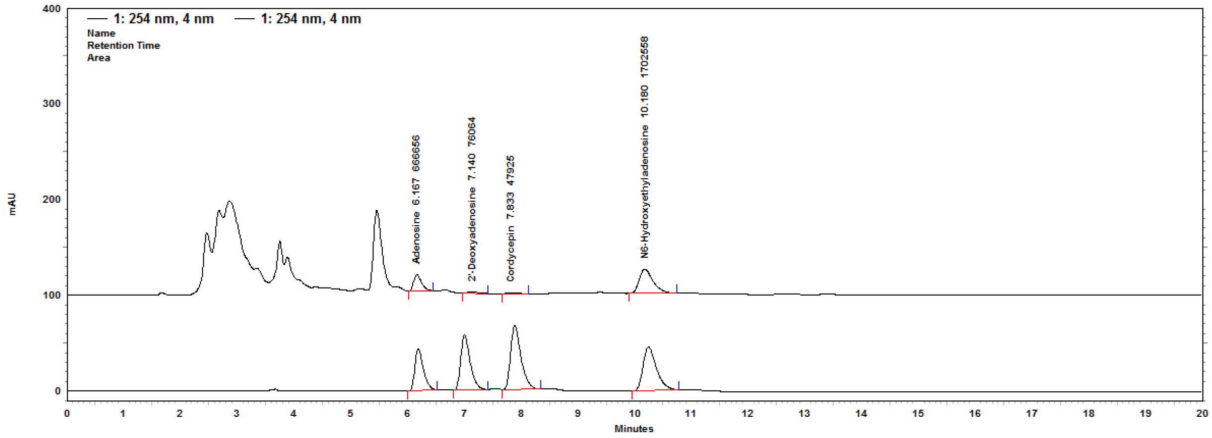

FIG. 1: High-performance liquid chromatogram of N^6-(2-hydroxyethyl)-adenosine and adenosine standard sample (bottom) and powder of *Cordyceps cicadae* submerged mycelial culture (top). Retention time of adenosine and N^6-(2-hydroxyethyl)-adenosine is 6.167 and 10.180 min, respectively.

significantly higher than the yield obtained in a previous study.[20] This difference can probably be attributed to the different growth media selected in this study. Furthermore, *C. cicadae* mycelial culture was analyzed for the presence of heavy metals, and such values were all within the daily intake levels (data not shown).

B. General Observations

No abnormal clinical signs or mortality related to the administration of *C. cicadae* were observed in both sexes of rats. The overall feed consumption was not significantly different among the 4 groups (data not shown). There was no significant difference in the body weights (Fig. 2) and relative organ weights of rats of either sex in any of the 4 groups (Table 1). Ophthalmologic exanimations, even of mice receiving the highest dosage, indicated that no abnormality related to the administration of *C. cicadae* submerged mycelial culture powder occurred.

C. Serum Biochemistry

In female animals, all serum biochemical parameters were normal except the concentrations of albumin, globulin, and creatinine, which were lower ($P < 0.05$) in *C. cicadae*–treated groups when compared with the control group (Table 2). Albumin, synthesized in the

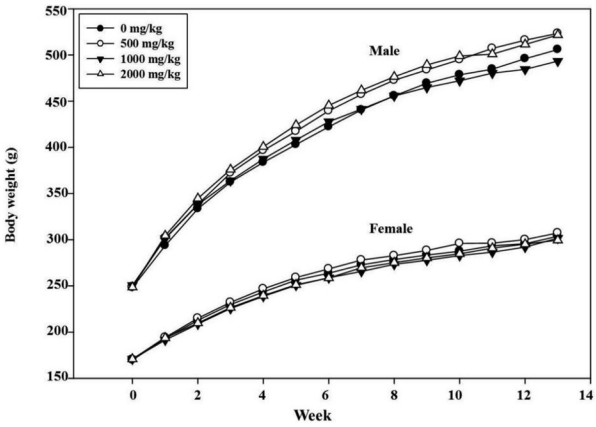

FIG. 2: Group mean body weights of males and females over the course of the study.

liver, is the protein with the highest concentration in plasma.[21] Albumin transports many small molecules in the blood and is important for the maintenance of osmotic pressure. A diseased liver produces insufficient albumin, and diseased kidneys sometimes may lose large amounts of albumin into the urine. *Globulin* is a generic term used to describe a set of approximately 60 plasma proteins. There are 4 groups of globulins known as alpha-1, alpha-2, beta, and gamma proteins.[22] The liver produces most of the alpha and beta globulins. Normally, there is more albumin than globulins, giving a normal albumin-

TABLE 1: Relative Weights of Organs in Rats after Administration of *Cordyceps cicadae* Mycelia from 90-Day Submerged Culture

Item	Relative Weight (g/100 g Body Weight)			
	0	500	1000	2000
Female				
Brain	0.66 ± 0.05	0.64 ± 0.05	0.67 ± 0.05	0.66 ± 0.05
Heart	0.38 ± 0.03	0.37 ± 0.02	0.39 ± 0.04	0.37 ± 0.02
Liver	3.08 ± 0.14	3.11 ± 0.16	3.00 ± 0.14	2.99 ± 0.14
Kidneys	0.74 ± 0.05	0.78 ± 0.06	0.75 ± 0.05	0.71 ± 0.04
Spleen	0.17 ± 0.02	0.18 ± 0.01	0.17 ± 0.02	0.19 ± 0.01
Ovaries	0.026 ± 0.003	0.028 ± 0.003	0.027 ± 0.005	0.025 ± 0.005
Male				
Brain	0.43 ± 0.04	0.41 ± 0.05	0.44 ± 0.04	0.42 ± 0.04
Heart	0.35 ± 0.03	0.34 ± 0.03	0.34 ± 0.03	0.34 ± 0.03
Liver	2.88 ± 0.19	2.81 ± 0.11	2.78 ± 0.21	2.76 ± 0.17
Kidneys	0.74 ± 0.05	0.73 ± 0.06	0.72 ± 0.05	0.71 ± 0.06
Spleen	0.17 ± 0.02	0.17 ± 0.02	0.16 ± 0.01	0.15 ± 0.01
Testes	0.72 ± 0.09	0.66 ± 0.07	0.70 ± 0.08	0.68 ± 0.07

Data are mean ± standard deviation (n = 10).

to-globulin ratio (A:G) of slightly more than 1.0. Since disease states affect the relative amounts of albumin and globulin, the A:G may provide a clue as to the cause of the change in protein concentrations. Our results show that the decreases in the concentrations of albumin and globulin in females are not dose-dependent, which indicates that these findings are not related to treatment. Furthermore, the total protein concentration and A:G remain unaffected when compared with the control group, indicating that *C. cicadae* is not influencing liver function. Creatinine is a breakdown product of creatine phosphate in muscle and is produced at a constant rate in the body. Serum creatinine is an important parameter for the evaluation of renal function.[23] The creatinine concentration is interpreted in conjunction with BUN to diagnose impaired renal function. Our results show that the decreases in the concentrations of creatinine in females are not dose-dependent, which indicates that these findings are not related to treatment. In addition, these values were found to be within the normal range of rats, thus indicating the result of normal variation among animal groups.[24] All creatinine and BUN concentrations were normal, suggesting that long-term *C. cicadae* ingestion does not cause renal damage. In male animals, the concentrations of sodium in the group treated with 1000 mg/kg were lower ($P < 0.05$) than those in other groups. However, such values were in the range of normal values,[24] which suggests normal organ function.

D. Hematology

Hematological analysis revealed a lower ($P < 0.05$) MCV in males receiving 1000 mg/kg *C. cicadae* submerged mycelial culture powder, but the values in the 500 and 2000 mg/kg groups were not different from those of the control. No treatment-related changes in other parameters were noticed (Table 3). MCV is a measure of "average" RBC volume, which is calculated as follows[25]: (total volume of packed RBCs ÷ total number of RBCs) × 10. Although the males in the 1000 mg/kg group had lower MCVs, the number of RBCs was not different from those of the other 3 groups. Nonetheless, the decrease is not dose dependent, indicating no expressive changes in general metabolism after consumption of *C. cicadae* by rats.

TABLE 2: Serum Biochemistry of Rats after Administration of *Cordyceps cicadae* Mycelia from 90-Day Submerged Culture

Item	Dose (mg/kg Body Weight/Day)			
	0	500	1000	2000
Female				
AST (U/L)	83.6 ± 15.1	85.0 ± 12.5	85.4 ± 13.4	85.4 ± 16.1
ALT (U/L)	37.5 ± 5.70	37.7 ± 9.0	39.7 ± 9.6	37.9 ± 9.8
ALP (U/L)	47.6 ± 16.7	64.0 ± 18.9	55.7 ± 13.3	49.9 ± 13.8
Total protein (g/dL)	8.1 ± 0.4	7.7 ± 0.2	7.7 ± 0.3	7.5 ± 0.4
Albumin (g/dL)	5.2 ± 0.3	4.9 ± 0.1*	4.9 ± 0.2*	4.8 ± 0.3*
Globulin (g/dL)	2.9 ± 0.1	2.8 ± 0.1*	2.7 ± 0.2*	2.7 ± 0.2*
BUN (mg/dL)	14.7 ± 1.4	14.7 ± 1.6	14.0 ± 2.4	13.0 ± 1.7
Creatinine (mg/dL)	0.56 ± 0.05	0.51 ± 0.04*	0.51 ± 0.07*	0.49 ± 0.04*
Glucose (mg/dL)	162.3 ± 13.7	156.0 ± 27.1	162.2 ± 33.3	172.1 ± 43.0
Triglyceride (mg/dL)	57.8 ± 20.9	47.2 ± 10.8	49.6 ± 11.5	46.2 ± 11.0
Cholesterol (mg/dL)	90.4 ± 11.9	89.1 ± 16.8	91.7 ± 13.6	88.2 ± 20.0
Sodium (mEq/L)	147.9 ± 2.3	147.8 ± 1.1	147.4 ± 1.4	147.2 ± 2.2
Potassium (mEq/L)	5.7 ± 0.2	5.8 ± 0.4	5.9 ± 0.3	6.2 ± 0.8
Calcium (mEq/L)	11.9 ± 0.4	11.8 ± 0.3	11.7 ± 0.3	11.8 ± 0.4
Chloride (mEq/L)	100.7 ± 1.7	100.7 ± 1.5	100.0 ± 1.8	100.3 ± 1.1
Phosphorus (mg/dL)	7.4 ± 0.7	7.8 ± 0.8	7.6 ± 0.6	7.8 ± 0.9
Male				
AST (U/L)	108.5 ± 14.8[a]	108.0 ± 12.6	98.1 ± 14.8	105.5 ± 20.6
ALT (U/L)	45.8 ± 7.4	46.6 ± 7.1	41.4 ± 6.0	42.0 ± 6.2
ALP (U/L)	110.1 ± 23.8	106.0 ± 21.1	93.7 ± 11.9	95.7 ± 17.5
Total protein (g/dL)	6.9 ± 0.4	7.0 ± 0.5	6.7 ± 0.4	6.7 ± 0.2
Albumin (g/dL)	4.2 ± 0.2	4.3 ± 0.3	4.2 ± 0.2	4.1 ± 0.2
Globulin (g/dL)	2.7 ± 0.2	2.7 ± 0.3	2.6 ± 0.1	2.5 ± 0.2
BUN (mg/dL)	14.2 ± 2.6	13.0 ± 0.8	12.9 ± 0.9	13.5 ± 1.4
Creatinine (mg/dL)	0.46 ± 0.05	0.46 ± 0.05	0.43 ± 0.04	0.46 ± 0.03
Glucose (mg/dL)	174.1 ± 31.1	191.7 ± 44.1	176.8 ± 25.0	178.0 ± 34.6
Triglyceride (mg/dL)	50.1 ± 16.3	63.6 ± 34.2	54.5 ± 18.1	56.5 ± 22.4
Cholesterol (mg/dL)	57.9 ± 17.8	64.7 ± 10.1	54.5 ± 13.4	62.0 ± 18.3
Sodium (mEq/L)	150.5 ± 1.7	149.3 ± 2.2	148.6 ± 1.4*	150.6 ± 1.4
Potassium (mEq/L)	6.2 ± 0.5	6.1 ± 0.5	6.0 ± 0.4	5.9 ± 0.4
Calcium (mEq/L)	11.5 ± 0.4	11.7 ± 0.6	11.6 ± 0.2	11.5 ± 0.4
Chloride (mEq/L)	100.9 ± 1.4	101.2 ± 1.9	101.9 ± 1.9	101.3 ± 1.9
Phosphorus (mg/dL)	9.3 ± 0.6	9.0 ± 0.4	9.1 ± 0.6	8.6 ± 0.5*

Data are mean ± standard deviation (n = 10). ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; BUN, blood urea nitrogen.

*Significantly different from the control group ($P < 0.05$).

TABLE 3: Hematology of Rats after Administration of *Cordyceps cicadae* Mycelia from 90-Day Submerged Culture

Item	Dose (mg/kg Body Weight/Day)			
	0	**500**	**1000**	**2000**
Female				
WBC (10^3/μL)	8.7 ± 2.3	8.4 ± 1.7	7.6 ± 2.2	9.0 ± 2.4
RBC (10^6/μL)	8.4 ± 0.3	8.6 ± 0.5	8.5 ± 0.5	8.4 ± 0.5
Hemoglobin (g/dL)	15.7 ± 0.8	15.8 ± 0.9	15.8 ± 1.0	15.6 ± 0.9
Hematocrit (%)	51.1 ± 2.4	51.9 ± 3.2	51.3 ± 3.5	50.6 ± 3.1
MCV (fL)	60.7 ± 1.7	60.3 ± 1.4	60.6 ± 2.3	60.3 ± 1.6
MCH (pg)	18.7 ± 0.5	18.4 ± 0.4	18.6 ± 0.6	18.6 ± 0.4
MCHC (g/dL)	30.8 ± 0.5	30.5 ± 0.6	30.8 ± 0.5	30.8 ± 0.5
Platelet (10^3/μL)	840.6 ± 103.9	866.0 ± 72.9	833.7 ± 83.6	780.5 ± 102.1
Neutrophil (%)	13.3 ± 4.0	13.6 ± 2.2	13.2 ± 4.0	13.9 ± 3.9
Lymphocyte (%)	80.9 ± 4.2	80.0 ± 2.7	79.4 ± 5.6	80.1 ± 5.4
Monocyte (%)	4.5 ± 1.1	5.0 ± 1.0	5.9 ± 1.9	4.6 ± 1.7
Eosinophil (%)	1.2 ± 0.5	1.3 ± 0.4	1.4 ± 0.6	1.2 ± 0.3
Basophil (%)	0.14 ± 0.07	0.15 ± 0.07	0.18 ± 0.09	0.16 ± 0.12
Reticulocyte (%)	1.8 ± 0.7	1.6 ± 0.6	1.7 ± 0.7	1.7 ± 0.3
PT (sec)	10.0 ± 0.2	10.1 ± 0.2	10.0 ± 0.2	10.2 ± 0.6
Male				
WBC (10^3/μL)	8.8 ± 5.8	10.9 ± 5.4	13.1 ± 2.9	10.0 ± 3.0
RBC (10^6/μL)	9.1 ± 0.3	9.0 ± 0.6	9.0 ± 0.3	8.8 ± 0.4
Hemoglobin (g/dL)	16.2 ± 0.8	16.3 ± 0.9	15.8 ± 0.5	15.9 ± 0.4
Hematocrit (%)	52.8 ± 2.6	52.7 ± 3.0	50.6 ± 2.2	51.4 ± 1.3
MCV (fL)	58.3 ± 2.3	58.9 ± 1.6	56.3 ± 1.2*	58.3 ± 2.3
MCH (pg)	17.8 ± 0.7	18.2 ± 0.5	17.5 ± 0.4	18.0 ± 0.6
MCHC (g/dL)	30.7 ± 0.7	30.9 ± 0.7	31.2 ± 0.4	30.9 ± 0.5
Platelet (10^3/μL)	833.4 ± 116.2	890.2 ± 209.2	863.6 ± 82.5	810.1 ± 86.5
Neutrophil (%)	18.4 ± 6.0	15.3 ± 3.7	15.8 ± 3.4	16.9 ± 4.0
Lymphocyte (%)	75.5 ± 7.5	78.4 ± 4.1	77.1 ± 4.6	77.1 ± 4.3
Monocyte (%)	4.9 ± 2.1	5.1 ± 1.1	5.8 ± 1.3	4.8 ± 1.3
Eosinophil (%)	1.1 ± 0.4	1.0 ± 0.5	1.2 ± 0.5	1.0 ± 0.4
Basophil (%)	0.17 ± 0.11	0.11 ± 0.09	0.15 ± 0.05	0.17 ± 0.16
Reticulocyte (%)	2.4 ± 0.9	2.0 ± 0.5	2.0 ± 1.1	2.2 ± 0.9
PT (sec)	13.6 ± 1.0	13.5 ± 1.3	14.0 ± 1.6	13.8 ± 1.0

Data are mean ± standard deviation (n = 10). MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, red blood cell; WBC, white blood cell.

*Significantly different from the control group ($P < 0.05$).

TABLE 4: Urinalysis of Rats after Administration of *Cordyceps cicadae* Mycelia from 90-Day Submerged Culture

Parameter	Result	Dose (mg/kg Body Weight)							
		Female				Male			
		0	500	1000	2000	0	500	1000	2000
Color	Yellow	5	7	7	5	1	2	4	2
	Pale yellow	1	1	1	0	1	1	0	0
	Amber	3	2	2	5	8	6	6	8
	Brown	1	0	0	0	0	1	0	0
Clarity	Clear	10	8	7	7	4	7	9	6
	Light turbid	0	2	3	3	6	3	1	4
Glucose	Negative	10	10	10	10	10	10	10	10
	Trace	0	0	0	0	0	0	0	0
Bilirubin	Negative	9	9	10	10	8	10	9	8
	+1	1	1	0	0	2	0	1	2
Ketone	Negative	9	7	7	5	3	1	5	2
	Trace	1	3	3	5	6	8	3	4
	1+	0	0	0	0	1	1	2	3
	2+	0	0	0	0	0	0	0	1
Specific gravity	≤1.015	3	2	2	1	0	1	1	1
	1.016–1.020	1	3	3	2	0	0	1	0
	1.021–1.025	2	1	1	0	1	1	1	0
	1.026–1.030	3	1	1	3	2	2	4	0
	>1.030	1	3	3	4	7	6	3	9
pH	6.0	0	0	0	0	0	0	0	0
	6.5	3	2	4	3	1	0	0	1
	7.0	7	8	6	7	8	8	8	9
	8.0	0	0	0	0	1	2	1	0
	9.0	0	0	0	0	0	0	1	0
Protein	Negative	9	9	10	9	2	4	5	2
	Trace	1	1	0	1	4	4	3	6
	1+	0	0	0	0	3	2	2	2
	2+	0	0	0	0	1	0	0	0
Urobilinogen	0.1	9	10	10	10	6	9	10	10
	1	1	0	0	0	4	1	0	0
Nitrite	Negative	8	10	10	10	10	10	10	10
	Positive	2	0	0	0	0	0	0	0
Blood	Negative	9	5	5	6	0	2	4	1
	Trace	1	4	5	3	9	7	5	5
	1+	0	1	0	1	1	1	1	4
RBC	0–1 hpf	10	8	10	10	9	9	10	9
	2–5 hpf	0	2	0	0	1	1	0	1
WBC	0–1 hpf	9	9	9	10	9	9	10	9
	2–5 hpf	1	1	1	0	1	1	0	1
EP	0–1 hpf	10	10	10	10	10	10	10	10
	2–5 hpf	0	0	0	0	0	0	0	0

There were 10 rats in each group. RBC, red blood cell; WBC, white blood cell; EP, epithelial cell; hpf, high-power field.

TABLE 5: Histopathological Changes of Rats after Administration of *Cordyceps cicadae* Mycelia from 90-Day Submerged Culture

Organ	**Lesions**	**Group***			
		Control		**High Dose**	
		Male	**Female**	**Male**	**Female**
Adrenal		—	—	—	—
Aorta		—	—	—	—
Brain		—	—	—	—
Brain stem		—	—	—	—
Bone, femur		—	—	—	—
Bone marrow		—	—	—	—
Cerebellum		—	—	—	—
Cervix		N	—	N	—
Epididymis		—	N	—	N
Esophagus		—	—	—	—
Eyes		—	—	—	—
Harderian gland	Infiltration, mononuclear cell, focal, slight†	—	2	1	1
Heart	Infiltration, mononuclear cell, focal, minimal to slight	1	1	3	1
Intestine, small					
Duodenum		—	—	—	—
Jejunum		—	—	—	—
Ileum		—	—	—	—
Intestine, large					—
Cecum		—	—	—	—
Colon		—	—	—	—
Rectum		—	—	—	—
Kidney	Cyst, tubule, focal, minimal to slight	—	—	—	2
	Fibrosis, interstitial, focal, minimal	1	—	2	—
	Mineralization, tubule, focal, minimal to slight	—	7	—	9
Liver		—	—	—	—
Lung	Inflammation, focal, minimal	—	1	—	3
Lymph node		—	—	—	—
Mammary gland		N	—	N	—
Optic nerve		—	—	—	—
Ovary		N	—	N	—
Pancreas		—	—	—	—
Parathyroid		—	—	—	—
Pituitary		—	—	—	—
Prostate gland	Infiltration, mononuclear cell, focal, minimal to slight	2	—	1	—
Salivary gland		—	—	—	—
Sciatic nerve		—	—	—	—
Skin		—	—	—	—
Seminal vesicle		—	N	—	N
Spinal cord	Cyst, lumbar, focal, slight	1	—	—	—
Spleen		—	—	—	—
Stomach		—	—	—	—

TABLE 5: (Continued)

Testes	Atrophy, seminiferous tubule, artifact, moderately severe	10	N	10	N
Thigh muscle	Infiltration, mononuclear cell, focal, minimal	1	—	—	—
Thymus		—	—	—	—
Thyroid		—	—	—	—
Tongue		—	—	—	—
Trachea		—	—	—	—
Urinary bladder		—	—	—	—
Uterus	Dilation, focal, slight to moderate	N	8	N	3
Vagina		N	—	N	—

*There were 10 mice in each group. The numerals indicate the number of animals that showed lesions.
†The degree of lesion was graded depending on severity: minimal (<1%), slight (1–25%), moderate (26–50%), moderate/severe (51–75%), and severe/high (76–100%).
—, No animal showed lesions; N, not available.

E. Urinalysis

The results of urinalysis (presented in Table 4) indicate that all the findings for both sexes are not dose-dependent.

F. Histopathology

Histopathologic findings are presented in Table 5. High-dose treatment increased the incidence of minimal to slight tubule cysts (2 of 10 in the high-dose group versus 0 of 10 in the control group) and focal mineralization (9 of 10 in the high-dose group versus 7 of 10 in the control group) in the kidneys of females, and the incidence of minimal interstitial fibrosis in the kidneys of males (2 of 10 in the high-dose group versus 1 of 10 in the control group). Treatment with a high dose also increased the findings of minimal to slight infiltration of mononuclear cells in the hearts of males (3 of 10 in the high-dose group versus 1 of 10 in the control group) and minimal inflammation in the lungs of females (3 of 10 in the high-dose group versus 1 of 10 in the control group). Because the alterations were also observed in the control group, these findings are considered to be not related to treatment.

IV. CONCLUSIONS

The results of this study show that dietary exposure to powder of *C. cicadae* submerged mycelial culture for 90 days at concentrations up to 2000 mg/kg was tolerated by SD rats with relatively minimal indication of systemic toxicity. Therefore, the no-observed-adverse-effect-level of powder of *C. cicadae* submerged mycelial culture is determined to be >2000 mg/kg for both sexes of SD rats. This information provides evidence supporting the use of *C. cicadae* fermentation product as a safe agent for functional foods.

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