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Acute and Sub-Chronic Oral Toxicity of Mycoprotein Product in Wistar Rats

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ABSTRACT Alternative proteins are becoming more popular with consumers nowadays. Various alternative proteins, such as those from plants, insects, and even microorganisms, are widely available and distributed. In the past, Thailand could not produce protein from microorganisms and had to import it from abroad. Currently, Thailand can successfully develop the technology for producing mycoprotein from the fungus *Aspergillus oryzae* strain BCC7051 found in the country. However, oral toxicology studies of this product have never been undertaken. The safety evaluation has to be conducted on animals before the human studies to obtain safety information on the products for consumers. This study aimed to assess the acute and sub-chronic oral toxicity of mycoprotein in Wistar rats. The results showed that mycoprotein at 300 and 2,000 mg/kg body weight (BW) did not cause acute toxicity and mortalities in the rats, indicating that mycoprotein was classified in GHS (Globally Harmonised System for Classification and Labeling of Chemicals) category 5 or unclassified, with the LD_{50} cut-off at 5,000 mg/kg BW or over. In the sub-chronic oral toxicity testing, mycoprotein was administered consecutively for 90 days at doses of 500, 1,000, and 2,000 mg/kg BW, with a 14-day recovery period. It was concluded that the no-observed-adverse-effect level (NOAEL) of mycoprotein was 2,000 mg/kg BW in Wistar rats.

Keywords: Mycoprotein, *Aspergillus oryzae*, Acute and sub-chronic oral toxicity in rat

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Introduction

An alternative protein derived from fungal fermentation is shaped into many food products as a meat substitute called "mycoprotein". Mycoprotein can be produced from the fermentation process of microorganisms of the fungal group. It has the appearance and shape of cells like filaments with a high amount of protein and contains various nutrients that have nutritional value,[1,2] but has no toxins from mold or mycotoxins. Mycoprotein has been reported to be produced from filamentous fungi of various strains[3], and it is an alternative protein source and meat substitute responding to the needs of consumer groups such as vegetarians and flexitarians. Production of mycoprotein from fungi has many advantages because it needs less space to produce, uses less water, and emits less greenhouse gas than raising animals, moreover, this can be expanded more easily and its quality can be controlled. Mycoprotein is expected to be the food for the future (future food) which helps reduce environmental impacts[4] and meet the increasing global protein demand sustainably.

Mycoprotein produced from *Fusarium venenatum* has been registered with the UK Food and Drug Administration, and in 2001, was classified as a safe food. It has been on the market since 2002 and is accepted as a safe food ingredient[5]. Earlier, Thailand did not produce mycoprotein by itself and needed to import it from abroad. The Functional Ingredients and Food Innovation Research Group at the National Science and Technology Development Agency (NSTDA), Thailand, has successfully developed the technology for producing this protein using the liquid fermentation process from *Aspergillus oryzae* strain BCC7051, which is found in the country and is considered a Thai innovation. The fungus *A. oryzae* is used in the production of various food products available on the market, such as tempeh, hamanatto, miso, and shoyu[6]. The strains of *A. oryzae* are safe and classified as food-grade microbes that have remarkable features such as high protein production and hypha formation to get a better texture that is nutritious and safe,[7] in addition, there are no toxins or mycotoxins[8] harmful to consumers. Generally, it is used as semi-industrial liquid fermentation technology under Codex Good Hygiene Practices (GHPs), and Hazard Analysis and Critical Point System (HACCP) standards without chemicals or antibiotics (data not shown). The mycoprotein has high nutritional values like that from eggs which contains 39.65–42.29% total protein dry weight, no cholesterol (data not shown), and all essential amino acids as shown in Table 1, including fiber, vitamins, and beta-glucan (data not shown). However, the toxicological testing for this mycoprotein has never been carried out, thus the safety evaluation had to be conducted in animals to provide toxicity data before human studies. In addition, the results of safety studies can be used as supporting data for commercial production and registration with Thailand's Food and Drug Administration (Thai FDA) and to confirm product safety for consumers.

The purpose of this study was to identify the chemical hazard classification of the mycoprotein produced according to the GHS (Globally Harmonised System for Classification and Labeling of Chemicals) category, which complied with the Organization for Economic Co-operation and Development (OECD)

Guidelines for the Testing of Chemicals 423. Moreover, studies of acute oral toxicity using the acute toxic class method[9] and sub-chronic oral toxicity were performed to confirm the edible safety and evaluate the No-Observed-Adverse-Effect Level (NOAEL) for long-term use of mycoprotein following the methodology of sub-chronic oral toxicity modified from the OECD Guidelines for Testing of Chemicals 408, Repeated Dose 90-Day Oral Toxicity Study in Rodents[10].

Materials and Methods

Specification of test item

The test item was mycoprotein, the finished product from *Aspergillus oryzae* BCC7051 as a brownish-yellow powder, obtained from the BIOTEC Bioprocessing Facility, National Science and Technology Development Agency (NSTDA), Thailand. Its nutrition facts label shown as g/100 g as protein 44.69,[11] ash 6.52,[12] fat 5.94,[13] total dietary fiber 33.18,[14] sodium 52.55,[15] carbohydrate 37.55,[16] calories from fat 53.46,[16] and 382.42 Kcal/100g[16]. For microbiological analysis, its estimated aerobic plate counts (EAPC) were fewer than 2.5×10^2 cfu/g for total plate count,[17] and fewer than 10 cfu/g for *Escherichia coli*[18] and coliforms,[18] while *Salmonella* spp. (per 25 g),[19] heavy metals (mg/kg) as arsenic,[20] cadmium,[21] and mercury,[22] and mycotoxin (μg/kg) such as aflatoxin,[23,24] ochratoxin A,[25] etc., were not detected. In addition, amino acid contents were measured as shown in Table 1. The stability was one year after the manufacturing date, and it was kept in an aluminum foil vacuum bag at room temperature (22±3°C). The level of microbial contamination based on aerobic plate count (APC) was less than 10 cfu/g, which was monitored by the Laboratory of the Quality Control Office, National Laboratory Animal Center, Mahidol University, Thailand, before administering the test item orally to Wistar rats. For the production process of mycoprotein, it is unrevealed due to a commercial secret.

Table 1 Amino acid contents of the mycoprotein from *A. oryzae* strain BCC7051

Amino acid	Content (mg/100g)
Histidine	0.28
Isoleucine	0.46
Leucine	0.78
Lysine	0.71
Methionine	0.16
Threonine	0.53
Valine	0.56
Alanine	0.62
Arginine	0.67
Aspartic acid	1.08
Cysteine	0.11
Glutamic acid	1.70
Glycine	0.50
Ornithine	0.06

Table 1 Amino acid contents of the mycoprotein from *A. oryzae* strain BCC7051 (continued)

Amino acid	Content (mg/100g)
Proline	0.41
Serine	0.54
Taurine	0.05
Tyrosine	0.40

Test animals

Both male and female Wistar rats (*Rattus norvegicus*, strain Mlac:WR) were obtained from the Office of Laboratory Animal Production, National Laboratory Animal Center, Mahidol University, Thailand. For acute oral toxicity, only female Wistar rats with body weight 186-196 grams, 7 weeks of age were used, while sub-chronic oral toxicity, both sexes were used, 5 weeks of age, 153-178 grams for males, and 146-163 grams body weight for females.

Animal husbandry conditions

There were one and two Wistar rats per stainless steel cage for acute oral toxicity and sub-chronic toxicity, respectively, with animal feed (082, Perfect Companions, Thailand) and 5–7 ppm chlorinated water ad libitum, including maintaining the standard condition of a 12:12 light/dark cycle at 22±3°C and 30–70% RH, respectively. The acclimatization was at least 5 days before the study. The guidelines were followed throughout the study, using the Guide for the Care and Use of Laboratory Animals.[26]

Acute oral toxicity testing

Twelve Wistar rats at the age of 8 weeks were divided into three female rats per group per each mycoprotein dose and fasted overnight (15–18 hours) with water ad libitum before dose administration. Following the fasting, mycoprotein was prepared by weighing and mixing with sterile water in a single dose of 300 and 2,000 mg/kg body weight (BW) orally administered to the rats using a gavage needle. After 3–4 hours of administration, the rats were fed with normal feed.

Observation: the rats were observed individually after administration for 30 minutes, and special attention was given during the first 4 hours and periodically during the first 24 hours. The feed and water consumption were measured and recorded daily after each dose administration until necropsy day. Body weights were measured and recorded on days 1, 7, and 14 after dose administration.

Necropsy examination: at the end of acute oral toxicity testing (day 14), surviving rats were euthanized using CO_2 inhalation,[27] and the evaluation for positions, shapes, sizes, and colors of internal organs was performed.

Sub-chronic oral toxicity testing

104 Wistar rats consisting of 52 males and 52 females at 6 weeks were weighed and distributed into five groups (20 animals of 10 per sex in each group). The control group was administered with sterile water while the test (Main) groups were administered with mycoprotein as 500, 1,000, and 2,000 mg/kg BW, respectively, and the Recovery group (12 animals of 10 per sex), were identified as

Groups 1, 2, 3, 4, and 5, respectively. Mycoprotein was weighed and calculated to the body weight of animals on the day of dosing at a constant volume of 1 mL per 500 g BW. All animals were administered the test items once daily for 90 days via oral route, while the Recovery group had a recovery period of 14 days without dosing after 90 days of administration.

Observation: Observation of sub-chronic oral toxicity was recorded weekly, and the Recovery group was continuously observed in the recovery period of 14 days after 90 days of dose administration. Cage side observation included evaluation of skin, fur or coat, eyes, and mucous membrane (occurrence of secretions and excretions), autonomic nervous activity (lacrimation, piloerection, pupil size, and respiratory pattern), changes in gait, posture, and response to handling, the presence of clonic and tonic movements, and stereotype or bizarre behavior such as excessive grooming, repetitive cycling, self-mutilation, and walking backwards. Ophthalmological examination was performed once during the acclimatization period and within a week before the scheduled necropsy in the eyes of animals in the high-dose and control groups. In addition, sub-chronic oral toxicity was assessed neurologically once a week, starting in week 11, including sensory reactivity (auditory, visual, proprioception, fore- and hind-limb grip strength tests, and motor activity assessments).

Necropsy examination: the animals were fasted overnight but with sterile water (15–18 hours) and euthanized using CO_2 inhalation[27] on the last day of each group. The positions, shapes, sizes, and colors of internal organs were evaluated, and then the cardiac puncture method was used to collect blood samples. The blood samples were separated into two tubes, one containing EDTA for hematological analysis (200 µL) and the other without EDTA for clinical biochemistry analysis (800 µL). Hematology analyses were performed on whole blood using an automated analyzer (Procyte Dx™, IDEXX Laboratories, Westbrook, Maine, USA) to evaluate the following parameters: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), white blood cell count (WBC), neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), eosinophil (EO), and basophil (BASO). The clinical biochemistry analyses were conducted for the determination of sodium (Na), potassium (K), chloride (Cl), glucose (GLC), cholesterol (CHOL), triglyceride (TG), uric acid (UA), blood urea nitrogen (BUN), creatinine (CR), total protein (TP), albumin (ALB), globulin (GLO), high-density lipoprotein (HDL), low-density lipoprotein (LDL), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) using an automated blood analyzer (Cobas® c311, Roche Diagnostics, Basel, Switzerland). The organs, such as the liver, kidneys, lung, adrenals, heart, spleen, brain, thymus, testes, and epididymis for males, and the uterus and ovaries for females, were cut and weighed to determine the relative organ's weight per 100 g of body weight. Histopathological examinations used the paraffin section technique for cutting, embedding, and sectioning the organs. Then the scoring of the

organs of the control and high-dose groups were conducted comparatively.

Statistical analysis

All the quantitative results were expressed as mean ± standard deviation. The data with normality and homogeneity of variances were analyzed using the Kolmogorov-Smirnov test and Levene's test.[28] ANOVA and the Mann-Whitney U test were used to compare the data between the control group and each treatment group. In addition, significant difference analyses were performed with SPSS® Statistical software version 18.0.0 ($p \leq 0.05$).

Ethical approval

The study was approved by the National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), Mahidol University, Thailand (No. RA2021-34 on September 10, 2021, for acute oral toxicity testing and RA2022-31 on October 19, 2022, for sub-chronic oral toxicity testing).

Results

Acute oral toxicity testing

From the acute oral toxicity testing, body weights and body weight changes of the groups of control, 300 and 2,000 mg/kg BW mycoprotein were shown in Table 2. Health observation results, including feed, and drinking-water consumptions of all animals were normal except one animal at the dose of 300 mg/kg BW that was observed to have hydronephrosis.

Sub-chronic oral toxicity testing

The sub-chronic toxicity studies were carried out on the test item. General clinical observation, health examination, and ophthalmological examination results of all the rats were not found abnormal. In addition, the neurological examination results of the animals did not show any neurological signs for both sexes. The motor activity assessment scores of only male rats in the 1,000 mg/kg BW mycoprotein group (weeks 12 and 13) were significantly higher than those of the control group ($p \leq 0.05$). The fore-limb grip strength in males of the 500 (week 11) and females of the 2,000 mg/kg BW mycoprotein groups (week 12) were significantly higher and lower than the control group ($p \leq 0.05$), respectively. The hind-limb grip strength of the Recovery group for both sexes (males in week 14 and females in week 15) was significantly lower than the control ($p \leq 0.05$). The body weights of the rats continued to be gained throughout the study, as shown in Figure 1, with no differences when compared with the control groups for both sexes in the Main and Recovery groups.

Figure 2 shows the feed consumption of both sexes in the Main and Recovery groups. In the Main group at 500 (weeks 3, 4, and 5), 1,000 (weeks 2 and 5), and 2,000 mg/kg BW mycoprotein (weeks 2 to 8) of male rats, the significant difference was lower than the control ($p \leq 0.05$), but in the Recovery group (weeks 5, 6, 8, 10, and 12 to15), it was higher than the control ($p \leq 0.05$). Female animals in the Main group at 500 (week 4), 1,000 (weeks 3 and 4), 2,000 mg/kg BW mycoprotein (week 4), and the Recovery group (weeks 4, 5, 9, 10, and 13 to 15) had significant differences higher than control ($p \leq 0.05$).

The water consumption of both sexes of the animals in the Main and Recovery groups was significantly higher than the control

($p \leq 0.05$). In the Main group, 500 (weeks 2, 4 to 12 for males and week 8, 10 for females), 1,000 (weeks 4 to 13 for males and week 5 for females), 2,000 mg/kg BW mycoprotein (weeks 4 to 13 for males and week 2, 3, 6 to 12 for females), and the Recovery group (weeks 1 to 15 for males and weeks 1, 3 to 15 for females) as shown in Figure 3.

The relative organ weights are summarized in Table 3. The left adrenal glands of male rats in the 500 and 1,000 mg/kg BW mycoprotein groups showed significant differences higher than the control group ($p \leq 0.05$). In female animals, the left and right thyroid and parathyroid glands in the 2,000 mg/kg BW mycoprotein group, including the heart in the 1,000 mg/kg BW mycoprotein group, showed significant differences higher than the control group ($p \leq 0.05$).

Hematological analysis results in Table 4 showed that the averages of HGB, HCT, MCV with 500, WBC, LYMPH with 1,000, and HGB, HCT, WBC, LYMPH with 2,000 mg/kg BW mycoprotein of male animals had significant differences higher than the control group, while the average numbers of NEUT of 1,000, MCHC, NEUT, EO of 2,000 mg/kg BW mycoprotein, and MCHC of the Recovery group had significant differences lower than the control group ($p \leq 0.05$). In female animals, only the average WBC in the Main group of 2,000 mg/kg BW mycoprotein and the Recovery group showed significant differences higher than the control ($p \leq 0.05$). Only WBC, NEUT, and LYMPH of male animals were directly variable to the dose levels of mycoprotein.

In the clinical biochemistry analysis (Table 5), the averages of GLU in the 1,000 and 2,000 mg/kg BW mycoprotein groups and Na in the Recovery group of male animals were significantly higher than in the control group ($p \leq 0.05$). In contrast, the averages of Cl (1,000 and 2,000 mg/kg BW mycoprotein), CREA, and Na (2,000 mg/kg BW mycoprotein) were significantly lower than the control group ($p \leq 0.05$). The averages of TP and ALB in the 1,000 mg/kg BW mycoprotein group were significantly higher than the control group ($p \leq 0.05$) in female animals. Additionally, the averages of ALT (1,000 and 2,000 mg/kg BW mycoprotein), AST, and Cl (2,000 mg/kg BW mycoprotein) were significantly lower than the control group ($p \leq 0.05$). Only GLU and Cl of male animals and ALT of female animals were proportional to the dose levels of mycoprotein.

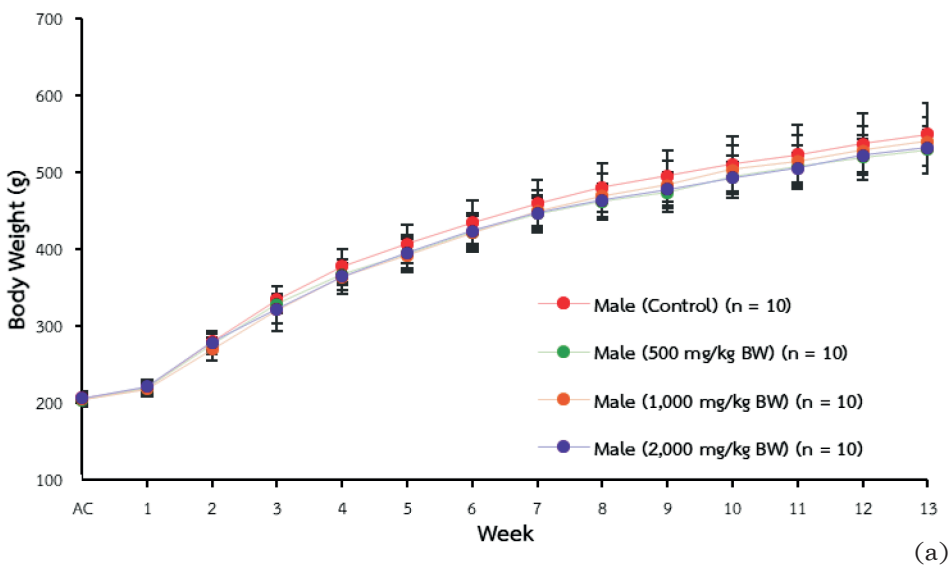
For macroscopic findings, multifocal thymic hemorrhage was found in all study groups, and clear fluid retention in the uterus was found in all female rats. Microscopic findings were observed in the kidney as a tubular hyaline cast of the control, 2,000 mg/kg BW mycoprotein, and the Recovery groups of male animals, and focal tubular mineralization in the females of the 2,000 mg/kg BW mycoprotein group. Focal micro-vesicular fatty degeneration in the liver and focal mononuclear cell infiltration in the heart were shown in males of the Recovery and 2,000 mg/kg BW mycoprotein groups, respectively. Ultimobranchial cyst (females in the 2,000 mg/kg BW mycoprotein group), congenital thyroid cyst (males in the Recovery group), and ectopic thymic tissue (females in the Recovery group) of thyroid and parathyroid glands were found. In addition, it was found that multifocal thymic hemorrhage in all study groups of both sexes was consistent with the macroscopic lesions mentioned above. Uterine

luminal dilation (proestrus) of all groups and small uterine lumen (diestrus) of the 500, 1,000 mg/kg BW mycoprotein, and Recovery groups were observed only in female rats.

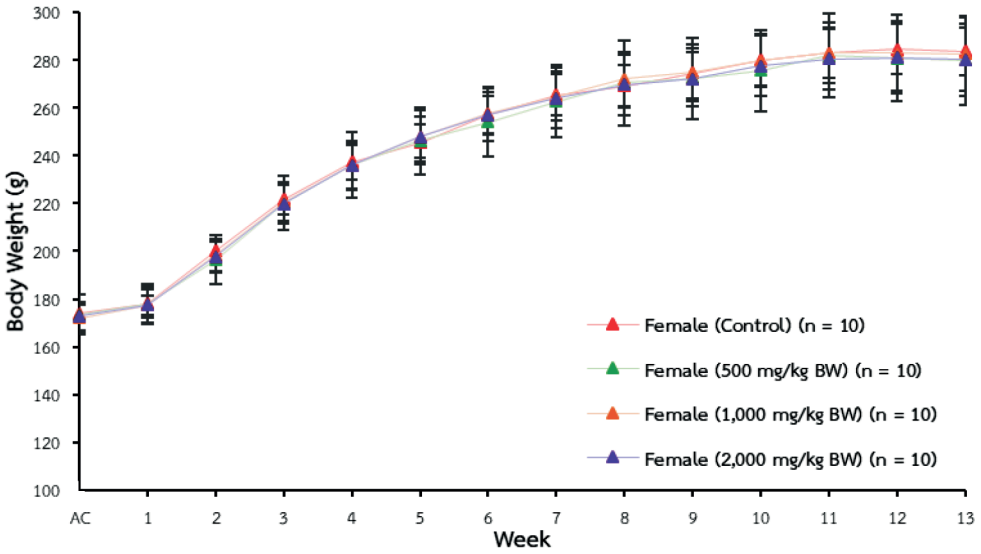
Based on the results, it was concluded that mycoprotein was classified in GHS category 5 or unclassified, the LD_{50} cut-off at 5,000 mg/kg BW or above, and the NOAEL of mycoprotein was considered to be 2,000 mg/kg BW per day.

Table 2 Body weight (g) of Wistar rats in acute oral toxicity testing after administrations of 300 and 2,000 mg/kg BW mycoprotein.

Mycoprotein (mg/kg BW)	Animal no.	Body weight (g)						BW change (%)
		Quarantine	Acclimatization	Day 0	Day 7	Day 14	Terminated	
300	1	196	206	210	234	257	251.35	22.38
	2	194	203	202	228	242	238.77	19.80
	3	192	198	201	223	234	231.43	16.42
	4	191	200	205	232	263	253.69	28.29
	5	190	198	210	234	249	248.64	18.57
	6	190	202	205	230	245	246.17	19.51
2,000	7	189	195	207	229	248	244.25	19.81
	8	189	202	212	236	250	246.60	17.92
	9	189	201	215	245	259	253.88	20.47
	10	187	189	203	234	245	239.64	20.69
	11	187	190	198	231	242	234.24	22.22
	12	186	203	214	244	260	251.78	21.50



(a)



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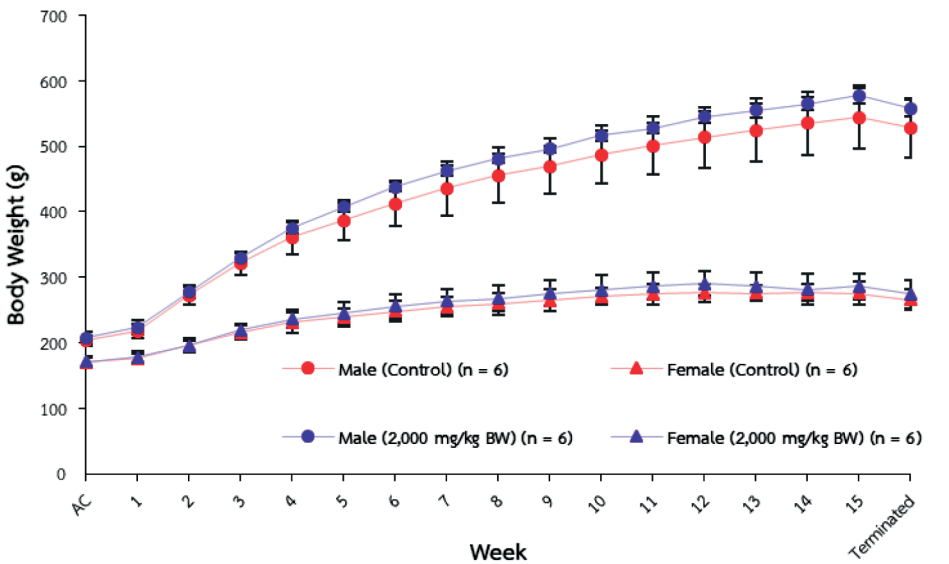
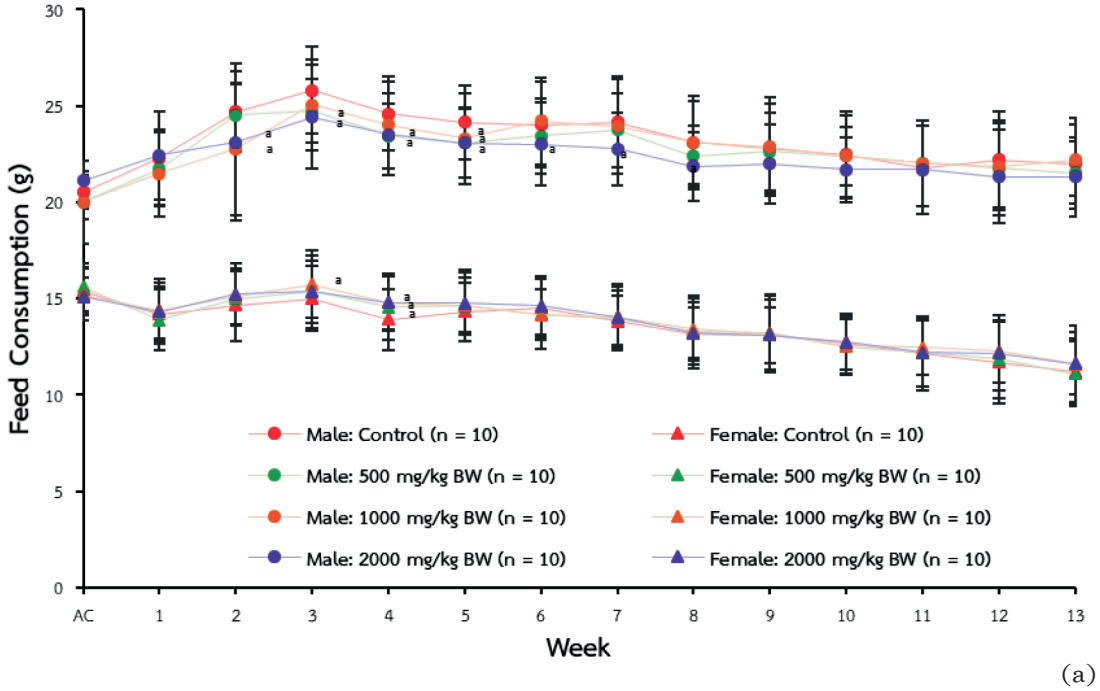
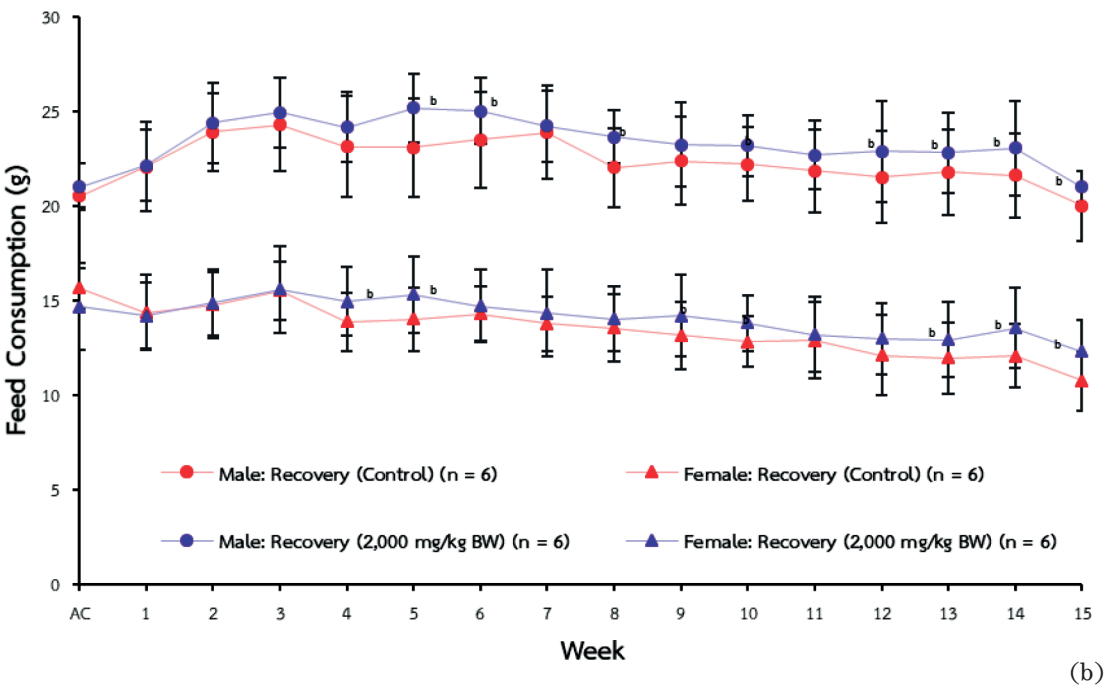


(c)

Figure 1 Body weight (g) of male and female Wistar rats administered with mycoprotein for 90 days. (a) Control and treated male rats with 500, 1,000, and 2,000 mg/kg BW mycoprotein, (b) Control and treated female rats with 500, 1,000, and 2,000 mg/kg BW mycoprotein, and (c) the Recovery group of male and female rats. AC = acclimatization.



(a)



(b)

Figure 2 Feed consumptions (g) in male and female Wistar rats administered with mycoprotein for 90 days. (a) Control and treated male and female rats with 500, 1,000, and 2,000 mg/kg BW mycoprotein, and the average is significantly different from the control group ($p \leq 0.05$). (b) The Recovery group of male and female rats, and the average is significantly different from the Control-Recovery group ($p \leq 0.05$). AC = acclimatization.

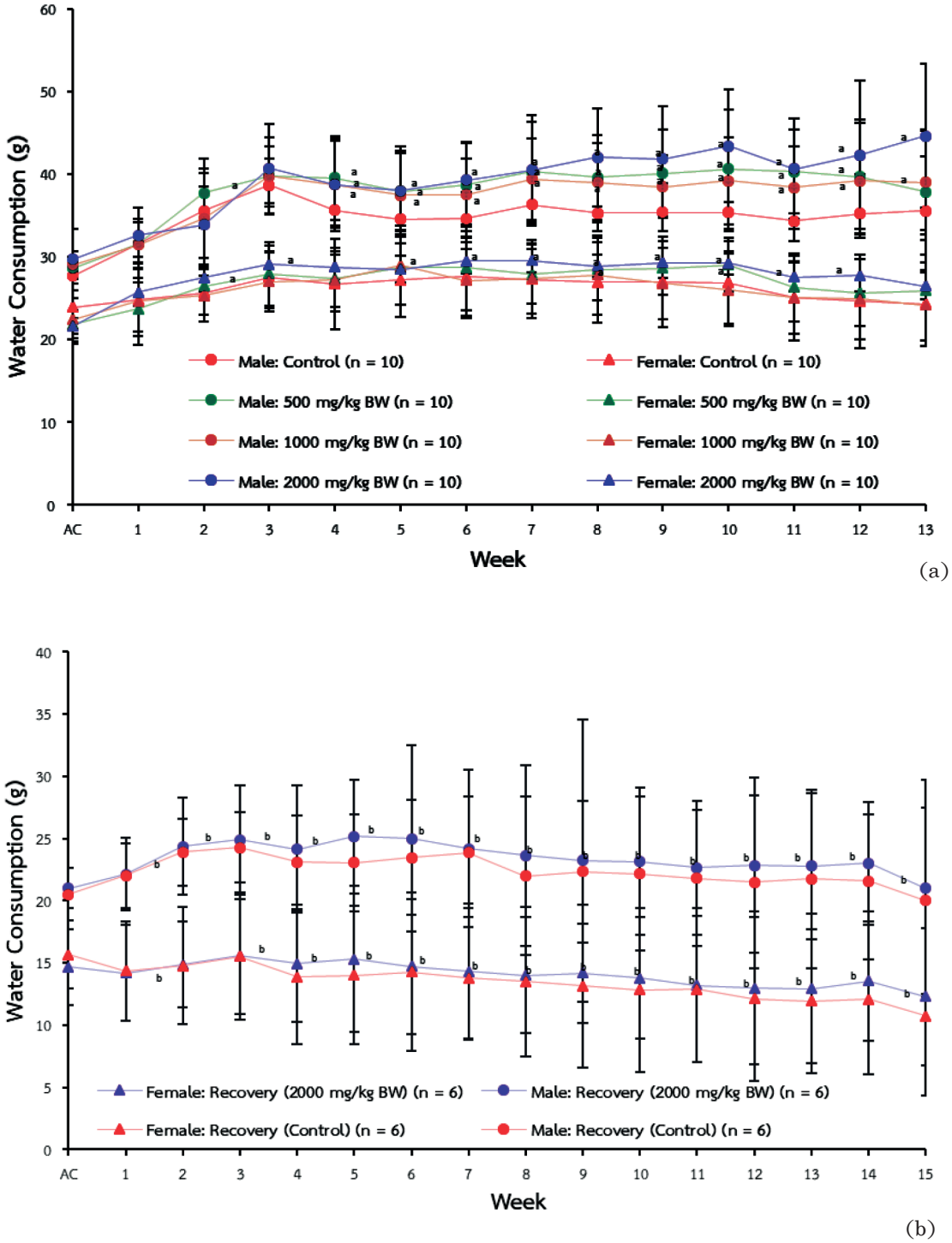


Figure 3 Water consumption (g) in male and female Wistar rats treated with mycoprotein for 90 days. (a) Control and treated male and female rats with 500, 1,000, and 2,000 mg/kg BW mycoprotein, and the average is significantly different from the control group ($p \leq 0.05$). (b) The Recovery group of male and female rats, and the average is significantly different from the Control-Recovery group ($p \leq 0.05$). AC = acclimatization.

Table 3 Relative organ weights in Wistar rats treated with 500, 1,000, and 2,000 mg/kg BW mycoprotein for 90 days.

Organ	Main Group (organ weight in grams after administration with mycoprotein)							
	Control Sterile water (n = 20)		500 mg/kg BW (n = 20)		1,000 mg/kg BW (n = 20)		2,000 mg/kg BW (n = 20)	
	Male	Female	Male	Female	Male	Female	Male	Female
Liver	2.6653±0.14	2.5589±0.26	2.6165±0.14	2.5207±0.16	2.7516±0.14	2.6909±0.15	2.7491±0.11	2.6072±0.16
Right Kidney	0.2601±0.02	0.2865±0.01	0.2584±0.01	0.2879±0.01	0.2473±0.02	0.2922±0.01	0.2528±0.02	0.2942±0.01
Left Kidney	0.2465±0.01	0.2728±0.01	0.2364±0.06	0.2754±0.01	0.2491±0.02	0.2851±0.01	0.2426±0.01	0.2797±0.01
Heart	0.3053±0.01	0.3463±0.01	0.3067±0.02	0.3519±0.02	0.3004±0.01	0.3690±0.02[a]	0.2931±0.01	0.3600±0.02
Spleen	0.1798±0.02	0.2421±0.03	0.1877±0.02	0.2490±0.02	0.1788±0.02	0.2512±0.03	0.1777±0.02	0.2468±0.03
Pituitary	0.0022±0.00	0.0047±0.00	0.0022±0.00	0.0047±0.00	0.0024±0.00	0.0049±0.00	0.0021±0.00	0.0051±0.00
Brain	0.4110±0.03	0.7491±0.04	0.4310±0.03	0.7664±0.05	0.4191±0.03	0.7457±0.05	0.4197±0.02	0.7586±0.04
Right Thyroid, Para	0.0023±0.00	0.0031±0.00	0.0027±0.00	0.0047±0.00[a]	0.0025±0.00	0.0038±0.00	0.0023±0.00	0.0039±0.00[a]
Left Thyroid, Para	0.0022±0.00	0.0032±0.00	0.0027±0.00	0.0041±0.00[a]	0.0025±0.00	0.0036±0.00	0.0021±0.00	0.0031±0.00
Right Adrenal	0.0073±0.00	0.0167±0.00	0.0082±0.00	0.0168±0.00	0.0078±0.00	0.0176±0.00	0.0078±0.00	0.0172±0.00
Left Adrenal	0.0080±0.00	0.0177±0.00	0.0093±0.00[a]	0.0189±0.00	0.0093±0.00[a]	0.0196±0.00	0.0087±0.00	0.0185±0.00
Right Testis / Right Ovary, oviduct	0.3722±0.03	0.0266±0.00	0.3821±0.02	0.0278±0.00	0.3779±0.03	0.0267±0.00	0.3764±0.02	0.0254±0.00
Left Testis / Left Ovary, oviduct	0.3773±0.03	0.0261±0.00	0.3892±0.02	0.0279±0.00	0.3844±0.03	0.0261±0.00	0.3865±0.02	0.0264±0.00
Right Epididymis	0.1170±0.01	-	0.1199±0.01	-	0.1171±0.01	-	0.1193±0.01	-
Left Epididymis	0.1201±0.01	-	0.1200±0.01	-	0.1184±0.01	-	0.1208±0.01	-
Prostate / Uterus	0.0852±0.02	0.1923±0.05	0.0886±0.01	0.1876±0.05	0.0859±0.01	0.2230±0.08	0.0846±0.01	0.2468±0.08
Thymus	0.0578±0.01	0.0871±0.02	0.0532±0.01	0.0908±0.02	0.0616±0.01	0.0955±0.01	0.0637±0.01	0.0911±0.01

Note: Main group: mycoprotein was administered once daily for 90 days via oral route, values are average ± standard deviation. [a] The average is significantly different from the control group ($p \leq 0.05$).

Table 3 Relative organ weights in Wistar rats treated with 500, 1,000, and 2,000 mg/kg BW mycoprotein for 90 days. (continued)

Organ	Recovery Group (organ weight in grams after administration with mycoprotein)			
	Control			
	Sterile water (n = 12)		2,000 mg/kg BW (n = 12)	
	Male	Female	Male	Female
Liver	2.4485±0.10	2.4920±0.15	2.5003±0.16	2.6407±0.17
Right Kidney	0.2445±0.02	0.3019±0.01	0.2499±0.02	0.2963±0.01
Left Kidney	0.2326±0.02	0.2798±0.02	0.2468±0.02	0.2802±0.02
Heart	0.3075±0.03	0.3626±0.01	0.2977±0.01	0.3640±0.02
Spleen	0.1777±0.02	0.2421±0.03	0.1723±0.01	0.2404±0.03
Pituitary	0.0021±0.00	0.0052±0.00	0.0021±0.00	0.0051±0.00
Brain	0.4233±0.04	0.7686±0.05	0.4049±0.01	0.7508±0.05
Right Thyroid, Para	0.0021±0.00	0.0037±0.00	0.0022±0.00	0.0031±0.00
Left Thyroid, Para	0.0020±0.00	0.0035±0.00	0.0022±0.00	0.0033±0.00
Right Adrenal	0.0078±0.00	0.0180±0.00	0.0062±0.00	0.0173±0.00
Left Adrenal	0.0085±0.00	0.0201±0.00	0.0083±0.00	0.0187±0.00
Right Testis/Right Ovary, oviduct	0.3728±0.05	0.0250±0.00	0.3746±0.03	0.0268±0.00
Left Testis/Left Ovary, oviduct	0.3851±0.06	0.0259 ±0.00	0.3792±0.03	0.0260±0.00
Right Epididymis	0.1213±0.01	-	0.1166±0.01	-
Left Epididymis	0.1239±0.02	-	0.1188±0.01	-
Prostate / Uterus	0.0851±0.01	0.2134±0.04	0.0858±0.02	0.1975±0.04
Thymus	0.0510±0.01	0.0828±0.01	0.0590±0.01	0.0772±0.01

Note: Recovery group: mycoprotein was administered once daily for 90 days via oral route, and recovery was in 14 days without dosing.

Table 4 Hematological analysis results in Wistar rats treated with 500, 1,000, and 2,000 mg/kg BW mycoprotein for 90 days.

Parameter	Main Group (after administration with mycoprotein)							
	Control Sterile water (n = 20)		500 mg/kg BW (n = 20)		1,000 mg/kg BW (n = 20)		2,000 mg/kg BW (n = 20)	
	Male	Female	Male	Female	Male	Female	Male	Female
RBC (M/μL)	9.74±0.43	9.85±0.38	9.98±0.23	9.74±0.30	10.05±0.42	9.77±0.49	10.10±0.35	9.72±0.55
HGB (g/dL)	16.92±0.65	17.90±0.55	17.68±0.42[a]	18.07±0.43	17.44±0.29	17.91±1.11	17.48±0.41[a]	17.93±0.96
HCT (%)	52.69±2.32	55.65±1.84	55.30±1.38[a]	56.24±1.57	54.58±1.30	55.88±3.72	55.16±1.35[a]	55.91±3.10
MCV (fL)	54.07±0.84	56.52±1.15	55.41±1.07[a]	57.74±1.17	54.34±1.49	57.19±1.43	54.63±1.40	57.56±1.24
MCH (pg)	17.38±0.25	18.19±0.43	17.72±0.42	18.56±0.36	17.36±0.55	18.35±0.37	17.31±0.52	18.45±0.41
MCHC (g/dL)	32.12±0.34	32.17±0.29	31.97±0.28	32.13±0.31	31.96±0.35	32.07±0.32	31.69±0.26[a]	32.07±0.14
PLT (K/μL)	689.20±38.70	746.70±45.32	724.60±59.72	728.10±62.52	680.13±66.18	715.60±58.55	684.78±57.83	737.90±76.25
WBC (K/μL)	6.15±0.59	4.56±0.71	6.64±1.04	4.61±0.76	7.47±1.17[a]	5.35±1.23	7.83±1.48[a]	5.79±0.86[a]
NEUT (%)	12.90±1.56	9.20±2.19	12.86±1.31	8.95±2.02	10.94±1.44[a]	8.57±1.63	10.66±0.90[a]	7.51±1.35
LYMPH (%)	81.68±1.89	85.76±1.89	81.81±1.18	85.64±2.41	84.19±1.66[a]	85.58±1.97	84.97±2.46[a]	86.72±2.12
MONO (%)	3.97±0.70	4.19±0.90	4.00±1.41	4.28±0.71	3.66±0.86	5.19±1.61	3.53±1.70	5.11±1.66
EO (%)	1.24±0.39	0.62±0.29	0.96±0.20	0.88±0.46	0.99±0.20	0.42±0.13	0.64±0.25[a]	0.38±0.19
BASO (%)	0.21±0.14	0.23±0.13	0.37±0.20	0.25±0.21	0.23±0.19	0.24±0.12	0.20±0.18	0.28±0.12

Note: Main group: mycoprotein was administered once daily for 90 days via oral route. Values are average ± standard deviation. [a] The average is significantly different from the control group ($p \leq 0.05$).

Table 4 Hematological analysis results in Wistar rats treated with 500, 1,000, and 2,000 mg/kg BW mycoprotein for 90 days. (Continued)

Parameter	Recovery Group (after administration with mycoprotein)			
	Control Sterile water (n = 12)		2,000 mg/kg BW (n = 12)	
	Male	Female	Male	Female
RBC (M/μL)	10.38±0.58	9.70±0.28	10.37±0.24	9.55±0.56
HGB (g/dL)	18.18±0.81	18.03±0.44	17.74±0.37	17.93±0.95
HCT (%)	56.85±2.94	56.22±1.16	56.52±1.54	56.30±3.03
MCV (fL)	54.78±1.38	58.00±0.73	54.48±0.99	59.00±1.88
MCH (pg)	17.53±0.51	18.60±0.18	17.14±0.36	18.80±0.64
MCHC (g/dL)	32.00±0.35	32.07±0.30	31.38±0.38[b]	31.85±0.24
PLT (K/μL)	688.00±48.82	760.83±61.77	718.00±63.45	723.33±52.00
WBC (K/μL)	7.07±2.14	4.64±0.84	7.19±0.96	5.85±0.97[b]
NEUT (%)	16.08±10.65	12.33±4.62	11.48±2.78	9.13±1.75
LYMPH (%)	78.45±10.41	82.23±4.68	82.58±3.53	85.18±1.64
MONO (%)	4.27±0.72	4.53±0.81	4.68±1.21	4.88±0.77
EO (%)	0.85±0.28	0.62±0.17	1.00±0.42	0.55±0.24
BASO (%)	0.35±0.24	0.28±0.16	0.26±0.18	0.25±0.16

Note: Recovery group: mycoprotein was administered once daily for 90 days via oral route, and recovery was in 14 days without dosing. Values are average ± standard deviation. [b]The average is significantly different from the control recovery group ($p \leq 0.05$).

Table 5 Clinical biochemistry analysis results in Wistar rats treated with 500, 1,000, and 2,000 mg/kg BW mycoprotein for 90 days.

Parameter	Main Group (after administration with mycoprotein)							
	Control Sterile water (n = 20)		500 mg/kg BW (n = 20)		1,000 mg/kg BW (n = 20)		2,000 mg/kg BW (n = 20)	
	Male	Female	Male	Female	Male	Female	Male	Female
GLU (mg/dL)	328.06±22.26	205.81±45.86	354.69±64.61	166.19±56.01	381.69±33.26[a]	220.36±91.14	388.93±48.51[a]	244.56±47.27
BUN (mg/dL)	19.59±1.94	17.91±1.73	19.86±1.70	18.24±1.98	21.10±1.15	18.08±1.98	20.08±1.61	17.94±1.85
CREA (mg/dL)	0.38±0.03	0.41±0.03	0.40±0.03	0.45±0.03	0.36±0.03	0.41±0.03	0.35±0.02[a]	0.39±0.04
UA (mg/dL)	6.55±0.62	4.36±0.46	6.74±0.88	4.33±0.42	7.46±0.58	4.76±0.83	7.19±1.05	4.92±0.65
CHOL (mg/dL)	76.60±10.52	94.16±9.78	82.77±5.15	81.53±10.21	84.84±6.82	94.68±17.86	82.89±6.58	94.71±10.79
TRIGL (mg/dL)	98.27±22.69	70.00±19.31	97.57±15.08	63.27±6.53	108.06±13.10	75.62±21.51	110.99±25.63	76.08±18.29
LDL (mg/dL)	9.28±2.78	8.17±1.70	11.00±2.63	6.86±2.11	11.54±2.21	8.95±3.73	11.44±2.93	8.22±1.88
AST (U/L)	98.66±32.12	83.87±6.95	102.48±24.22	83.63±6.73	103.99±29.38	80.59±5.10	79.62±15.79	73.48±4.40[a]
ALT (U/L)	73.98±44.17	46.95±8.02	73.78±33.34	41.73±4.41	84.63±32.38	38.95±6.73[a]	53.44±13.92	37.12±5.13[a]
ALP (U/L)	84.10±13.90	44.90±5.82	87.60±8.85	40.90±4.51	91.25±8.15	42.90±3.51	85.44±8.35	41.80±3.26
TP (g/dL)	7.02±0.14	7.15±0.17	7.06±0.11	7.18±0.26	7.15±0.25	7.42±0.25[a]	7.08±0.21	7.21±0.21
ALB (g/dL)	5.06±0.11	5.40±0.11	5.13±0.09	5.50±0.19	5.08±0.15	5.60±0.18[a]	5.10±0.11	5.51±0.17
HDL (mg/dL)	55.47±8.42	74.59±8.39	59.45±4.84	64.60±8.20	62.34±5.51	74.73±12.61	60.64±4.40	76.05±8.62
Na (mmol/L)	148.40±0.84	149.20±1.14	148.20±1.40	148.50±1.72	147.50±0.93	148.80±1.23	147.00±0.50[a]	148.00±1.56
K (mmol/L)	8.92±0.53	9.87±0.72	9.26±0.80	10.55±0.84	9.00±0.52	10.06±0.83	8.82±0.63	10.20±0.91
Cl (mmol/L)	102.40±1.00	106.40±1.49	102.60±1.45	107.67±1.57	100.94±1.13[a]	105.50±2.15	100.99±0.69[a]	104.26±1.53[a]
GLO (mg/dL)	1.96±0.12	1.74±0.11	1.93±0.10	1.68±0.11	2.08±0.12	1.83±0.15	1.97±0.11	1.70±0.08

Note: Main group: mycoprotein was administered once daily for 90 days via oral route. Values are average ± standard deviation. a The average is [a] significantly different from the control group ($p \leq 0.05$).

Table 5 Clinical biochemistry analysis results in Wistar rats treated with 500, 1,000, and 2,000 mg/kg BW mycoprotein for 90 days. (continued)

Parameter	Recovery Group (after administration with mycoprotein)			
	Control Sterile water (n = 12)		2,000 mg/kg BW (n = 12)	
	Male	Female	Male	Female
GLU (mg/dL)	332.80±59.93	220.25±31.06	348.48±28.00	206.80±70.80
BUN (mg/dL)	17.33±1.46	15.70±1.37	17.86±2.36	16.52±1.58
CREA (mg/dL)	0.37±0.03	0.40±0.02	0.38±0.03	0.43±0.02
UA (mg/dL)	6.12±1.36	4.00±0.58	6.66±0.54	3.92±0.42
CHOL (mg/dL)	82.20±9.19	93.08±12.98	75.14±8.71	99.03±11.29
TRIGL (mg/dL)	97.15±27.84	61.07±10.67	85.54±11.18	50.83±8.03
LDL (mg/dL)	10.83±3.61	7.90±2.14	8.76±1.76	10.03±2.74
AST (U/L)	92.38±17.40	79.32±16.64	113.90±46.84	80.05±6.39
ALT (U/L)	61.45±11.44	40.43±7.72	84.38±65.67	36.65±5.02
ALP (U/L)	78.67±11.64	40.17±4.02	85.00±12.25	42.00±4.56
TP (g/dL)	6.93±0.23	7.24±0.30	7.18±0.09	7.00±0.24
ALB (g/dL)	4.86±0.09	5.38±0.23	4.93±0.06	5.13±0.15
HDL (mg/dL)	58.20±7.61	74.77±10.44	51.88±6.93	78.30±8.08
Na (mmol/L)	146.33±1.03	144.67±1.03	147.80±0.45[b]	145.67±1.51
K (mmol/L)	9.13±0.58	9.86±0.92	9.51±0.44	10.40±0.53
Cl (mmol/L)	101.37±1.34	103.08±1.35	100.72±0.46	103.17±0.95
GLO (mg/dL)	2.07±0.17	1.86±0.15	2.25±0.08	1.87±0.18

Note: Recovery group: mycoprotein was administered once daily for 90 days via oral route, and recovery was in 14 days without dosing. Values are average ± standard deviation. [b]The average is significantly different from the control recovery group ($p \leq 0.05$).

Discussion

The toxicity study of mycoprotein in Wistar rats started with acute oral toxicity with a single dose of mycoprotein as the test substance at 300 and 2,000 mg/kg BW. All animals did not show signs of toxic effects, moribundity, or mortality except one rat at a dose of 300 mg/kg BW that was observed to have hydronephrosis. This lesion was found in a Wistar rat because it was associated with naturally occurring background lesions,[29] indicating that this study did not find abnormal gross findings related to the test item. Thus, this result suggested that mycoprotein was classified in GHS category 5 or unclassified, with the LD_{50} cut-off at 5,000 mg/kg BW or over.

Then, sub-chronic oral toxicity was performed by oral administration of mycoprotein at doses of 500, 1,000, and 2,000 mg/kg BW for a total of 90 days. The clinical signs of toxicity

were not observed and did not cause animal mortalities up to the dose of 2,000 mg/kg BW for this test item. All animals had changes in the eye, compared between before and after dosing for ophthalmological examination. In addition, there was little alopecia and scaling skin in health examinations but they were unrelated to the test items. There was a significant difference in the results of the animal motor activity assessment and fore-limb grip strength test. The results were normal transient changes and not related to doses of the test items. The animals continued to gain weight throughout the study, and there were significant differences in feed and water consumption that were not related to the test item because the consumption was a normal transient change. Only WBC, NEUT, and LYMPH of male rats in hematological analysis results, and GLU and Cl of the male and ALT of female rats in clinical biochemistry analysis results were related to the doses of mycoprotein tested. However, it was still in the range of historical control data, which was based on (Mlac:WR),[30] RccHan™:WIST, and Crl:WI(Han).[31] The lesions were observed in the study that commonly occurred as background lesions for the kidney[29] and heart,[32] and physiological changes[33–35] during the estrus cycle for the uterus, attributed to euthanasia or necropsy technique, and considered a dissection-induced artifact[36] for the thymus, congenital anomaly[37] for the thyroid gland, which showed no relation with other groups[38] for the liver. Some studies of sub-chronic oral toxicity of different protein products such as the pea (*Pisum sativum*) protein isolate performed with dietary levels of 25,000, 50,000, and 100,000 ppm in Wistar rats,[39] lyophilized apoaequorin protein (isolated from jellyfish) conducted with doses of 1,000, 2,000, and 4,000 mg/kg BW in Sprague Dawley rats,[40] and a protein derived from *Xanthobacter* sp. SoF1[41] at doses of 375, 750, and 1,500 mg/kg BW with an additional group at 1,290 mg/kg BW as a positive control in Wistar rats.[41] For those results of the studies mentioned, neither mortalities nor induced toxicological changes were found. Moreover, in this study, no treatment-related adverse effect was observed after 90 days of consecutive administration of mycoprotein by considering the results of body-weight change, feed and water consumption, hematological, clinical biochemistry, clinical pathology, or histopathological examination. The results of our study were consistent with those found in other previous studies.[39-41] However, chronic preclinical toxicological investigations of mycoprotein for a longer period are recommended to confirm this observation and to provide adequate safety data for future clinical trials. There should also be a study of efficiency or other related aspects to support this protein product.

Conclusion

In conclusion, the acute oral toxicity study of mycoprotein was observed hydronephrosis at a dose of 300 mg/kg BW, which was just a background lesion of animals. In addition, it showed no effect on body weight, food, or water consumption. Thus, it could be classified in GHS category 5 or unclassified with the LD_{50} cut-off at 5,000 mg/kg BW or over. The sub-chronic oral toxicity study of mycoprotein was relatively safe. The NOAEL of mycoprotein was considered to be 2,000 mg/kg BW per day for Wistar rats under experimental conditions, which was

confirmed by the results of histopathology (hematological and biochemical assays), body-weight change, food and water consumptions, and organ weights.

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ความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรังทางปากของผลิตภัณฑ์ Mycoprotein ในหนูแรทสายพันธุ์ Wistar

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บทคัดย่อ โปรตีนทางเลือกกำลังเป็นที่นิยมในผู้บริโภคมากขึ้น จึงมีผลิตภัณฑ์โปรตีนทางเลือก เช่น โปรตีน จากพืช แมลง และจุลินทรีย์ออกมาจำหน่ายจำนวนมาก ที่ผ่านมาประเทศไทยยังไม่สามารถผลิตโปรตีนจากจุลินทรีย์ได้เองต้องมีการนำเข้าจากต่างประเทศ ปัจจุบันประเทศไทยสามารถพัฒนาเทคโนโลยีการผลิตโปรตีน mycoprotein ได้สำเร็จจากเชื้อรา *Aspergilus oryzae* สายพันธุ์ BCC7051 และยังไม่มีการศึกษาพิษวิทยาทางปากของผลิตภัณฑ์ดังกล่าว ซึ่งการประเมินความปลอดภัยต้องดำเนินการในสัตว์ทดลองก่อนที่จะศึกษาในมนุษย์ เพื่อให้ได้ข้อมูลความปลอดภัยของผลิตภัณฑ์ต่อผู้บริโภค การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรังทางปากของ mycoprotein ในหนูแรทสายพันธุ์ Wistar ผลการทดสอบพบว่า mycoprotein ขนาด 300 และ 2,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักสัตว์ทดลอง ไม่ก่อให้เกิดความเป็นพิษเฉียบพลันและการตายในสัตว์ทดลอง แสดงให้เห็นว่า mycoprotein จัดอยู่ในระบบการจัดกลุ่มสารเคมี การติดฉลาก และการแสดงรายละเอียดบนเอกสารข้อมูลความปลอดภัยสากลหมวดที่ 5 หรือไม่ระบุประเภท มี LD_{50} ที่ 5,000 มิลลิกรัมต่อกิโลกรัมขึ้นไปของน้ำหนักตัวสัตว์ทดลอง ในการทดสอบความเป็นพิษทางปากแบบกึ่งเรื้อรัง การให้โปรตีนชนิดนี้ติดต่อกันเป็นเวลา 90 วัน ที่ขนาด 500 1,000 และ 2,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักสัตว์ทดลอง และในกลุ่มพักฟื้นที่มีเวลาพักฟื้น 14 วัน สรุปได้ว่าขนาด mycoprotein ที่มากที่สุดที่ได้รับต่อเนื่องเป็นเวลานาน โดยไม่ก่อให้เกิดอันตรายที่ร้ายแรงต่อร่างกายต่อวันในสัตว์ทดลอง คือ 2,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง

คำสำคัญ: Mycoprotein, *Aspergillus oryzae*, การทดสอบความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรังในหนู