Master of Science

Evaluation of L-tryptophan associated Eosinophilia-Myalgia Syndrome in Rat 90-Day Repeated Dose Toxicity Study

The Graduate School
of the University of Ulsan
Department of Medical Science
Soo Min Ko

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Supervisor: Woo-Chan Son

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Soo Min Ko

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This certifies that the master's thesis of Soo Min Ko is approved.

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Committee Chair Dr. Hyeong-Seok Lim

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Committee Member Dr. Sang-Nyun Kim

_____

Committee Member Dr. Woo-Chan Son

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Abstract

Eosinophilia-myalgia syndrome (EMS) is a rare disease that was observed in the human who took L-tryptophan as a dietary supplement in 1989 in New Mexico. Since the L-tryptophan is an essential amino acid for not only humans, but also in animals, L-tryptophan had used as a feed additive in livestock. However, after the outbreak of EMS, the safety evaluation of L-tryptophan and other amino acids has been required by the regulatory. Dried L-tryptophan fermentation product is a part of materials of feed additive produced by fermentation with metabolically engineered *Corynebacterium glutamicum*. The safety of the dried L-tryptophan fermentation product was evaluated by a subchronic toxicity study in Sprague Dawley (SD) rats. The product was administered daily by oral gavage at doses of 0, 500, 1000, and 2000 mg/kg/day to groups of 10 male and 10 female SD rats for 13 weeks. For the groups that were administered doses of 0 and 2000 mg/kg/day, an additional 5 male and 5 female SD rats were tested as a recovery group. Histochemical and immunohistochemical staining analysis were conducted to evaluate the L-tryptophan-associated EMS on selected organs.

No adverse effects associated with the test substance were observed in male or female SD rats after 13 weeks of administration at dosages of 0, 500, 1000, and 2000 mg/kg/day and 4 weeks of recovery at dosages of 0 and 2000 mg/kg/day. Furthermore, there were no statistically significant changes in histochemical and immunohistochemical examination in both sexes of administration groups given 0 and 2000 mg/kg/day. Therefore, the no-observed-adverse-effect level of the dried L-tryptophan fermentation product was determined to be 2000 mg/kg/day in both sexes and significant histopathological evidence on EMS was not observed by the administration of the dried L-tryptophan.

Introduction

Eosinophilia-myalgia syndrome (EMS) is a multi-organ inflammatory disease that causes varying degrees of clinical symptoms. A number of organs may be affected in EMS, including the skin, muscles, and lungs. EMS is characterized by eosinophilia in the peripheral blood, skin induration, subacute myalgia, chronic neuropathy, and other clinical signs with pneumonia or pneumonitis with or without dyspnea, fatigue, rash, pulmonary vasculitis, arthralgia, and edema of the limbs (J. Allen & Varga, 2014). EMS was first recognized in New Mexico in several patients in whom eosinophilia, myalgia, abdominal pain, skin rashes, muscle weakness, and elevated serum level of aldolase were observed (Kugler, 2020). Histopathological examination reveals hyperplasia of eosinophil precursors in the bone marrow and perivascular inflammatory cell infiltration in the muscles (Kilbourne et al., 1990). Patients with EMS also show eosinophilic fasciitis-like features. Collagen bundles in the fascia, at all levels of the dermis, and in septa of the subcutaneous fat are thickened, showing homogeneous hyaline-like changes observed in scleroderma. The degree of inflammation is variable and the inflammatory infiltrate is a mixture of various cell types, with eosinophils only sometimes observed. Lymphatic edema and dilation occur in some cases (Troy, 1991).

The Centers for Disease Control (CDC) suggested three criteria for EMS: (i) absolute eosinophil count ≥1000 cells/mm^3, (ii) severity of myalgia limiting normal daily activities, and (iii) histopathologic evidence of perimyositis, perivasculitis, or unspecified fasciitis in muscle biopsy sample under the non-infection or non-neoplasm condition. As of July 1991, the CDC had received reports of 1543 cases of EMS, of which 30 resulted in death (CDC, 1989). Toxicoepidemiologic studies showed a correlation between EMS and dietary supplements containing L-tryptophan produced using genetically engineered strains of

bacteria (J. A. Allen et al., 2011). Most of the cases of EMS were thought to be caused by contamination during the manufacturing process of L-tryptophan at the Showa Denko K.K. plant in Japan (Blackburn, 1997). According to Mayeno et al. (1990), high-performance liquid chromatography showed that the trace contaminant was 1,1'ethylidenebis (tryptophan), peak E (EBT), which led to the initial conclusion that EBT was the causal contaminant of the EMS outbreak. However, a study conducted by the Minnesota group asserted that the research methodology of Mayeno et al. (1990) was impaired, such as the exclusion of some early cases from case-control analysis, so EBT could not be considered the definite cause of EMS (Kilbourne, 1992). Although there is a correlation between EMS and the level of EBT, there is still a lack of statistical significance. Therefore, it has been argued that either the effectiveness of EBT was altered by other contaminants generated during the manufacturing process or that a completely different compound caused EMS (Hendler & Rorvik, 2008).

Tryptophan is one of the essential amino acids that must be supplied as a supplementary form because it cannot be synthesized in the body. L-Tryptophan, the L-isomer of tryptophan, is used in protein synthesis and can pass through the blood–brain barrier. Serotonin (5-hydroxytryptamine), melatonin, quinolinic acid, and kynurenic acid are produced by the metabolism of tryptophan; accordingly, tryptophan is regarded as an important neuromodulator that regulates hemodynamics, gastrointestinal function, and appetite by controlling the secretion of insulin-like growth factor 1, cortisol, corticosterone, and the heat shock protein 70 (Fouad et al., 2021; Le Floc'h, Otten, & Merlot, 2011).

L-Tryptophan has long been used as a feed additive; however, toxicological data for long-term administration are insufficient. A 13-week toxicity study of L-tryptophan in rats found no toxicological changes in ophthalmology, clinical pathology, necropsy, organ weight, or histopathology between the control and treatment groups (Shibui et al., 2018). Toxicologically significant changes in body weight gain and food intake were observed

throughout the administration period in the male 2.5% dose group and in both sexes in the 5.0% dose group. The body weight gain was reduced by up to 8.8% and 17.5% for the males in the 2.5% and 5.0% groups, respectively, and by up to 14.3% for the females in the 5.0% group. However, these changes were not observed after a 5-week recovery period. Consequently, the no observed adverse effect level (NOAEL) of L-tryptophan was determined to be 1.25% for males (779 mg/kg/day) and 2.5% for females (1765 mg/kg/day).

The test substance of the current study, a dried L-tryptophan fermentation product, is directly added as an L-tryptophan supplemental nutrient to feeds, complementary feeds, and premixtures for animals. The dried L-tryptophan fermentation product is produced by fermentation with metabolically engineered *C. glutamicum* to improve the production yield of L-tryptophan. The product contains approximately 60% L-tryptophan as an active substance and other components derived from the biomass of the fermentation broth. Metabolically engineered *C. glutamicum* biomass is not expected to negatively impact the bioavailability of L-tryptophan in the dried L-tryptophan fermentation product (Wensley et al., 2020). However, because the dried L-tryptophan fermentation product is a novel type of amino acid, a toxicity study of this substance is necessary to verify its safety. The potential mutagenicity, clastogenic effect, and acute oral toxicity of the dried L-tryptophan fermentation product have been previously reported (Kang et al., 2020). The product shows no evidence of mutagenicity or chromosomal aberrations. Additionally, the results of acute oral toxicity tests demonstrated that the product was not toxic and had a median lethal dose >2,000 mg/kg/day; therefore, the product was classified as Category 5/unclassified according to the Globally Harmonized System (GHS) (OECD, 2002).

A 90-day repeated dose toxicity study was performed in Sprague Dawley (SD) rats to investigate the potential risks to cause EMS and subchronic toxicity of the L-tryptophan fermentation product. The study was conducted in compliance with the Organization for

Economic Cooperation and Development (OECD) principles of good laboratory practice (GLP) and OECD test guideline 408 (OECD TG 408; 1998, 2018; Redfern et al. 2010). To determine the reversibility of the test-substance-related changes, a 4-week recovery study was conducted.

Materials and Methods

This study was conducted at the Korea Testing & Research Institute (KTR, Hwasun, Korea) according to the OECD TG 408 (updated in 2018) 'Repeated Dose 90-Day Oral Toxicity Study in Rodents' in compliance with the OECD Principles of GLP (1997) ENV/MC/CHEM(98)17. The study protocol was approved by the regulations of the Institutional Animal Care and Use Committee at the KTR based on the Animal Protection Act. No. 16075 and the Laboratory Animal Act. No. 15944 (IACUC No. IAC2019-0789).

1 Test substances

The dried L-tryptophan fermentation product (CJ CheilJedang Corporation, Suwon, Korea) was produced via the fermentation of metabolically engineered *C. glutamicum* (TRP Pro). The product contains 61.09% L-tryptophan as an active substance and other components derived from the biomass of the fermentation broth. The substance was weighed and suspended using sterile distilled water as a dosing vehicle to obtain a final concentration of 200 mg/mL as a high dose. The low and medium doses were prepared using sterile distilled water to obtain final concentrations of 100 and 50 mg/mL, respectively. The dose volume used in this study was 10 mL/kg of body weight.

The test substance was homogenized by stirring and remained stable for 6 days (data not shown). Samples were prepared two or more times per week.

2 Animals

Crl: CD SD rats (55 males and 55 females) were obtained from Orient Bio Co., Ltd. (Gapyeong, Korea). Upon receipt, the male and female rats were 5 weeks old and weighed

137.24–145.26 and 94.22–122.22 g, respectively. Each rat was caged and labeled individually on the tail with a permanent marker pen. After acclimation for 7 days, the rats were re-weighed and randomly assigned to four dose groups, with 10 rats of each sex for the low- and medium-dose groups and 15 rats of each sex for the control and high-dose groups, respectively. At the initial TRP Pro administration, male and female body weights were in the ranges of 188.26–214.01 and 147.02–180.31 g, respectively.

The temperature and relative humidity of the study room was controlled within the range of 20–23 °C and 49%–60%, respectively, with a 12-h light/12-h dark cycle (from 08:00 to 20:00 h), 150–300 lux of luminous intensity, and 10–20 air changes every hour. Rats were fed Rodent Diet 20 5053 (Labdiet, USA), and R/O water was provided *ad libitum*.

3 Dose determination

The dosages used in this study were determined based on a 4-week dose range-finding (DRF) toxicity study in SD rats (internal study No. TBK-2019-002906). The DRF study applied doses of 250, 500, 1000, and 2000 mg/kg/day to SD rats and found no test substance-related effects in any group based on clinical observations, body weight, food consumption, ophthalmological examination, urinalysis, hematologic examination, clinical biochemistry, necropsy, or organ weight. Based on the results, a high dose of 2000 mg/kg/day of the test substance was selected. A common ratio of 2 was set for the low and medium dosages of the test substance. The control group was treated with sterile distilled water, which was the injection vehicle.

4 Test substance administration

The test substance was directly administered into the stomach via oral gavage through a stomach tube (sonde) and provided once a day, 7 days/week, for 13 weeks until the day before the scheduled necropsy. During the recovery period, the test substance was not administered. The individual dose volume for each rat was quantified based on the most recently measured body weight.

5 Serial observation

5.1 Clinical observation

All animals were monitored visually twice a day during the administration period (before/after administration) and once a day during the recovery period to demonstrate ill-health or reaction to treatment by checking clinical signs such as mortality, morbidity, general appearance, and behavioral changes.

5.2 Functional observation

Grip strength, motor activity, and sensory reactivity to stimuli of all rats were observed once toward the end of the exposure period. Grip strength for the forelimbs and hindlimbs was calculated as the average value of three checks using a grip strength measurement device (1027 CSX Grip Strength Meter, Columbus Instruments, OH, USA). Motor activity tests for all rats were conducted every 10 min for 1 h using a motor activity observation system (Opto-Varimex-4 Auto-Track System 4.90, Columbus Instruments). Sensory reactivity to stimuli was determined based on the visual, auditory, touch, and pain responses and righting reflex.

5.3 Body weight

All animals were weighed on the day before the administration of the test substance commenced (day 1), weekly during the administration period, and before necropsy.

5.4 Food consumption

Food consumption was recorded once a week on the first day of administration. Weighed food was provided to each cage using a wire lid with a polycarbonate cage, and the remainder was weighed once a week to calculate the mean daily consumption (g/animal/day).

5.5 Ophthalmological examination

The appearance of the eyes was macroscopically observed for all rats in the control and high-dose groups before the first exposure and in the week prior to necropsy. The pupils were dilated with 1% Tropicamide (Mydriacyl, Alcon Couvreur, Puurs, Belgium). The ocular fundus was examined using a fundus camera (Genesis, Kowa, Japan).

5.6 Urinalysis

In the last week of observation, urine was collected from 5 animals/sex/group in the principal treatment group, and all animals in the recovery group were individually housed in a metabolic cage.

A test strip (Multistix 10SG, Siemens Healthcare Diagnostics, Inc., NY, USA) was soaked in approximately 1 mL of the collected urine. The colors of the collected urine samples were observed visually, and further analysis was conducted using an automatic urinary chemistry analyzer (Clinitek Advantus, Siemens Healthcare Diagnostics, Inc., NY, USA). Several chemical parameters were measured, including glucose, bilirubin, ketone bodies, blood, specific gravity, pH, protein, urobilinogen, nitrite, and leukocytes.

5.7 Hematological examination and clinical biochemistry

Blood was taken from the aorta after fasting overnight (16–24 h) under the influence of isoflurane-induced anesthesia. Blood samples were transferred to CBC bottles (EDTA K2, BD bioscience, CA, USA) and vacutainers (9NC sodium citrate, BD) for hematological and

coagulation tests, respectively. Blood samples were analyzed using a hematology system (ADVIA 2120i, Siemens Healthcare Diagnositcs, Eschborn, Germany) with the following parameters: white blood cell count, differential leukocyte count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocytes, and platelets. Methemoglobin was evaluated using a microplate reader (Synergy HT, Bio Tek, VT, USA).

The coagulation test was conducted using plasma isolated via centrifugation at 3000 rpm (4 °C) for 10 min. Prothrombin time and activated partial thromboplastin time were measured using a coagulation analyzer (ACL Elite Pro, Instrumentation Laboratory, MA, USA).

After taking blood samples for the hematologic and coagulation tests, the remnant samples were placed in serum separation tubes. The tubes were maintained at room temperature without the coagulant. The serum was isolated by centrifugation at 3000 rpm (4 °C) for 10 min. The samples were analyzed using a biochemistry analyzer (TBA-120FR, Toshiba Medical System Co. Ltd., Tokyo, Japan) for the following parameters: total protein, albumin, albumin/globulin ratio, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatinine, blood urea nitrogen, total cholesterol, triglyceride, glucose, calcium, inorganic phosphorus, γ-glutamyl transpeptidase, high-density lipoprotein, low-density lipoprotein, creatine kinase, bile acid, sodium, potassium, chloride, cholinesterase, and urea. Hormone levels in the serum samples were analyzed using a hormone analyzer (Immulite 2000xpi, Siemens Healthcare Diagnositcs, Eschborn, Germany) for the following parameters: thyroxine, triiodothyronine, and thyroid-stimulating hormone.

6 Necropsy and histology

6.1 Necropsy and organ weights

After blood sampling under deep anesthesia induced by isoflurane, the animals were sacrificed via exsanguination from the aorta. External abnormalities in each animal were carefully observed. The organs were removed and examined following the examination of abnormalities in the abdominal, thoracic, and cranial cavities. The following organs and tissues were examined macroscopically: liver, kidneys, adrenal gland, heart/aorta, lung/bronchus, brain, pituitary gland, spleen, seminal vesicle with coagulating glands, testes, ovaries, epididymis, prostate gland, uterus with cervix, vagina, tongue, trachea, esophagus, thyroid gland, parathyroid gland, thymus, stomach, small/large intestine, urinary bladder, skin, mammary gland, sublingual/parotid/submandibular gland, pancreas, eyeball/accessory organs, sternum, femur/bone marrow, spinal cord (cervical, mid-thoracic, and lumbar), submandibular/mesenteric lymph nodes, skeletal muscles, and sciatic nerves. Except for the testes/epididymides (Bouin's fixative solution) and eyes (Davidson's fixative solution), all internal organs were placed individually in 10% neutral-buffered formalin for more than 24 h. Following necropsy, the absolute and relative (organ-to-body weight ratio) weights of the liver, kidneys, spleen, brain, heart, thymus, adrenal glands, pituitary gland, thyroid, testes, epididymides, prostate gland, uterus, and ovaries were obtained. For bilateral organs (e.g., the kidneys, adrenal gland, testes, ovaries, and epididymides), the weights of the left and right organs were measured, and the combined weight was calculated.

6.2 Histopathological examination

Tissue slices of internal organs were individually stored in 10% neutral-buffered formalin, except for the testes/epididymides (fixed with Bouin's fixative solution) and eyes (fixed with Davidson's fixative solution) for more than 24 h. Histopathological examinations were conducted on the fixed organs of all animals in the control and high-dose groups. A

vaginal smear at necropsy provided information on the stage of the rat estrous cycle at the time of the humane killing and facilitated the histological assessment of estrogen-sensitive tissues.

7 Histochemical and immunohistochemical staining

7.1 Histochemical staining

To assess the amount of fascial collagen deposition as one of the clinical features seen in EMS, formalin-fixed paraffin-embedded (FFPE) blocks for the skin tissues were sectioned to 3 μm thickness using a microtome (RM2255, Leica Microsystem, Wetzlar, Germany) and then stained with Masson's trichrome. Using ImageJ (Version 1.52a, National Institutes of Health, MD, USA) collagen intensity in the skin was quantified for the selected five views of a sectioned slide at 200× magnification. The skin tissues were stained with Toluidine blue O (8544-4125, Daejung Chemicals & Metals Co., Siheung, Korea) to examine the number of mast cells. For Toluidine blue staining, five views of a sectioned slide were selected for each section of stained skin tissue, and the mast cells were counted with the naked eye. The FFPE blocks for skeletal muscle were stained for reticular fibers using a Reticulum Stain Kit (ab236473, Abcam, Cambridge, UK). A detailed inspection was conducted following the method recommended by the manufacturer. Congo red (0339-1330, Showa Chemical Industry Co., Ltd., Tokyo, Japan) was used for differentiating eosinophilic infiltration in the lung.

7.2 Immunohistochemical staining

FFPE blocks for the lung tissues were sectioned to 3 μm thickness and immunohistochemically stained with eotaxin antibody (CCL11; LS-C409169, LifeSpan BioSciences, Inc., WA, USA) with a dilution factor of 1:500. Benchmark XT (Ventana

Medical Systems, Inc., Tucson, AZ, USA) autostainer and UltraView DAB IHC Detection Kit (Ventana Medical Systems, Inc.) was used for immunohistochemical staining. Stained slides were scanned with a digital slide scanner (Motic Easy Scan Pro 6-FS, Motic Digital Pathology, SF, USA) at 40× magnification. The proportion of positive cells in the total cells of whole slides images were quantified using the QuPath software (Version 0.3.0., University of Edinburgh, Edinburgh, UK)

8 Statistical analyses

The body weight, food consumption, grip strength, motor activity, organ weights, and hematological and biochemical data were analyzed using the SPSS program (Version 19.0, IBM Corp., NY, USA). The homogeneity of the variances was analyzed using Levene's test, and a one-way ANOVA was conducted to evaluate the significant differences. Scheffe's multiple comparison test was conducted as a post hoc test when the homogeneity of variance and the significance of the difference were demonstrated. Dunnett's T3 test was performed if there was no homogeneous variation. The recovery group was analyzed using *t*-tests. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA, USA) was used to analyze the histochemical and immunohistochemical data, and the multiple unpaired *t*-test procedure of Benjamini, Krieger, and Yekutieli was conducted to evaluate the significance.

Results

1 Clinical observation

The clinical signs of all the animals were examined daily during the treatment period of administration and recovery. Dried L-tryptophan fermentation product-related mortality did not occur in the male or female groups at any dose. Slight salivation was found in one female rat in the high-dose group on days 25, 26, 27, and 32; however, this sign was incidental and unrelated to the test substance (data not shown).

2 Functional observation

There were no dried L-tryptophan fermentation product-related sensory reactivity changes in either sex of all treatment groups (data not shown). There were also no statistically significant differences in grip strength between the control and treatment groups of both sexes (Table 1). Conversely, statistically significant decreases ($p < 0.05$) in motor activity were observed in the male high-dose group compared to the control group at time points 20–30, 30–40, and 40–50 minutes (Figure 1). In addition, motor activity decreased significantly ($p < 0.05$) at time points 40–50 in the male medium-dose group compared to the control group. In the female middle-dose group, statistically significant increases ($p < 0.05$) in motor activity were observed at time points 10–20, 20–30, 30–40, 40–50, and 50–60. Motor activity decreased significantly in the female high-dose group ($p < 0.05$) compared to the control group at time points 10–20 and 50–60.

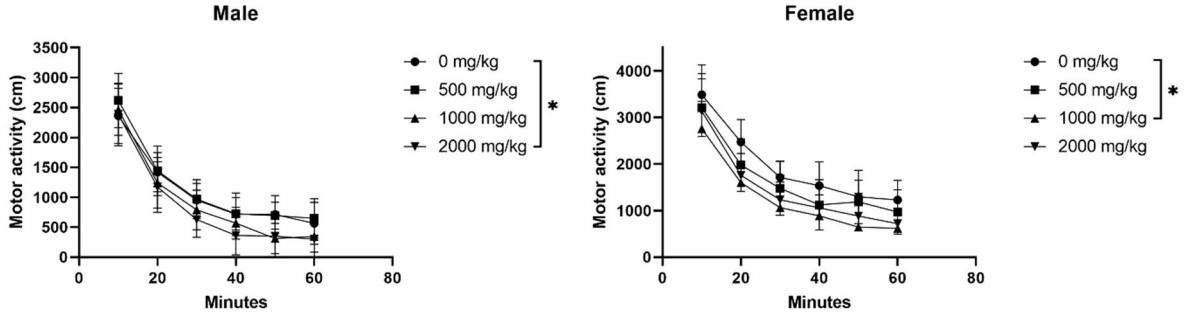

Figure 1 Motor activity examination of male and female rats administered dried L-tryptophan fermentation product for 90 days.

There were significant differences in the male group given 2000 mg/kg and the female group given 1000 mg/kg compared to their respective control groups. Changes seen in the motor activity were not considered test substance-related because there was no dose response observed, the low magnitude of changes, no correlation between sexes, and no histological correlation with central nerve tissues.

Table 1. Functional observation of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

Parameters		Dosage groups (mg/kg/day)							
		Males				Females			
		0 (n=15)	500 (n=10)	1000 (n=10)	2000 (n=15)	0 (n=15)	500 (n=10)	1000 (n=10)	2000 (n=15)
Forelimb grip strength		793±175	901±188	831±169	868±208	554±91	578±85	592±92	569±72
Hindlimb grip strength		305±101	373±156	324±157	342±160	203±66	244±103	254±116	212±77
Motor activity (cm)	0-10 min	2361±460	2618±452	2462±428	2384±523	3491±640	3212±619	2759±586	3136±804
	10-20 min	1419±328	1443±414	1240±424	1171±419	2472±486	1977±564	1600±410*	1755±472*
	20-30 min	953±282	970±325	789±330	628±299*	1710±346	1478±582	1062±547*	1232±533
	30-40 min	715±358	725±270	566±267	364±329*	1535±509	1122±540	885±454*	1059±448
	40-50 min	712±315	692±225	312±256*	351±357*	1295±569	1188±467	647±534*	883±487
	50-60 min	565±350	649±326	346±263	299±293	1227±422	970±477	615±436*	719±491*
	Total	6725±1484	7097±1108	5716±1285	5197±1448*	11730±1696	9947±2729	7568±2202*	8784±2595*

*: significant difference compared with the control ($p < 0.05$)

3 Body weight and food consumption

No dried L-tryptophan fermentation product-related body weight changes were found in either sex during the administration or recovery period (Table 2). The body weight gains of the control and test substance-treated groups in both sexes were not statistically significant (Figure 2A).

Statistically significant differences in food consumption were sporadically observed in the treatment groups compared to the control group in the male high-dose group at week 1, the male medium-dose group at weeks 8 and 11, the male low-dose group at week 11, and the female medium-dose group at week 1 (Figure 2B). Increased food intake was also observed in the male medium- and high-dose groups at week 1 and in the female high-dose group at weeks 2 and 13 (Table 3). These differences were not observed during the recovery period. Sporadic changes in food consumption are incidental because of a lack of a dose–response relationship.

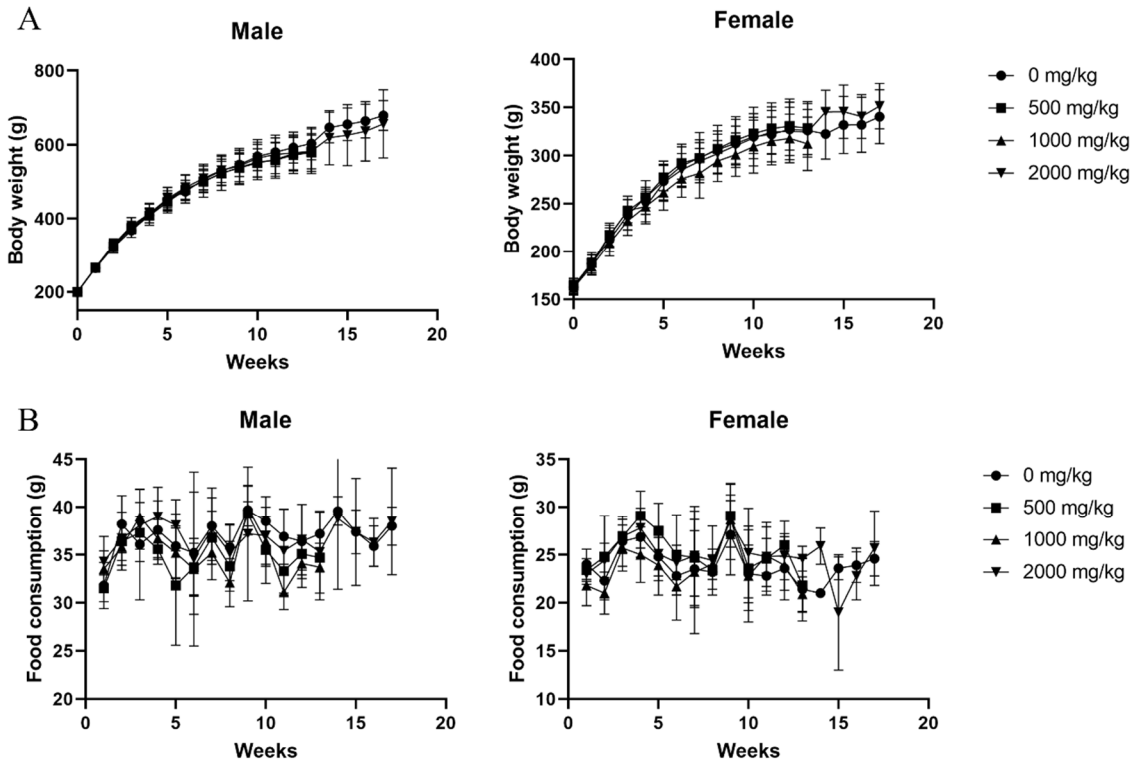

Figure 2 Body weight and food consumption of male and female rats

A) Body weight change during the administration and recovery period of male and female rats. There was no significant body weight change during the whole study period. B) Food consumption changes during the whole study period. Statistically significant changes were sporadically observed in the administration group in either sex; however, these changes were not correlated with the body weight changes. *$p < 0.05$.

Table 2. Group mean body weight of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

Week	Dosage groups (mg/kg/day)							
	Males				Females			
	0	500	1000	2000	0	500	1000	2000
0	199.2±7.5	199.8±6.0	200.6±7.3	199.8±6.4	162.7±7.4	164.1±7.8	162.2±7.7	163.3±8.6
1	264.5±10.0	265.7±9.9	266.0±11.5	266.4±7.7	187.1±11.5	188.1±8.6	184.0±9.0	186.7±9.4
2	322.0±16.2	325.2±10.9	327.4±18.0	329.1±14.9	212.7±12.7	217.5±12.3	208.8±13.5	216.7±11.2
3	368.2±18.9	373.2±14.6	376.2±26.9	380.9±21.9	238.4±15.7	242.4±12.0	232.2±14.1	243.0±15.1
4	409.4±20.0	407.9±19.9	411.7±29.8	414.3±25.3	256.6±17.6	256.5±10.7	247.1±15.9	257.1±18.0
5	452.2±23.5	444.8±24.7	450.4±34.5	454.1±30.5	273.6±20.8	277.4±13.8	261.7±18.3	272.2±17.9
6	473.0±24.2	477.8±26.0	480.6±38.3	484.6±33.2	289.4±22.6	292.3±17.0	276.2±19.3	285.4±17.2
7	506.0±28.1	501.2±28.9	500.1±42.1	508.9±38.4	298.0±26.1	297.6±19.0	281.7±26	294.3±19.4
8	529.2±31.7	523.0±31.9	522.4±45.8	530.8±41.2	306.4±25.2	307.1±18.6	294.4±21.3	302.2±21.1
9	546.0±32.4	537.6±37.3	538.6±45.9	542.6±47.6	312.8±25.1	316.2±24.4	301.2±22.5	310.5±20.2
10	568.6±38.4	550.5±38.7	553.0±48.1	563.4±50.4	319.9±30.5	323.1±20.7	309.4±27.5	318.4±21.4
11	580.0±38.6	559.9±45.0	557.9±49.1	573.9±51.8	322.1±28.6	328.2±26.7	315.3±24.8	324.6±22.4
12	592.0±42.1	575.9±43.8	572.4±49.9	577.6±48.7	327.3±31.6	330.7±24.8	318.0±25.2	325.4±25.0
13	602.9±44.1	580.6±45.6	578.7±50.9	582.3±60.8	326.3±27.8	328.7±27.5	312.4±27.7	324.7±23.4
14	646.7±41.5	-	-	618.7±73.2	322.7±26.1	-	-	345.6±22.5
15	654.9±44.1	-	-	626.0±82.5	331.9±29.6	-	-	345.8±27.8
16	663.6±45.9	-	-	636.1±80.2	332.1±28.6	-	-	340.7±22.8
17	679.1±40.3	-	-	656.1±92.2	340.6±28.1	-	-	351.5±23.6

Table 3. Group mean feed consumption of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

Week	Dosage groups (mg/kg/day)							
	Males				Females			
	0	500	1000	2000	0	500	1000	2000
1	31.8±2.4	31.5±1.4	33.5±1.9	34.3±2.6*	24.0±1.6	23.4±1.2	21.8±2.1*	23.0±1.1
2	38.2±3.0	36.4±2.0	35.8±2.4	36.7±2.8	22.3±2.0	24.8±4.3	21.0±2.2	24.4±1.8*
3	36.1±5.8	37.3±1.7	39.0±1.5	38.1±3.8	26.4±2.6	26.9±0.5	25.7±2.4	26.9±1.8
4	37.6±3.1	35.6±1.6	36.7±2.0	39.0±3.1	26.7±2.1	29.1±2.6	25.0±2.9	27.8±2.1
5	35.9±3.4	31.8±6.2	35.2±2.6	38.1±2.7	24.8±2.0	27.5±2.9	23.9±3.1	25.1±2.8
6	35.2±6.4	33.7±3.0	33.5±2.7	34.6±9.1	22.8±2.0	25.0±4.2	21.7±3.5	24.2±1.8
7	38.0±4.0	36.8±2.8	35.2±2.8	37.7±3.3	23.5±4.0	24.7±4.0	23.2±6.4	24.9±5.2
8	35.8±2.4	33.8±2.6	32.1±2.5*	35.2±2.9	23.2±1.8	23.4±0.5	24.0±1.6	24.4±3.6
9	39.7±2.6	39.3±1.3	39.9±2.3	37.2±7.0	27.1±4.2	29.1±3.3	28.7±2.0	28.5±4.0
10	38.5±2.6	35.5±3.5	36.4±2.6	37.0±3.0	23.0±3.0	23.6±4.4	22.8±4.8	25.2±4.7
11	36.9±2.9	33.3±2.5*	31.1±1.8*	35.4±4.4	22.8±1.6	24.6±3.8	24.9±1.8	24.8±3.1
12	36.4±3.9	35.1±1.7	34.1±2.5	36.6±3.7	23.6±3.3	26±1.2	23.9±1.5	24.9±3.6
13	37.2±2.3	34.7±1.5	33.7±3.4	35.3±4.3	21.4±2.0	21.8±2.8	20.9±1.8	24.6±1.3*
14	39.6±1.5			38.8±7.4	21.0±0.4			25.9±1.9*
15	37.4±2.3			37.4±5.6	23.6±1.2			19.0±6.0
16	35.9±2.1			36.3±2.5	23.9±1.8			22.8±2.5
17	38.0±2.0			38.5±5.6	24.6±1.8			25.7±3.9

*: significant difference compared with the control (p <0.05)

4 Ophthalmological examination

No abnormal ophthalmological effects of dried L-tryptophan fermentation product were observed in either sex during the administration or recovery period (data not shown).

5 Urinalysis

Most urinalysis parameters showed that there was no dried L-tryptophan fermentation product-related effect between the treatment groups and the control group in either sex of the treatment or recovery groups. Table 4 presents the urinalysis results. Bilirubin and urobilinogen tended to increase proportionally with the dose in all-female treatment groups. Additionally, pH tended to decrease with the dose in all treatment groups of both sexes. However, the pH value analyzed in all treatment groups was in the normal range for urine pH. The abovementioned parameters of the high-dose and control groups were recovered at the end of the recovery period.

Table 4. Urinalysis results of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

(a) Administration group

Parameters		Dosage groups (mg/kg/day)							
		Males (n=5)				Females (n=5)			
		0	500	1000	2000	0	500	1000	2000
Color	Yellow	5	5	5	5	5	5	5	5
	Dark yellow	0	0	0	0	0	0	0	0
Glucose	Negative	5	5	5	5	5	5	5	5
	Trace	0	0	0	0	0	0	0	0
Bilirubin	Negative	5	5	5	4	5	5	3	2
	1+	0	0	0	0	0	0	1	2
	2+	0	0	0	1	0	0	1	1
Ketone body (mg/dL)	-	3	3	3	0	5	5	2	2
	≤ 5	1	2	2	3	0	0	3	2
	≤ 15	1	0	0	2	0	0	0	1
Blood	-	4	5	5	5	5	5	5	5
	±	1	0	0	0	0	0	0	0
Specific gravity	≤ 1.005	0	0	0	0	1	0	0	1
	1.010	4	4	4	2	3	3	2	2
	1.015	1	1	1	1	1	2	2	0
	1.020	0	0	0	1	0	0	0	1
	1.025	0	0	0	1	0	0	0	0
	≥ 1.030	0	0	0	0	0	0	1	1
pH	6.5	0	0	0	0	0	0	1	1
	7.0	0	0	0	1	0	1	0	1
	7.5	0	1	2	2	1	1	2	0
	8.0	1	1	1	1	1	2	1	2
	8.5	4	3	2	1	3	1	1	1
	≥ 9.0	0	0	0	0	0	0	0	0
Protein (mg/dL)	Negative	1	1	1	2	4	5	2	2
	Trace	4	4	4	2	0	0	1	2
	≤ 30	0	0	0	1	0	0	1	1
	≤ 100	0	0	0	0	1	0	1	0
Urobilinogen (E.U./dL)	0.2	5	5	5	4	5	5	3	3
	1.0	0	0	0	1	0	0	2	2
Nitrite	Negative	5	5	4	5	3	2	3	2
	Positive	0	0	1	0	2	3	2	3
Leukocyte	Negative	3	1	4	4	4	5	5	4
	Trace	2	4	1	1	0	0	0	1
	1+	0	0	0	0	1	0	0	0
	2+	0	0	0	0	0	0	0	0

E.U.: Ehrlich's unit

(b) Recovery group

Parameters		Dosage groups (mg/kg/day)			
		Males (n=5)		Females (n=5)	
		0	2000	0	2000
Color	Yellow	5	5	5	5
	Dark yellow	0	0	0	0
Glucose	Negative	5	5	5	5
	Trace	0	0	0	0
Bilirubin	Negative	5	5	5	5
	1+	0	0	0	0
	2+	0	0	0	0
Ketone body (mg/dL)	-	3	1	5	4
	≤ 5	1	2	0	1
	≤ 15	1	2	0	0
Blood	-	5	5	5	5
	±	0	0	0	0
Specific gravity	≤ 1.005	0	1	0	0
	1.010	3	1	2	2
	1.015	1	2	1	1
	1.020	1	1	2	2
	1.025	0	0	0	0
	≥ 1.030	0	0	0	0
pH	6.5	0	0	0	0
	7.0	0	0	0	0
	7.5	1	0	1	1
	8.0	0	0	1	0
	8.5	3	2	1	2
	≥ 9.0	1	3	2	2
Protein (mg/dL)	Negative	1	0	4	4
	Trace	3	1	1	1
	≤ 30	1	4	0	0
	≤ 100	0	0	0	0
Urobilinogen (E.U./dL)	0.2	5	5	5	5
	1.0	0	0	0	0
Nitrite	Negative	5	5	4	5
	Positive	0	0	1	0
Leukocyte	Negative	2	1	5	5
	Trace	2	2	0	0
	1+	1	0	0	0
	2+	0	2	0	0

E.U.: Ehrlich's unit

6 Hematology and clinical biochemistry

Table 5 summarizes the analytical data of the hematology and coagulation values of the male and female rats administered dried L-tryptophan fermentation product for 90 days. In the male high-dose group, the prothrombin time increased significantly ($p < 0.05$) during the administration period, but prothrombin time did not change during the recovery period.

In the male high-dose recovery group, monocytes (%) showed a statistically significant decrease ($p < 0.05$) relative to the control group (Figure 3A). In the female high-dose recovery group, the white blood cell count, lymphocyte count, and reticulocytes (%) were significantly different ($p < 0.05$) compared to the control group. However, these changes were not observed during the administration period, and the values were within the normal histological background range.

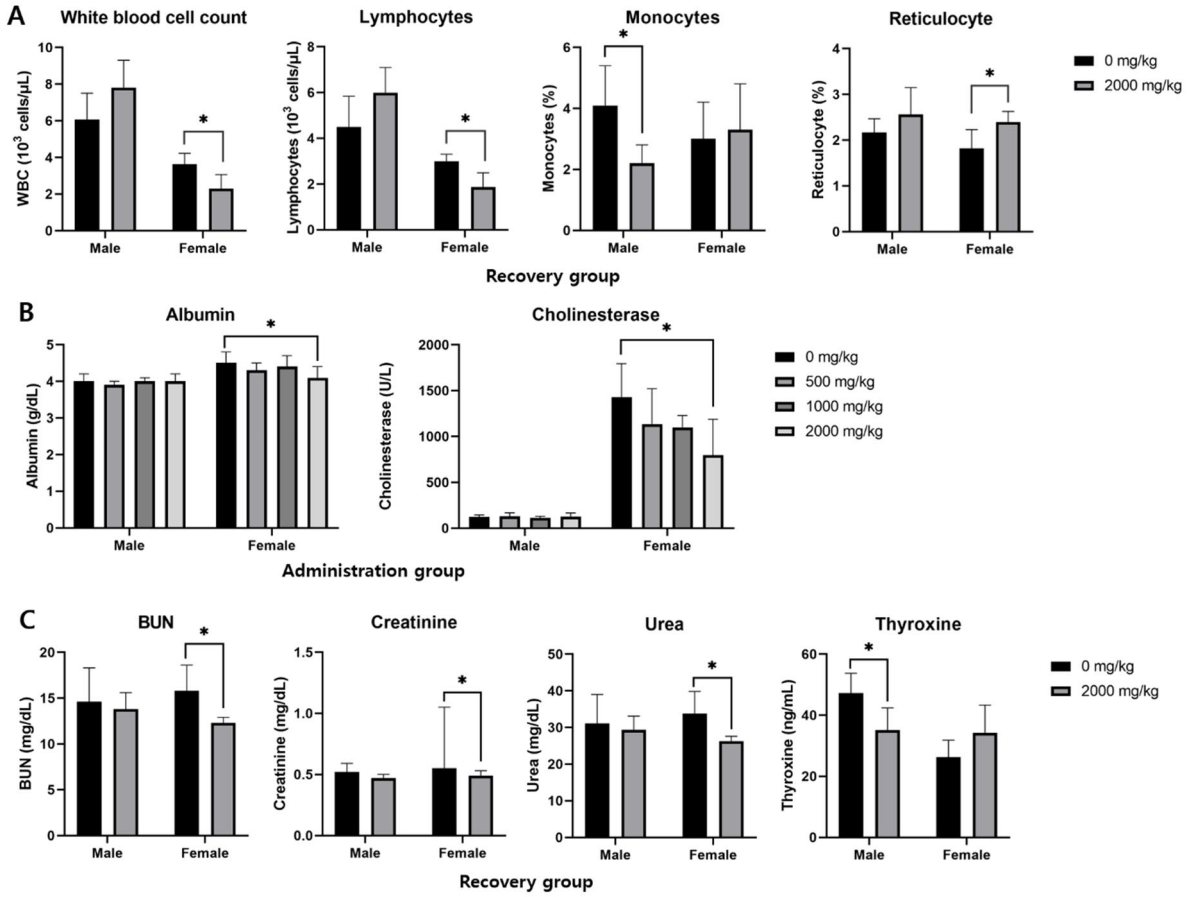

Figure 3 Changes in blood hematology, clinical biochemistry, and hormones of male and female rats administered dried L-tryptophan fermentation product for 90 days.

(**A**) Significant decreases in the white blood cell (WBC), lymphocytes, monocytes, and reticulocytes were observed in recovery groups given 2000 mg/kg compared to the control group in both sexes. These changes were considered low severity and magnitude, and their values were within the historical background data. Clear dose–response and histological correlations with bone marrow were not seen either. (**B**) On clinical biochemistry of terminal sacrifice in the female administration group given 2000 mg/kg, significant decreases in albumin and cholinesterase were observed. For reasons similar to the above, these changes were not considered test substance-related. Their values were within the historical background data and recovered in the recovery group. Clear dose-response and histological correlations with the kidney or liver were not observed. (**C**) Clinical biochemistry and hormone analyses of the recovery group showed significant decreases in blood urea nitrogen

(BUN), creatinine, and urea in the female group and significant decreases in thyroxine in the male group. These changes were not considered test substance-related because they were within the historical background data with low severity and histological correlation with the kidney and thyroid gland. *$p < 0.05$.

Table 5. Group mean hematologic and coagulation values of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

(a) Administration group

Parameters		Dosage groups (mg/kg/day)							
		Males (n=10)				Females (n=10)			
		0	500	1000	2000	0	500	1000	2000
White blood cell count (10^3 cells/μL)		8.98±2.92	7.61±1.91	7.66±1.48	8.68±1.91	3.01±0.87	3.18±1.05	3.78±1.58	4.21±1.47
Differential leucocyte count (10^3 cells/μL)	Neut	1.29±0.41	0.97±0.41	0.93±0.15	1.18±0.54	0.53±0.30	0.47±0.18	0.5±0.14	0.55±0.16
	Lymph	7.23±2.8	6.21±1.59	6.28±1.41	6.99±1.60	2.58±0.50	2.98±0.90	3.21±0.91	4.13±1.81
	Mono	0.20±0.06	0.17±0.07	0.19±0.06	0.25±0.09	0.07±0.02	0.08±0.04	0.07±0.03	0.10±0.05
	Eos	0.11±0.04	0.13±0.06	0.14±0.06	0.12±0.04	0.07±0.02	0.07±0.02	0.08±0.03	0.08±0.03
	Baso	0.01±0.01	0.01±0.01	0.01±0.01	0.02±0.01	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Differential leucocyte count (%)	Neut	15.3±5.8	12.6±4.2	12.5±3.1	13.5±4.6	15.7±6.3	13.0±3.5	13.4±4.7	12.6±5.1
	Lymph	79.5±5.9	81.7±4.5	81.6±3.8	80.6±5.1	79.2±6.2	82.4±3.8	81.9±5.0	83.1±5.2
	Mono	2.3±0.8	2.3±0.5	2.5±0.7	2.9±0.8	2.3±0.4	2.3±0.8	1.9±0.7	2.0±1.0
	Eos	1.3±0.4	1.7±0.9	1.9±1.1	1.5±0.5	2.2±0.8	1.8±0.5	2.0±0.8	1.8±0.5
	Baso	0.2±0.1	0.1±0.0	0.2±0.1	0.2±0.1	0.1±0.1	0.1±0.1	0.1±0.1	0.0±0.1
Red blood cell count (10^6 cells/μL)		8.16±0.37	8.13±0.27	8.3±0.47	8.41±0.36	7.94±0.31	7.84±0.29	7.82±0.3	7.86±0.32
Hemoglobin (g/dL)		14.2±0.5	14.5±0.7	14.8±0.4	14.7±0.5	14.5±0.5	14.6±0.6	14.3±0.7	14.7±0.6
Hematocrit (%)		42.7±1.5	43.6±1.9	43.7±1.7	43.9±1.6	42.4±1.4	43.3±1.6	42.2±1.7	43.5±1.2
Mean corpuscular volume (fL)		52.3±1.2	53.7±2.1	52.7±2.0	52.3±1.5	53.4±1.5	55.3±1.9	54.0±1.7	55.3±2.0
Mean corpuscular hemoglobin (pg)		17.5±0.4	17.8±0.7	17.8±0.9	17.4±0.5	18.2±0.5	18.6±0.7	18.3±0.7	18.8±0.8
Mean corpuscular hemoglobin concentration (g/dL)		33.4±0.3	33.2±0.6	33.8±0.6	33.3±0.5	34.1±0.6	33.7±0.6	33.8±0.6	33.9±0.8
Reticulocyte (10^9 cells/L)		178.3±42.4	170.4±26.3	152.2±19.9	155.2±20.0	130.3±11.6	149.3±21.4	120.5±14.9	130.1±20.4
Reticulocyte (%)		2.19±0.53	2.1±0.31	1.84±0.23	1.85±0.24	1.64±0.12	1.91±0.31	1.54±0.19	1.66±0.29
Platelet (10^3 cells/μL)		842±218	913±87	860±103	874±94	1007±115	980±132	1036±61	974±133
Prothrombin time (sec)		15.7±1.2	16.1±0.6	16.5±0.7	17±0.8*	14.9±0.4	15.2±0.5	15.1±0.8	15.6±0.8
Activated partial thromboplastin time (sec)		15.7±2.8	16.5±1.8	16.4±1.0	16.4±1.0	16.7±1.2	16.1±1.2	16.2±1.6	16.0±0.6
Methemoglobin (%)		0.2±0.43	0.59±0.92	0.5±0.28	0.33±0.25	0.53±0.71	0.12±0.19	0.48±0.22	0.53±0.62

*: significant difference compared with the control ($p < 0.05$)

Neut: Neutrophils; Lymph: Lymphocytes; Mono: Monocytes; Eos: Eosinophils; Baso: Basophils

(b) Recovery group

Parameters		Dosage groups (mg/kg/day)			
		Males (n=5)		Females (n=5)	
		0	2000	0	2000
White blood cell count (10^3 cells/μL)		6.07±1.43	7.81±1.49	3.63±0.59	2.30±0.76*
Differential leucocyte count (10^3 cells/μL)	Neut	1.14±0.38	1.42±0.44	0.39±0.20	0.27±0.10
	Lymph	4.50±1.34	6.0±1.09	2.99±0.31	1.87±0.62*
	Mono	0.23±0.05	0.18±0.07	0.11±0.06	0.08±0.05
	Eos	0.09±0.04	0.11±.0.02	0.06±0.03	0.04±0.01
	Baso	0.01±0.01	0.01±0.01	0.01±0.01	0.00±0.00
Differential leucocyte count (%)	Neut	19.2±5.5	18.0±3.2	10.4±3.9	12.0±1.8
	Lymph	73.6±7.1	77.0±3.9	83.0±5.6	81.5±3.8
	Mono	4.1±1.3	2.2±0.6*	3.0±1.2	3.3±1.5
	Eos	1.5±0.6	1.5±0.5	1.7±0.7	1.9±0.5
	Baso	0.1±0.1	0.1±0.1	0.2±0.1	0.1±0.1
Red blood cell count (10^6 cells/μL)		8.33±0.36	8.32±0.45	7.58±0.31	7.40±0.22
Hemoglobin (g/dL)		15.0±0.6	14.6±0.7	14.5±0.5	14.2±0.4
Hematocrit (%)		44.3±1.5	42.8±1.5	41.2±1.4	40.5±1.6
Mean corpuscular volume (fL)		53.2±1.9	51.6±2.2	54.4±1.2	54.6±1.2
Mean corpuscular hemoglobin (pg)		18.0±0.4	17.6±1	19.2±0.4	19.2±0.2
Mean corpuscular hemoglobin concentration (g/dL)		33.9±0.8	34.1±0.6	35.2±0.5	35.2±0.5
Reticulocyte (10^9 cells/L)		180.8±27.3	211.8±46.9	138.5±34.0	177.6±16.9
Reticulocyte (%)		2.17±0.31	2.56±0.59	1.82±0.41	2.40±0.23*
Platelet (10^3 cells/μL)		965±202	1088±128	753±431	875±503
Prothrombin time (sec)		16.3±0.9	16.1±0.7	15.5±0.9	15.3±0.4
Activated partial thromboplastin time (sec)		17.1±1.8	17.0±0.9	13.5±.0	13.9±2.8
Methemoglobin (%)		0.22±0.20	0.00±0.00	0.46±0.83	0.00±0.00

*: significant difference compared with the control ($p < 0.05$)

Neut: Neutrophils; Lymph: Lymphocytes; Mono: Monocytes; Eos: Eosinophils; Baso: Basophils

Table 6 presents the analytical data of the clinical biochemistry. No dried L-tryptophan fermentation product-related significant differences in clinical biochemistry were identified between the treatment groups and the control group during the administration period. Additionally, no statistically significant differences in the abovementioned parameters were observed throughout the recovery period. By contrast, albumin and cholinesterase decreased significantly ($p < 0.05$) in the female high-dose group compared to the control group during the administration period (Figure 2B). In the female high-dose recovery group, creatinine, blood urea nitrogen, and urea decreased significantly ($p < 0.05$) compared to the control group (Figure 2C). However, those changes were not observed in the treatment group, and the values were within the normal histological background range.

Table 6. Group mean biochemistry values of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

(a) Administration group

Parameters	Dosage groups (mg/kg/day)							
	Males (n=10)				Females (n=10)			
	0	500	1000	2000	0	500	1000	2000
Total protein (g/dL)	6.4±0.4	6.2±0.3	6.3±0.2	6.3±0.3	6.7±0.3	6.5±0.2	6.7±0.4	6.3±0.5
Albumin (g/dL)	4±0.2	3.9±0.1	4±0.1	4±0.2	4.5±0.3	4.3±0.2	4.4±0.3	4.1±0.3*
Albumin/globulin ratio	1.8±0.1	1.8±0.1	1.7±0.1	1.8±0.1	2±0.1	1.9±0.1	1.9±0.1	1.9±0.1
Total bilirubin (mg/dL)	0.02±0.02	0.03±0.02	0.02±0.02	0.03±0.01	0.06±0.02	0.05±0.01	0.05±0.03	0.06±0.02
Alkaline phosphatase (U/L)	492±94	472±87	450±146	517±132	150±16	154±24	151±46	205±79
Aspartate aminotransferase (U/L)	97±23	91±11	88±9	103±30	88±27	80±19	93±22	87±11
Alanine aminotransferase (U/L)	42±7	40±9	42±7	49±10	34±21	25±8	32±12	27±5
Creatinine (mg/dL)	0.44±0.05	0.44±0.03	0.43±0.04	0.42±0.05	0.5±0.04	0.48±0.03	0.48±0.03	0.48±0.05
Blood urea nitrogen (mg/dL)	14.9±1.1	14±1.7	15.6±1.3	14.9±2.2	16.8±1.7	15.5±1.8	17.4±2.6	17.2±4.2
Total cholesterol (mg/dL)	74±20	66±12	68±12	69±16	79±21	81±12	84±22	80±18
Triglyceride (mg/dL)	87±25	89±66	113±60	111±45	18±8	17±5	20±11	22±8
Glucose (mg/dL)	165±15	159±12	158±23	167±23	140±9	149±11	146±15	153±19
Calcium (mg/dL)	10.2±0.4	10±0.2	10.2±0.2	10.1±0.3	9.8±0.3	9.7±0.2	10±0.3	9.7±0.3
Inorganic phosphorous (mg/dL)	6.9±0.8	6.9±0.7	6.8±0.5	6.8±0.4	5±0.4	5.2±0.5	5.4±0.6	5.6±0.5
γ-glutamyltranspeptidase (IU/L)	0.05±0.17	0.2±0.31	0.14±0.17	0.21±0.4	0.28±0.34	0.23±0.29	0.31±0.43	0.57±0.64
High-density lipoprotein (mg/dL)	48±15	44±12	42±9	43±10	55±13	58±10	61±14	56±12
Low-density lipoprotein (mg/dL)	15±4	13±3	15±4	14±5	9±4	10±2	11±4	12±4
Creatine kinase (U/L)	429±296	350±129	251±87	334±182	202±89	207±119	237±142	222±105
Bile acid (μmol/L)	36.4±30.3	20.7±14.9	35±18.2	53.7±43.6	21.2±13.5	22.3±18.2	26.1±31.9	38.1±75.5
Sodium (mmol/L)	143.7±1.1	143.7±0.9	144.1±0.6	143.6±0.9	143.6±1.3	144.1±1.6	144.1±1.8	144.1±1.4
Potassium (mmol/L)	4.83±0.36	4.6±0.29	4.55±0.16	4.72±0.2	3.98±0.27	3.99±0.36	4.33±0.22	4.16±0.26
Chloride (mmol/L)	103.9±1.6	103.8±1.3	103.5±1.2	103.4±1.4	106.1±2.4	105.5±2.5	106.4±1.5	105.8±2
Cholinesterase (U/L)	124.2±20.1	129.2±38.3	114.1±15.5	127.2±38.6	1429.5±364.1	1136.6±384.6	1099.2±130.4	797.1±390.5*
Urea (mg/dL)	32±24	30±3.6	33.5±2.8	31.9±4.6	36±3.6	33.1±3.9	37.2±5.6	36.8±9.1

*: significant difference compared with the control ($p < 0.05$)

(b) Recovery group

Parameters	Dosage groups (mg/kg/day)			
	Males (n=5)		Females (n=5)	
	0	2000	0	2000
Total protein (g/dL)	6.0±0.3	6.0±0.3	6.5±0.3	6.2±0.5
Albumin (g/dL)	3.8±0.2	3.8±0.1	4.3±0.2	4.2±0.3
Albumin/globulin ratio	1.8±0.2	1.8±0.1	2.0±0.1	2.0±0.1
Total bilirubin (mg/dL)	0.03±0.02	0.05±0.02	0.07±0.02	0.05±0.03
Alkaline phosphatase (U/L)	226±29	299±141	85±16	149±74
Aspartate aminotransferase (U/L)	85±21	87±24	197±260	85±24
Alanine aminotransferase (U/L)	33±13	36±10	81±120	26±13
Creatinine (mg/dL)	0.52±0.07	0.47±0.03	0.55±0.5	0.49±0.04*
Blood urea nitrogen (mg/dL)	14.6±3.7	13.8±1.8	15.8±2.8	12.3±0.6*
Total cholesterol (mg/dL)	64±13	61±21	76±16	66±11
Triglyceride (mg/dL)	39±9	39±22	36±6	31±7
Glucose (mg/dL)	199±30	201±38	168±14	173±23
Calcium (mg/dL)	9.6±0.1	9.7±0.3	9.8±0.2	9.6±0.3
Inorganic phosphorous (mg/dL)	6.0±0.4	6.1±0.3	5.0±0.4	4.7±0.6
γ-glutamyltranspeptidase (IU/L)	0.44±0.27	1.25±1.50	0.61±1.08	0.39±0.41
High-density lipoprotein (mg/dL)	46±10	47±17	50±7	47±4
Low-density lipoprotein (mg/dL)	10±2	11±5	10±4	7±2
Creatine kinase (U/L)	159±86	203±158	311±157	345±304
Bile acid (μmol/L)	36.6±49.5	22.1±12.5	27.0±33.6	22.2±12.7
Sodium (mmol/L)	142.4±1.4	142.1±1.3	142.1±0.7	142.5±1.1
Potassium (mmol/L)	4.43±0.12	4.58±0.19	4.41±0.59	4.13±0.28
Chloride (mmol/L)	103.7±1.6	103.5±1.5	104.4±0.6	104.9±1.8
Cholinesterase (U/L)	133.5±40.5	114.8±22.8	1376.4±246.2	1370.1±598.6
Urea (mg/dL)	31.1±7.9	29.4±3.7	33.8±6.0	26.3±1.3*

*: significant difference compared with the control ($p < 0.05$)

The hormone analysis found no test substance-related changes in hormones for the females of the treatment or control groups (Table 7). Thyroxine decreased significantly ($p > 0.05$) in the male high-dose recovery group (Figure 3C). This change was not observed during the administration period, and the value was within the normal historical background range.

Table 7. Group mean hormone of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

(a) Administration group

Parameters	Dosage groups (mg/kg/day)							
	Males (n=10)				Females (n=10)			
	0	500	1000	2000	0	500	1000	2000
Triiodothyronine (ng/mL)	0.570±0.088[a]	0.531±0.136[b]	0.519±0.071[a]	0.561±0.100[c]	0.534±0.071	0.544±0.089[c]	0.515±0.068[b]	0.521±0.084[d]
Thyroxine (ng/mL)	67.4±13.4	62.6±10.7	64±10.1	64.7±10.0	40.9±8.6	39.4±7.2	37.3±9.1	45.6±10.2
Thyroid stimulating hormone (μIU/mL)	0.627±0.306	0.613±0.283	0.657±0.275	0.794±0.259	0.249±0.195	0.173±0.105	0.171±0.113	0.161±0.130

[a]: n=7, three animals were excluded from the statistical analysis due to the out-of-range values of triiodothyronine.
[b]: n=9, one animal was excluded from the statistical analysis due to the out-of-range values of triiodothyronine.
[c]: n=8, two animals were excluded from the statistical analysis due to the out-of-range values of triiodothyronine.
[d]: n=6, four animals were excluded from the statistical analysis due to the out-of-range values of triiodothyronine.

(b) Recovery group

Parameters	Dosage groups (mg/kg/day)			
	Males (n=5)		Females (n=5)	
	0	2000	0	2000
Triiodothyronine (ng/mL)	-[a]	0.431±0.019[b]	0.505±0.003[b]	0.534±0.138
Thyroxine (ng/mL)	47.2±6.5	35.1±7.3*	26.3±5.5	34.2±9.1
Thyroid stimulating hormone (μIU/mL)	0.321±0.176	0.358±0.256	0.121±0.057	0.207±0.133

*: significant difference compared with the control ($p < 0.05$)
[a]: n=0, five animals were excluded from the statistical analysis due to the out-of-range values of triiodothyronine.
[b]: n=2, three animals were excluded from the statistical analysis due to the out-of-range values of triiodothyronine.

7 Gross necropsy and organ weights

Adrenal gland discoloration was observed in one animal in the female high-dose group at necropsy (data not shown). Table 8 presents the relative organ weights of the male and female rats fed dried L-tryptophan fermentation product for 90 days. There were no dried L-tryptophan fermentation product-related changes between the male and control groups. The relative organ weights of the liver and kidneys (right and total) in the female high-dose group increased significantly ($p < 0.05$) during the administration period (Figure 4A). However, the weights of the liver and kidneys in the recovery group were not different from those in the control group. The relative organ weight of the uterus in the high-dose recovery group was notably higher ($p < 0.05$) than that in the control group (Figure 4B). However, these changes were considered to be sex cycle-related.

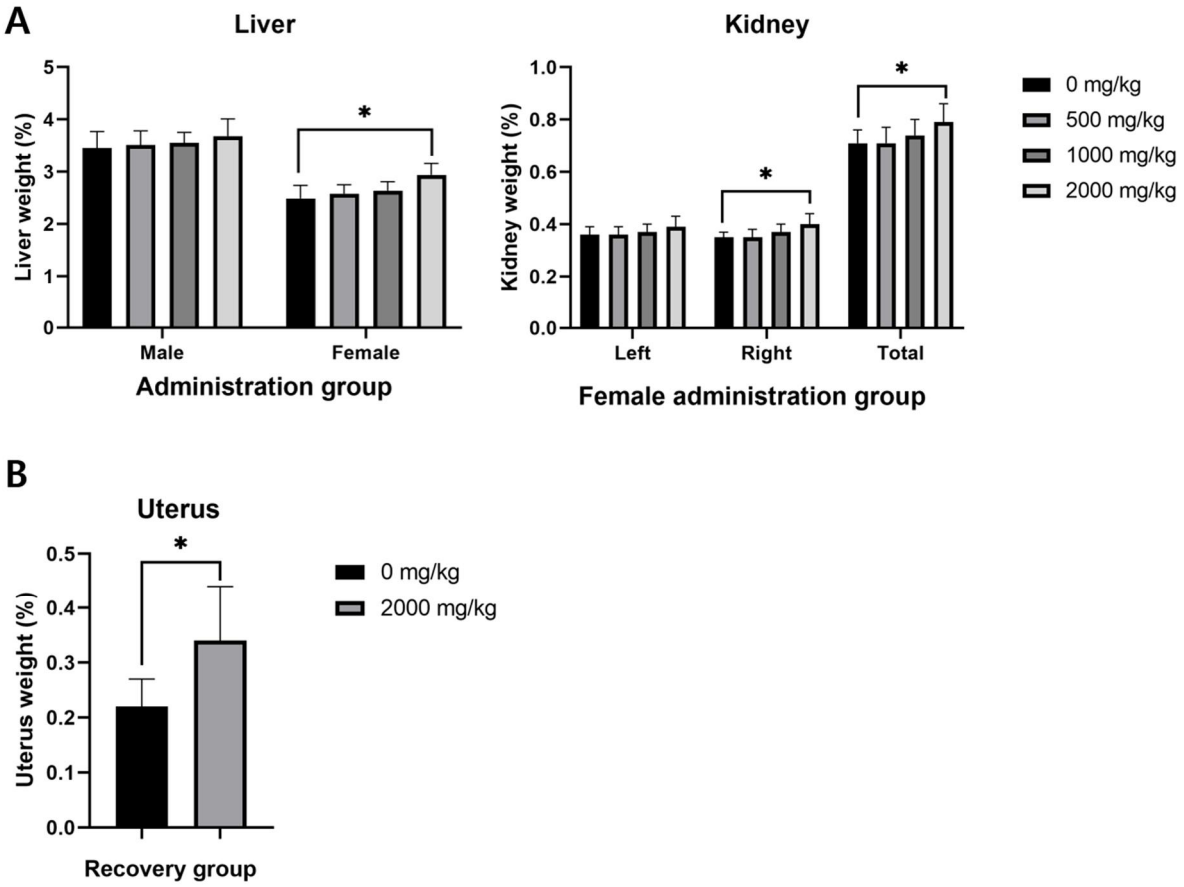

Figure 4. Changes in relative organ weight of male and female rats administered dried L-tryptophan fermentation product for 90 days.

(**A**) In the female administration group given 2000 mg/kg, significant increases were observed in the liver and kidney; however, these changes were recovered in the recovery group and had no microscopic correlation with the liver and kidney. (**B**) Significant increases were seen in the relative weight of the uterus in the female recovery group given 2000 mg/kg; however, it was considered sex cycle-related because of the absence of microscopic correlation in the uterus. *$p < 0.05$.

Table 8. Group mean relative organ weights of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

(a) Administration group

Parameters		Dosage groups (mg/kg/day)							
		Males (n=10)				Females (n=10)			
		0	500	1000	2000	0	500	1000	2000
Body weight (g)		561.1±41.2	549.9±44.3	547.3±47.3	558.0±47.7	313.5±32.0	309.8±26.5	298.4±26.4	301.7±23.9
Liver (%)		3.45±0.32	3.51±0.27	3.55±0.20	3.68±0.33	2.49±0.25	2.58±0.17	2.64±0.17	2.94±0.22*
Kidney (%)	Left	0.36±0.05	0.36±0.04	0.36±0.02	0.37±0.02	0.36±0.03	0.36±0.03	0.37±0.03	0.39±0.04
	Right	0.36±0.05	0.36±0.05	0.36±0.02	0.37±0.03	0.35±0.02	0.35±0.03	0.37±0.03	0.40±0.04*
	Total	0.72±0.09	0.72±0.08	0.72±0.04	0.74±0.05	0.71±0.05	0.71±0.06	0.74±0.06	0.79±0.07*
Spleen (%)		0.18±0.02	0.18±0.01	0.17±0.01	0.20±0.05	0.18±0.02	0.19±0.03	0.17±0.01	0.21±0.06
Brain (%)		0.43±0.05	0.42±0.02	0.41±0.02	0.42±0.05	0.68±0.06	0.67±0.04	0.69±0.05	0.69±0.06
Heart (%)		0.31±0.02	0.32±0.01	0.31±0.02	0.32±0.02	0.34±0.02	0.36±0.03	0.35±0.03	0.37±0.02
Thymus (%)		0.09±0.02	0.09±0.01	0.09±0.01	0.09±0.02	0.11±0.03	0.11±0.02	0.11±0.02	0.12±0.03
Adrenal gland (%)	Left	0.005±0.001	0.006±0.001	0.006±0.001	0.006±0.001	0.014±0.003	0.013±0.003	0.014±0.002	0.014±0.003
	Right	0.005±0.001	0.006±0.001	0.006±0.001	0.005±0.001	0.013±0.002	0.012±0.002	0.013±0.002	0.012±0.002
	Total	0.011±0.002	0.012±0.001	0.012±0.001	0.011±0.002	0.027±0.005	0.025±0.004	0.027±0.004	0.026±0.004
Pituitary gland (%)		0.003±0.000	0.002±0.000	0.002±0.000	0.002±0.000	0.007±0.001	0.007±0.002	0.008±0.001	0.008±0.002
Thyroid (%)	Left	0.003±0.000	0.003±0.001	0.003±0.001	0.003±0.001	0.004±0.001	0.004±0.001	0.004±0.001	0.004±0.001
	Right	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.001	0.004±0.001	0.004±0.001	0.004±0.001	0.004±0.001
	Total	0.007±0.001	0.006±0.001	0.006±0.002	0.006±0.001	0.008±0.001	0.007±0.002	0.007±0.002	0.007±0.001
Testis (%)	Left	0.30±0.04	0.32±0.02	0.31±0.02	0.34±0.05	-	-	-	-
	Right	0.29±0.05	0.32±0.02	0.32±0.02	0.33±0.07	-	-	-	-
	Total	0.59±0.09	0.65±0.04	0.63±0.03	0.66±0.12	-	-	-	-
Epididymis (%)	Left	0.16±0.02	0.16±0.02	0.16±0.03	0.15±0.02	-	-	-	-
	Right	0.16±0.02	0.16±0.01	0.16±0.02	0.15±0.01	-	-	-	-
	Total	0.31±0.04	0.32±0.03	0.32±0.04	0.30±0.03	-	-	-	-
P.S. (%)		0.74±0.13	0.73±0.07	0.68±0.05	0.69±0.09	-	-	-	-
Uterus (%)		-	-	-	-	0.24±0.06	0.30±0.19	0.23±0.08	0.27±0.09
Ovary (%)	Left	-	-	-	-	0.015±0.003	0.017±0.004	0.014±0.004	0.016±0.002
	Right	-	-	-	-	0.016±0.005	0.016±0.003	0.014±0.004	0.016±0.002
	Total	-	-	-	-	0.031±0.007	0.034±0.006	0.028±0.008	0.032±0.004

P.S.: Prostate+seminal vesicles with coagulating glands

*: significant difference compared with the control ($p < 0.05$)

(b) Recovery group

Parameters		Dosage groups (mg/kg/day)			
		Males (n=5)		Females (n=5)	
		0	2000	0	2000
Body weight (g)		636.7±39.1	611.5±77.9	308.9±23.2	320.8±18.8
Liver (%)		2.48±0.12	2.48±0.22	2.59±0.21	2.60±0.21
Kidney (%)	Left	0.30±0.03	0.34±0.04	0.36±0.03	0.39±0.03
	Right	0.31±0.03	0.34±0.03	0.36±0.04	0.38±0.03
	Total	0.61±0.06	0.69±0.07	0.72±0.07	0.77±0.06
Spleen (%)		0.16±0.01	0.16±0.01	0.20±0.01	0.21±0.02
Brain (%)		0.37±0.03	0.35±0.05	0.69±0.04	0.65±0.05
Heart (%)		0.30±0.02	0.30±0.02	0.37±0.04	0.37±0.03
Thymus (%)		0.08±0.02	0.08±0.01	0.11±0.02	0.11±0.02
Adrenal gland (%)	Left	0.005±0.000	0.006±0.001	0.014±0.002	0.014±0.002
	Right	0.005±0.000	0.006±0.001	0.013±0.001	0.013±0.001
	Total	0.010±0.001	0.012±0.001	0.027±0.002	0.027±0.002
Pituitary gland (%)		0.002±0.000	0.002±0.000	0.008±0.002	0.007±0.001
Thyroid (%)	Left	0.003±0.001	0.003±0.000	0.004±0.001	0.004±0.001
	Right	0.003±0.000	0.003±0.001	0.004±0.001	0.004±0.001
	Total	0.006±0.001	0.006±0.001	0.008±0.001	0.008±0.002
Testis (%)	Left	0.27±0.03	0.32±0.06	-	-
	Right	0.27±0.03	0.30±0.04	-	-
	Total	0.54±0.06	0.61±0.09	-	-
Epididymis (%)	Left	0.16±0.02	0.16±0.03	-	-
	Right	0.16±0.02	0.15±0.03	-	-
	Total	0.31±0.04	0.31±0.05	-	-
P.S. (%)		0.62±0.06	0.62±0.08	-	-
Uterus (%)		-	-	0.22±0.05	0.34±0.10*
Ovary (%)	Left	-	-	0.014±0.002	0.014±0.002
	Right	-	-	0.016±0.002	0.014±0.004
	Total	-	-	0.029±0.004	0.028±0.006

P.S.: Prostate+seminal vesicles with coagulating glands

8 Histopathological examination

Table 9 summarizes the histopathological observations of the representative organs of each system. The representative organs of the digestive, urogenital, cardiovascular, respiratory, reticuloendothelial/hematopoietic, and glandular systems were the liver, kidneys, heart, lung, spleen, and thymus, respectively. An increase in the frequency of multifocal alveolar macrophage infiltration into the lungs occurred in the male high-dose group during the administration period (Figure 5). However, this change was not considered a test substance-related adverse effect because the severity level was not severe, and lesions were observed at an increased frequency only in the male group. Additionally, the abovementioned condition was s a common incidental finding in SD rats, especially in the long-term toxicity study.

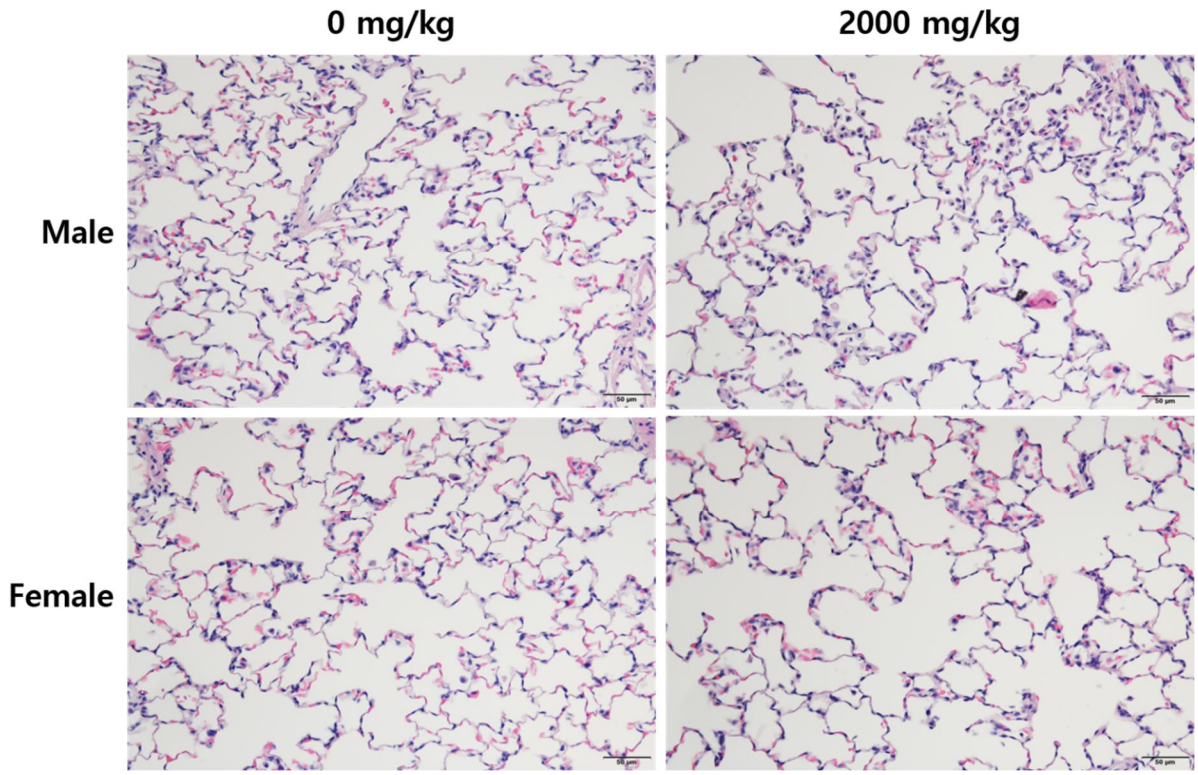

Figure 5 Findings seen in histopathology examination

H&E-stained slide of alveolar macrophages infiltration into lung tissue in male and female rats. This finding was not considered test substance-related because the increased frequency of lesions was only found in the male group the severity was low. Furthermore, alveolar macrophages were commonly observed in SD rats, especially in the long-term toxicity study. Original magnification, 200×, scale bar: 50 μm.

Table 9. Histopathological analysis of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks

System	Organ	Histopathological findings	Dosage groups (mg/kg/day)			
			Males (n=10)		Females (n=10)	
			0	2000	0	2000
Digestive	Liver	NAD	9	8	9	9
		Hepatocyte vacuolation				
		- Slight, Focal	1	0	0	0
		Inflammatory cell infiltration				
		- Minimal, Multifocal	1	0	0	1
		Hypertrophy, centrilobular				
		- Minimal, diffuse	0	1	0	0
		Inflammatory cell infiltration, centrilobular				
		- Minimal, Multifocal	0	0	1	0
		Focal necrosis				
		- Slight, multifocal	0	1	0	0
Urogenital	Kidney	NAD	5	4	6	5
		Basophilia, tubular				
		- Minimal, focal	1	0	0	0
		- Minimal, multifocal	2	4	0	0
		Inflammatory cell infiltration, interstitial				
		- Minimal, focal	1	0	1	1
		- Minimal, multifocal	1	0	1	2
		- Slight, focal	0	1	0	0
		- Slight, multifocal	1	1	0	0
		Scar, cortical				
		- Present	0	1	1	1
		Cyst, medullary				
		- Minimal, multifocal	0	0	0	1
		- Slight, multifocal	1	0	0	0
		Mineralization, medullary				
		- Minimal, focal	0	0	0	1
		Dilation, tubular				
		- Minimal, multifocal	1	0	0	0
		Cast, corticomedullary				
		- Slight, focal	0	0	1	0

Table 9. Histopathological analysis of male and female rats that were administered the dried L-tryptophan fermentation product for 13 weeks (Cont.)

System	Organ	Histopathological findings	Dosage groups (mg/kg/day)			
			Males (n=10)		Females (n=10)	
			0	2000	0	2000
Urogenital	Kidney	Cast, medullary				
		- Minimal, multifocal	0	0	1	0
		Mineralization, corticomedullary				
		- Minimal, multifocal	0	0	0	1
Cardiovascular	Heart	NAD	8	8	10	9
		Inflammatory cell infiltration, myocardial				
		- Minimal, multifocal	1	0	0	0
		- Slight, multifocal	1	2	0	1
Respiratory	Lung	NAD	8	3	8	8
		Macrophage infiltration, alveolar				
		- Minimal, multifocal	1	4	1	0
		- Slight, multifocal	0	2	0	0
		- Slight, diffuse	0	0	0	1
		Inflammatory cell infiltration, perivascular				
		- Minimal, multifocal	1	1	1	1
Reticuloendothelial/ hematopoietic	Spleen	NAD	10	10	10	10
Glandular	Thymus	NAD	10	10	10	10

NAD: no abnormality detected

9 Histochemical and immunochemical analysis

9.1 Mast cell count in skin tissue

Mast cells were counted from the 5 regions of interest after staining toluidine blue. As shown in Figure 6A, there was no significant change in the number of mast cells of treatment group in male and female compared to the control group. Furthermore, there was no statistical significance between control and treatment group (Figure 6B).

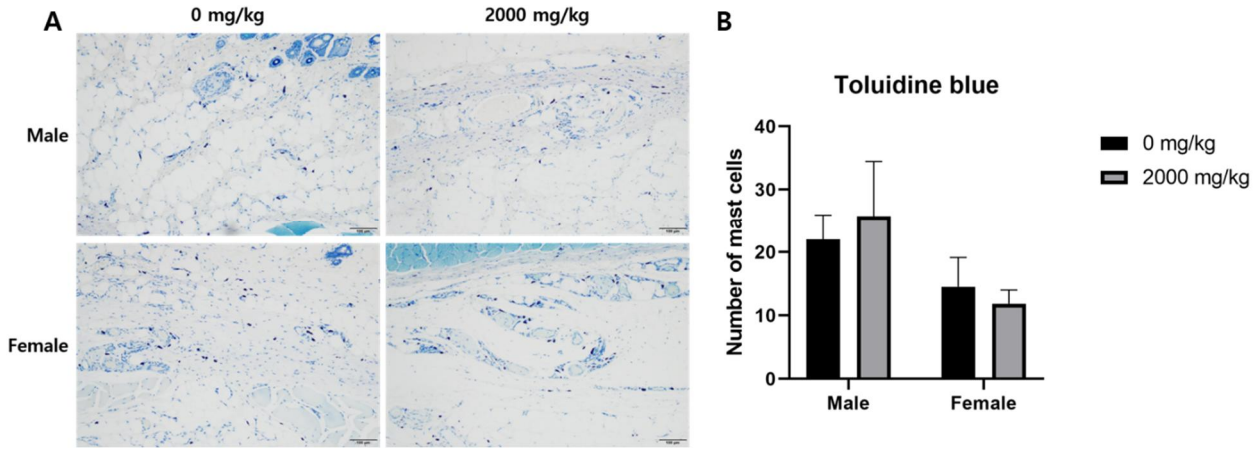

Figure 6. Mast cell staining with Toluidine blue in skin tissue

(**A**) Toluidine blue staining in skin tissue of rats. Dark purple to blue stained cells represented the mast cells. Original magnification, 200×, scale bar: 50 μm. (**B**) The number of mast cells in skin tissues. There was no statistically significant difference in the number of mast cells between control and 2000 mg/kg administration group in both sexes.

9.2 Collagen density analysis

The density of collagen in the skin was analyzed by Masson's trichrome staining (Figure 7A). The contents of collagen for the selected 5 views was compared by calculation of the proportion of the blue-stained area in the total area using ImageJ. There was no statistically significant difference in collagen density between the groups given 0 and 2000 mg/kg in either male or female rats (Figure 7B).

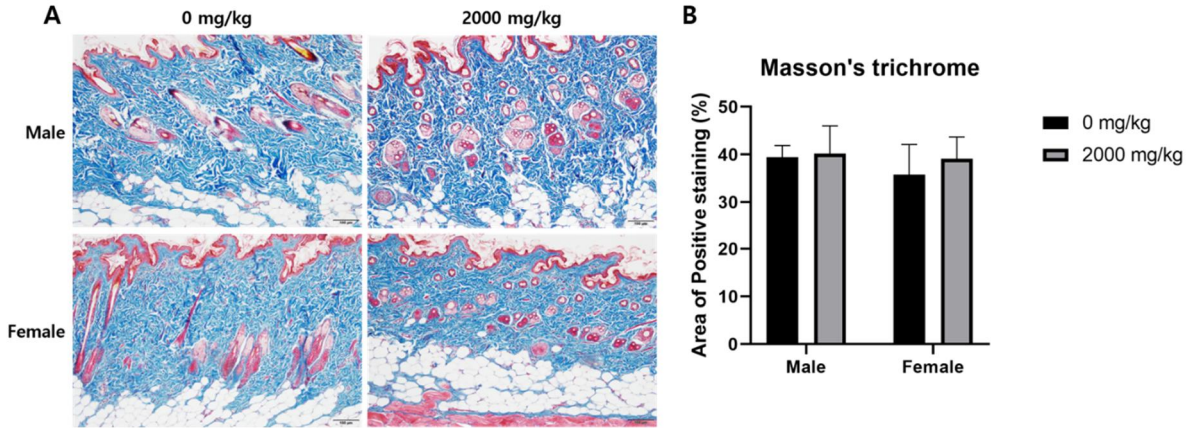

Figure 7. Masson's trichrome staining in skin tissue.

(**A**) Masson's trichrome staining in skin tissue of rats. Blue-stained area represented collagen of the dermis. Original magnification, 200×, scale bar: 50 μm. (**B**) The quantitative analysis on proportion of positive stained area. There was no statistically significant difference in the amount of collagen intensity of skin in the either sex.

9.3 Analysis of skeletal muscle tissue

Sections were stained by modified Gomori's reticulum method to examine the thickness, density, and cracking of muscles (Figure 8A). There was no difference in the density of muscle tissue between groups given 0 and 2000 mg/kg in both sexes, and no other statistically significant findings were observed (Figure 8B).

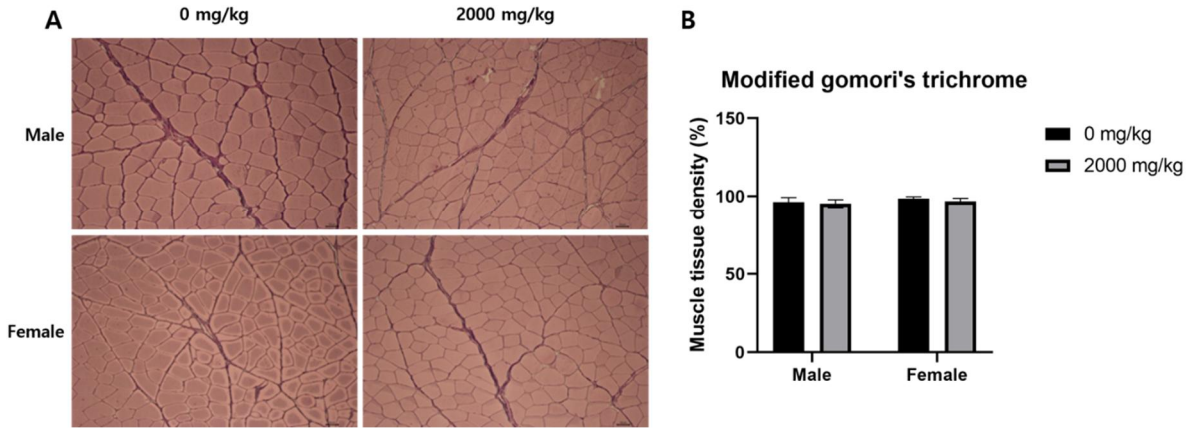

Figure 8 Modified gomori's trichrome staining in skeletal muscle tissue

(**A**) Micrograph of modified Gomori's trichrome staining in skeletal muscle tissue of rat. Reticulum fibers stained brown to black. Original magnification, 100×, scale bar: 100 μm. (**B**) The quantitative analysis on proportion of muscle tissue density. No statistically significant differences were observed in both groups.

9.4 Analysis of other eosinophil markers

Figure 9A represents the immunohistochemical staining (IHC) with eotaxin antibody in lung tissue. Specific positive-staining patterns were observed in the nuclei and/or cytoplasm of the bronchiolar epithelium. These patterns were not present in the negative control stained with antibody diluent without eotaxin antibody. Quantitative analysis of the proportion of positive cells in the total cells of whole slide images was conducted using QuPath (Figure 9B). There were no statistically significant differences between the control group and the group treated with 2,000 mg/kg in both sexes. In addition, lung tissue was stained with Congo red to detect the eosinophilic evidence on the positive cells shown in the immunohistochemical staining. No specific staining was observed in the Congo red-stained sections.

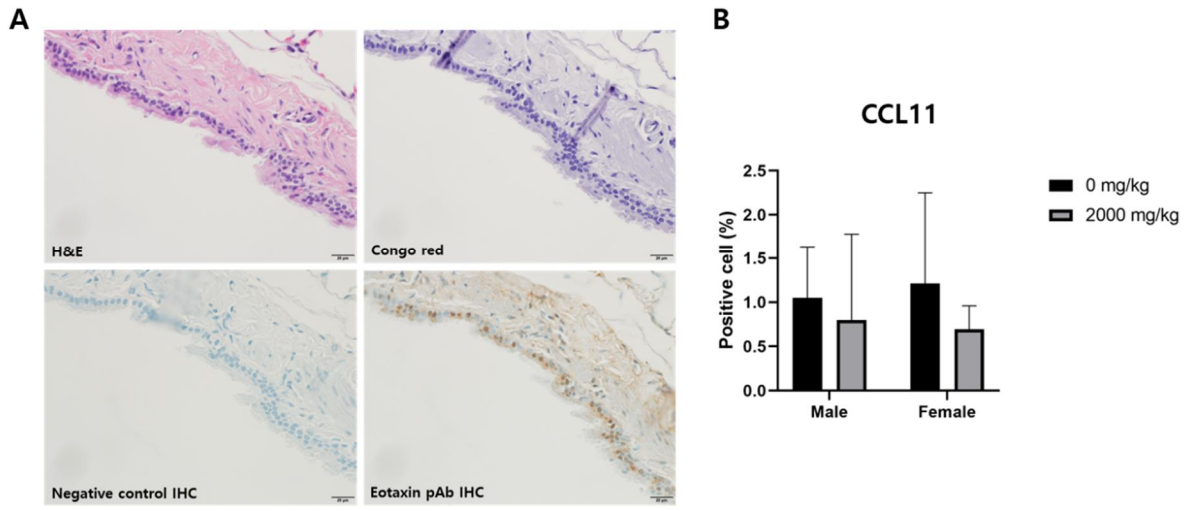

Figure 9. H&E, Congo red, negative control, and eotaxin polyclonal antibody (pAb) immunohistochemistry (IHC) in lung tissue.

(**A**) H&E-stained slide of lung bronchiolar epithelial in rat given 2000 mg/kg (animal no. 2402). Micrograph of the Congo red-stained section of bronchiolar epithelial showed no specific positive-stained eosinophils. IHC staining of antibody diluent only for negative control and rabbit anti-human eotaxin antibody (1:500) in the lung. Specific positive-stained cells were seen in the bronchiolar epithelial compared to the negative control. Original magnification, 400×, scale bar: 20 μm. (**B**) Quantitative analysis of the proportion of positive cells. There was no statistically significant difference in the proportion of positive cells in the total cells in lung tissue between the control and the group administered 2000 mg/kg in both sexes.

Discussion

L-Tryptophan is an essential amino acid for animal nutrition, and the dried L-tryptophan fermentation product is used as an L-tryptophan supplemental nutrient in animal feed additives. However, the safety of the product should be assessed due to the contents of L-tryptophan and other components derived from the fermentation broth of metabolically engineered *C. glutamicum*. Therefore, the subchronic toxicity of dried L-tryptophan fermentation product was evaluated, and NOAEL was investigated in SD rats. Dried L-tryptophan fermentation product was administered daily via oral gavage for 90 days at dose levels of 0, 500, 1000, and 2000 mg/kg/day.

No dried L-tryptophan fermentation product-related mortality was observed in any of the treatment groups. Additionally, body weight and ophthalmological tests showed no significant differences from the control animals. The lack of significant differences in body weight changes in this study was inconsistent with a previous 90-day study of the highly purified L-tryptophan in male and female SD rats (Shibui et al., 2018). This discrepancy could be caused by the difference in the amount of L-tryptophan administered. Shibui et al. (2018) found significant toxicological differences in body weight gain throughout the administration period in the male 2.5% dose group and the 5.0% dose group in both sexes. The converted mean values of the test substance intake in the 2.5% and 5.0% dose groups were 1566 mg/kg/day for males and 3045 and 3515 mg/kg/day for males and females, respectively. The high-dose of dried L-tryptophan fermentation product administered in the current study corresponded to an L-tryptophan intake of 1200 mg/body weight/day. Therefore, the dose in the study by Shibui et al. (2018) that showed a significant difference in body weight gain was much higher than that of the highest dose in the current study.

The significant changes demonstrated in the high-dose group in both sexes through functional observation of motor activity were not test substance-related, as no significant effects on grip strength measurement were noted; additionally, nerve tissue-related organ and histopathological lesions in the brain and spinal cord were not found. One animal each in the clinical observation and gross necropsy results showed EMS signs, which were not regarded as dried L-tryptophan fermentation product-related effects. Temporary slight salivation, commonly seen in rats, was observed in one animal in the female high-dose treatment group. Adrenal gland discoloration was observed in one female rat in the high-dose group during gross necropsy, and no associated effects were observed in the histopathologic or other clinical indicators.

During the study period, significant changes in food consumption were sporadically observed. These changes were regarded as unrelated to dried L-tryptophan fermentation product because the pattern of food consumption was irregular; additionally, the data were not correlated with body-weight gain.

Some hematologic parameters showed statistically significant differences compared to the control group. A significant decrease in monocytes (%) was observed in the male recovery high-dose group; however, no abnormal changes in white blood cells were observed, and the data were within the historical background range. The white blood cell count, lymphocyte count, and reticulocytes (%) were only significantly different in the female high-dose recovery group compared to the control group. These changes were not observed in the treatment group, and each value was within the normal historical background range. In addition, the histopathological examination of the bone marrow and thymus revealed no remarkable changes. Therefore, we concluded that the changes were not related to dried L-tryptophan fermentation product.

In the blood biochemistry analysis, the changes in albumin and cholinesterase levels in the female high-dose treatment group were within the historical background ranges. No remarkable findings in the histopathologic examination of the kidneys and liver were related to changes in albumin and cholinesterase levels. Therefore, we estimated that dried L-tryptophan fermentation product did not have any adverse effects. In addition, the changes in creatinine, blood urea nitrogen, and urea in the female high-dose recovery group showed no correlative histopathologic changes in the kidneys and the values were within the normal ranges of historical data. Therefore, it could be concluded that they were not affected by dried L-tryptophan fermentation product.

The hormone thyroxine decreased significantly in the male high-dose recovery group. However, this change was not considered test substance-related because no test substance-related effects were observed in histopathology; additionally, the weight of the thyroid gland was unchanged.

The organ weight measurements revealed that the relative weights of the liver and kidneys (right and total) increased significantly in the female high-dose group during the administration period. However, the analyzed values were within the normal range of historical background data; additionally, the weights of the liver and kidneys in the recovery group showed no difference compared to the control group. No adverse changes were observed during the clinical or histopathologic examination.

Histochemical and immunohistochemical analyses revealed no significant changes in the skin, skeletal muscle, and lung indicative of EMS. Mast cells are widely distributed in vertebrate connective tissue and induce various allergic or inflammatory reactions. Mast cell staining of skin tissue was examined in this study to determine whether dried L-tryptophan fermentation product triggers an allergic or inflammatory reaction. To observe any thickening of collagen bundles in fascia, dermis, and subcutaneous fat, as seen in human patients with

EMS, Masson's trichrome staining was used for analyzing collagen in the tissues. Modified Gomori's trichrome staining is a suitable method for observing the reticular fibers of the muscle, and it allows to investigate thickness, density, and crack of the muscle tissue. Eotaxin is a chemokine with potent and specific eosinophil chemoattractant activity. Up-regulation of the eotaxin mediated accumulation of eosinophils and induced development of inflammatory lung injury (Guo et al., 1999). As a result of quantified analysis of all staining sections, significant differences in the histopathological signs of EMS were not seen between the control and the groups given 2000 mg/kg in either sex in the number of mast cells, collagen of skin tissue, the proportion of muscle tissue density in modified Gomori's trichrome staining, and the proportion of eotaxin-positive cells.

However, in previous animal experiments, the correlation between the impurity of L-tryptophan and the occurrence of EMS was controversial. Some researchers reported oral gavage or daily intraperitoneal injection of contaminated L-tryptophan in rodents resulted in EMS-like features, including thickening of the fascia and perimysium fascia with inflammatory cell infiltration (Crofford et al., 1990; Silver et al., 1994). In another study, eosinophil infiltration and EMS-like clinical features or morphological changes were seen in the intestinal mucosa of Lewis rats given contaminated L-tryptophan (DeSchryver-Kecskemeti et al., 1991). However, a more recent study suggested that even though they treated impurity-proven L-tryptophan in rats in high doses for a long time, evidence of EMS was not found (Preuss, Echard, Talpur, Funk, & Bagchi, 2006).

From the NOAEL for rats determined in this study, the human equivalent dose (HED) could be calculated based on body surface area. To convert a rat dose to HED requires dividing the rat dose by 6.2 or multiplying the rat dose by 0.16 (Andrade et al., 2016). If the NOAEL in rats was determined to be 2000 mg/kg, the converted HED would be approximately 320 mg/kg. The daily nutritional requirement for L-tryptophan in humans is

estimated to be 3.5–6.0 mg/kg/day (Richard et al., 2009); however, many adults choose to consume doses of up to 60–70 mg/kg/day to improve mood or sleep (Fernstrom, 2012). In some case reports of EMS, L-tryptophan doses of 400–1500 mg/kg/day were concomitant with EMS-like features in humans (J. A. Allen et al., 2011; Grangeia, Schweller, Paschoal, Zambon, & Pereira, 2007). As the dose level was high enough and adversity was not seen in the current study, it is reasonable to conclude that the EMS-related safety concerns of dried L-tryptophan fermentation product, the test substance in this study, would be negligible when it consumed by humans through the meat product.

Conclusion

Male and female SD rats receiving daily oral administration of dried L-tryptophan fermentation product for 90 days were observed, followed by a 4-week recovery period. During the study, no dried L-tryptophan fermentation product-related adverse effects were observed. All parameter changes in this study were regarded as dried L-tryptophan fermentation product-unrelated. It was confirmed that no inflammatory reaction or tissue damage was found when dried L-tryptophan fermentation product was administered in rats for 90 days, and there was no difference between the control group and the group administered dried L-tryptophan fermentation product. In conclusion, under the conditions of this study, dried L-tryptophan fermentation product was not toxic, and the NOAEL was 2000 mg/kg/day in both sexes.

References

Allen, J., & Varga, J. (2014). Eosinophilia-Myalgia Syndrome. In *Encyclopedia of Toxicology: Third Edition* (pp. 419-425): Elsevier.

Allen, J. A., Peterson, A., Sufit, R., Hinchcliff, M. E., Mahoney, J. M., Wood, T. A., . . . Varga, J. (2011). Post-epidemic eosinophilia-myalgia syndrome associated with L-tryptophan. *Arthritis Rheum, 63*(11), 3633-3639. doi:10.1002/art.30514

Andrade, E., Bento, A., Cavalli, J., Oliveira, S., Schwanke, R., Siqueira, J., . . . Calixto, J. (2016). Non-clinical studies in the process of new drug development-Part II: Good laboratory practice, metabolism, pharmacokinetics, safety and dose translation to clinical studies. *Brazilian journal of medical and biological research, 49*.

Blackburn Jr, W. D. (1997). *Eosinophilia myalgia syndrome.* Paper presented at the Seminars in arthritis and rheumatism.

Crofford, L. J., Rader, J. I., Dalakas, M. C., Hill, R. H., Jr., Page, S. W., Needham, L. L., . . . et al. (1990). L-tryptophan implicated in human eosinophilia-myalgia syndrome causes fasciitis and perimyositis in the Lewis rat. *J Clin Invest, 86*(5), 1757-1763. doi:10.1172/JCI114902

DeSchryver-Kecskemeti, K., Gramlich, T., Crofford, L., Rader, J., Page, S., Needham, L., . . . Sternberg, E. (1991). MAST-CELL AND EOSINOPHIL INFILTRATION IN INTESTINAL-MUCOSA OF LEWIS RATS TREATED WITH L-TRYPTOPHAN IMPLICATED IN HUMAN EOSINOPHILIA-MYALGIA-SYNDROME. *Modern Pathology, 4*(3), 354-357.

Epidemiologic Notes and Reports Eosinophilia-Myalgia Syndrome and L-Tryptophan-Containing Products -- New Mexico, Minnesota, Oregon, and New York, 1989. (1989). Retrieved from https://www.cdc.gov/mmwr/preview/mmwrhtml/00001502.htm

Fernstrom, J. D. (2012). Effects and side effects associated with the non-nutritional use of tryptophan by humans. *J Nutr, 142*(12), 2236S-2244S. doi:10.3945/jn.111.157065

Fouad, A. M., El-Senousey, H. K., Ruan, D., Wang, S., Xia, W., & Zheng, C. (2021). Tryptophan in poultry nutrition: Impacts and mechanisms of action. *J Anim Physiol Anim Nutr (Berl)*. doi:10.1111/jpn.13515

Grangeia, T. d. A. G., Schweller, M., Paschoal, I. A., Zambon, L., & Pereira, M. C. (2007). Acute respiratory failure as a manifestation of eosinophilia-myalgia syndrome associated with L-tryptophan intake. *Jornal Brasileiro de Pneumologia, 33*, 747-751.

Guo, R.-F., Ward, P. A., Jordan, J. A., Huber-Lang, M., Warner, R. L., & Shi, M. M. (1999). Eotaxin expression in Sephadex-induced lung injury in rats. *The American journal of pathology, 155*(6), 2001-2008.

Hendler, S. S., & Rorvik, D. M. (2008). *PDR for nutritional supplements*: Thomson Reuters.

Iwuji, T., Akinmutimi, A., Ifeanyi PhD, O., Friday, E., & Odoemelam, V. (2014). ROLES OF TRYPTOPHAN IN MONOGASTRIC NUTRITION: A REVIEW. *Advances in Agriculture, Sciences and Engineering Research*.

Kang, K.-Y., Kim, M.-S., Lee, M.-S., Oh, J.-J., An, S., Park, D., . . . Kim, S.-D. (2020). Genotoxicity and acute toxicity evaluation of the three amino acid additives with Corynebacterium glutamicum biomass. *Toxicology reports, 7*, 241-253.

Kilbourne, E. M. (1992). Eosinophilia-myalgia syndrome: coming to grips with a new illness. *Epidemiologic reviews, 14*, 16-36.

Kilbourne, E. M., Swygert, L. A., Philen, R. M., Sun, R. K., Auerbach, S. B., Miller, L., . . . Falk, H. (1990). Interim guidance on the eosinophilia-myalgia syndrome. In: American College of Physicians.

Klarskov, K., Gagnon, H., Racine, M., Boudreault, P. L., Normandin, C., Marsault, E., . . . Naylor, S. (2018). Peak AAA fatty acid homolog contaminants present in the dietary supplement l-Tryptophan associated with the onset of eosinophilia-myalgia syndrome. *Toxicol Lett, 294*, 193-204. doi:10.1016/j.toxlet.2018.05.027

Le Floc'h, N., Otten, W., & Merlot, E. (2011). Tryptophan metabolism, from nutrition to potential therapeutic applications. *Amino Acids, 41*(5), 1195-1205. doi:10.1007/s00726-010-0752-7

Le Floc'h, N., & Seve, B. (2007). Biological roles of tryptophan and its metabolism: Potential implications for pig feeding. *Livestock Science, 112*(1-2), 23-32. doi:10.1016/j.livsci.2007.07.002

Mary Kugler, R. (2020). Eosinophilia-Myalgia Syndrome Symptoms and Treatment. Retrieved from https://www.verywellhealth.com/eosinophilia-myalgia-syndrome-2860325

Mayeno, A. N., Lin, F., Foote, C. S., Loegering, D. A., Ames, M. M., Hedberg, C. W., & Gleich, G. J. (1990). Characterization of" peak E," a novel amino acid associated with eosinophilia-myalgia syndrome. *Science, 250*(4988), 1707-1708.

OECD. (2002). *Guidance Document on Acute Oral Toxicity Testing*.

OECD. (2018). *Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents*.

OECD, G. (1998). OECD Principles of good laboratory practice (as revised in 1997).

Preuss, H. G., Echard, B., Talpur, N., Funk, K. A., & Bagchi, D. (2006). Does 5-hydroxytryptophan cause acute and chronic toxic perturbations in rats? *Toxicol Mech Methods, 16*(5), 281-286. doi:10.1080/15376520500195616

Proia, D. A., Smith, D. L., Zhang, J., Jimenez, J.-P., Sang, J., Ogawa, L. S., . . . Zhang, C. (2015). HSP90 inhibitor–SN-38 conjugate strategy for targeted delivery of topoisomerase I inhibitor to tumors. *Molecular cancer therapeutics, 14*(11), 2422-2432.

Redfern, W., Ewart, L., Hammond, T., Bialecki, R., Kinter, L., Lindgren, S., . . . Valentin, J. (2010). Impact and frequency of different toxicities throughout the pharmaceutical life cycle. *The Toxicologist, 114*(S1), 1081.

Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N., & Dougherty, D. M. (2009). L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. *Int J Tryptophan Res, 2*, 45-60. doi:10.4137/ijtr.s2129

Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N., & Dougherty, D. M. (2009). L-tryptophan: basic metabolic functions, behavioral research and therapeutic indications. *International Journal of Tryptophan Research, 2*, IJTR. S2129.

Shibui, Y., Matsumoto, H., Masuzawa, Y., Ohishi, T., Fukuwatari, T., Shibata, K., & Sakai, R. (2018). Thirteen week toxicity study of dietary l tryptophan in rats with a recovery period of 5 weeks. *Journal of Applied Toxicology, 38*(4), 552-563.

Silver, R. M., Ludwicka, A., Hampton, M., Ohba, T., Bingel, S. A., Smith, T., . . . Heyes, M. P. (1994). A murine model of the eosinophilia-myalgia syndrome induced by 1,1'-ethylidenebis (L-tryptophan). *J Clin Invest, 93*(4), 1473-1480. doi:10.1172/jci117125

Troy, J. L. (1991). Eosinophilia-Myalgia Syndrome. *Mayo Clinic Proceedings, 66*(5), 535-538. doi:10.1016/s0025-6196(12)62396-9

Wensley, M. R., Woodworth, J. C., DeRouchey, J. M., Dritz, S. S., Tokach, M. D., Goodband, R. D., . . . Haydon, K. D. (2020). Effects of amino acid biomass or feed-grade amino acids on growth performance of growing swine and poultry. *Translational Animal Science, 4*(1), 49-58.

Summary in Korean

호산구증가근육통증후군(Eosinophilia-myalgia syndrome, EMS)은 1989년에 뉴멕시코에서 L-트립토판(L-tryptophan)을 보조제로서 섭취한 사람에서 처음 발병한 희귀한 질환이다. 사람뿐만 아니라 동물에서도 L-트립토판은 필수 아미노산이며, 체내에서 자체적으로 생산되지 않아 동물에서도 사료 첨가제로서 사용되고 있다. EMS 발생 이후, L-트립토판과 다른 아미노산들에 대한 안전성 평가가 규제기관에서 필수적으로 시행되고 있다. 본 시험에서 사용된 건조 L-트립토판은 대사공학적으로 변형된 *Corynebacterium glutamicum*의 발효에 의해 생산되었으며, Sprague Dawley (SD) 랫드를 이용하여 90일 반복투여 아만성 독성 시험을 통해 L-트립토판의 안전성을 평가하였다. 매일 0, 500, 1000, 그리고 2000 mg/kg의 용량으로 경구투여 하였으며, 투여군은 군당 수컷 10마리, 암컷 10마리로 13주간 투여하였다. 0 및 2000 mg/kg으로 투여한 군에 대해서는 13주 투여가 끝난 후 관찰된 변화의 가역성을 확인하기 위해 4주간의 회복기간을 두었으며, 군당 수컷 5마리, 암컷 5마리를 통해 평가하였다. 추가적으로 0 및 2000 mg/kg 투여군의 전체 개체에 대하여 L-트립토판 섭취 시 나타날 수 있는 EMS의 영향에 대해 몇 개의 관련된 조직에 특수염색 및 면역조직화학염색을 시행하였다.

결과적으로 시험기간 전체 기간 및 회복 기간동안 모든 용량에서 L-트립토판과 관련된 부작용은 관찰되지 않았으며, 특수염색 및 면역조직화학염색법으로 시행한 분석에서도 통계학적으로 유의한 결과는 나타나지 않았다. 따라서 본 시험에서의 부작용이 관찰되지 않는 최대무독성량(No Observed Adverse Effect Level, NOAEL)은 2000 mg/kg인 것으로 사료되며, 본 시험에서 사용된 L-트립토판에서는 EMS에 대한 우려는 적을 것으로 판단된다.