Regulatory Toxicology and Pharmacology 77 (2016) 206–212

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Safety assessment of freeze-dried powdered *Tenebrio molitor* larvae (yellow mealworm) as novel food source: Evaluation of 90-day toxicity in Sprague-Dawley rats

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form
10 March 2016
Accepted 12 March 2016
Available online 16 March 2016

Keywords:
Tenebrio molitor
Edible insects
Subchronic toxicity
Food allergy

ABSTRACT

Worldwide demand for novel food source has grown and edible insects are a promising food sources for humans. *Tenebrio molitor*, as known as yellow mealworm, has advantages of being rich in protein, and easy to raise as a novel food source. The objective of this study was to evaluate subchronic toxicity, including potential hypersensitivity, of freeze-dried powdered *T. molitor* larvae (fdTML) in male and female Sprague-Dawley rats. The fdTML was administered orally once daily at dose levels of 0, 300, 1000 and 3000 mg/kg/day for 90 days. A toxicological assessment was performed, which included mortality, clinical signs, body and organ weights, food consumption, ophthalmology, urinalysis, hematology, serum chemistry, gross findings, histopathologic examination and allergic reaction. There were no fdTML-related findings in clinical signs, urinalysis, hematology and serum chemistry, gross examination, histopathologic examination or allergic reaction. In conclusion, the No Observed Adverse Effect Level (NOAEL) for fdTML was determined to be in excess of 3000 mg/kg/day in both sexes of rats under the experimental conditions of this study.

1. Introduction

Insects are a traditional food for humans and animals, especially in Asia, Africa, the Americas and Australia (Siemianowska et al., 2013). Demand for novel sources of food, in particular protein, is growing with the increase in the world population. From efficiency and environmental perspectives, there are many benefits to using insects as food. They are a more sustainable food source and alternative to animal protein (Oonincx et al., 2010; Yen, 2009). Insects have higher feed conversion efficiencies than homeothermic animals, because they are poikilotherms (Oonincx et al., 2010). Furthermore, insects are rich in protein and beneficial fatty acids (Heinrich and Prieto, 2008). The issue of edible insects has been discussed actively by the Food and Agriculture Organization of the United Nations (FAO) since 2003(Huis et al., 2013).

Tenebrio molitor larvae (yellow mealworm) are used widely as animal feed for reptiles, birds and monkeys (Huis et al., 2013; Jones et al., 1972; Ng et al., 2001). There have been many attempts to investigate *T. molitor* as food, as it is a commonly bred species worldwide (Bednářová et al., 2013; Mlcek et al., 2014), has a short life cycle and is easy to handle and raise (Ghaly and Alkoaik, 2009). Furthermore, several studies have shown that the nutritional content and composition of *T. molitor* is adequate for use as food (Ghaly and Alkoaik, 2009; Ravzanaadii et al., 2012; Yi et al., 2013; Yoo et al., 2013).

As a novel food, the nutritional value of *T. molitor* and its larva has been established (Ghaly and Alkoaik, 2009; Ravzanaadii et al., 2012; Yi et al., 2013; Yoo et al., 2013) and the genotoxicity and subchronic toxicity for 28 days has been assessed (Han et al., 2014); however, its long-term safety, including potential hypersensitivity for human consumption, has not been evaluated. The potential detrimental effects from eating *T. molitor* include microbial and chemical hazards, allergy and toxicity (Belluco et al., 2013; Mlcek et al., 2014). Moreover, it is possible to develop allergic sensitivity through long-term exposure in people with a history of atopy

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http://dx.doi.org/10.1016/j.yrtph.2016.03.006
0273-2300/

(allergic hypersensitivity) (Huis et al., 2013). This study examined potential hypersensitivity and subchronic toxicities in Sprague-Dawley (SD) rats administered freeze-dried powdered *T. molitor* larvae (fdTML) for 90 days. The study was performed in compliance with the Good Laboratory Practice (GLP) guidelines of the Organization for Economic Cooperation and Development (OECD, 1997).

2. Materials and methods

2.1. Preparation of freeze-dried powdered Tenebrio molitor larvae

T. molitor larvae were purchased from Sworm Farm (Cheonan, Chungcheongnam-do, Republic of Korea) and ground into a powder after a freeze-drying and sterilized using autoclave at 115 °C for 10 min by Worldway (Yeongi, Republic of Korea). The fdTML were examined for food poisoning pathogen contamination by assessing *Escherichia coli* O157:H7 and *Salmonella* spp. and for heavy metal content including Pb, Hg, As and Cd. They were found to be safe from food poisoning pathogens and Pb, As and Cd were undetectable (Yoo et al., 2013). Furthermore, the Hg concentration was 0.03 mg/kg, a concentration lower than the standard index for food (Yoo et al., 2013).

The general components were measured using the official analytical methods of the Association of Official Analytical Chemists and the marker compound was measured using gas chromatography (GC) (GC-2010 Plus, Shimadzu, Japan). Oleic acid comprised the highest proportion of fatty acids (51.4%) (Yoo et al., 2013); therefore, oleic acid was selected as the representative marker for the fdTML analysis. The nutritional components of *T. molitor* larvae are shown in Table 1.

2.2. Formulation and analysis of fdTML

For oral administration via gavage, fdTML was measured and suspended in distilled water at the concentration for the highest dose group. This suspension was diluted to prepare the lower doses.

Before administration, the fdTML analysis using GC was validated by the Korea Institute of Toxicology (KIT) (not published). The validation study included specificity, system suitability, linearity, calibration curve reproducibility, accuracy, precision, homogeneity and stability. The formulated fdTML was analyzed at the start (Day 1) and end (Week 13) of dosing for homogeneity and content.

Table 1
The nutritional components of *Tenebrio molitor* larvae.

General components	Contents (g/100 g)
Total carbohydrate	10.28
Protein	48.26
Fat	35.81
Moisture	2.47
Ash	3.17
Trans fat	0.11
Cholesterol	97.81
Saturated fat	8.80
Dietary fiber	5.89
Vitamin	**Contents (mg/100 g)**
Vitamin A	–[a]
Vitamin C	–
Vitamin D	–
Vitamin E (Tocopherol)	–
Vitamin B6 (Pyridoxine)	–
Vitamin B3 (Niacin)	7.83
Vitamin B5 (Pantothenic acid)	2.56
Mineral	**Contents (mg/kg or mg/100 g)**
Copper (Cu)	7.83/kg
Magnesium (Mg)	2388.00/kg
Manganese (Mn)	9.16/kg
Phosphorus (P)	680.80/100 g
Zinc (Zn)	106.70/kg
Iron (Fe)	4.65/100 g
Calcium (Ca)	37.02/100 g
Potassium (K)	656.90/100 g

[a] Not detected.

2.3. Experimental animals

This study was performed using male and female specific pathogen-free SD rats obtained from Orient Bio Co. (Seongnam-si, Republic of Korea) at 5 weeks of age. The animals were acclimatized for 7 days and healthy animals were selected for the study. Then, 50 male and 50 female rats were assigned randomly to four groups, one vehicle control and three treatment groups, using the Path/Tox system (ver. 4.2.2. Xybion Medical Systems Corp., Cedar Knolls, NJ, USA). A total of 10 male and 10 female rats were assigned to controls and the highest dose group served as recovery animals. Each group consisted of 15 (including recovery animals) or 10 rats of each sex. At the start of dosing, body weight ranged from 177.6 to 212.3 g [coefficient variation; CV (%) = 5.04] for males and 142.5 to 167.2 g [CV (%) = 4.30] for females.

Two or three animals were housed per stainless steel cage throughout the study period. Sterilized tap water and pellet food for rodents (PMI Nutrition International, Richmond, IN, USA) were provided *ad libitum*. The animal room was maintained at a temperature of 22 ± 3 °C, a relative humidity of ~30–70%, air ventilation of 10–20 times/h and light intensity of 150–300 lux with 12-h light–dark cycles. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the KIT and conducted in compliance with the Testing Guidelines for Safety Evaluation of Drugs (Notification No. 2012-86 issued by Korea Food and Drug Administration on August 24, 2012) and OECD Guidelines for Testing of Chemicals, Section 4, Health Effects, No. 408, Repeated Dose 90-Day Oral Toxicity Study in Rodents (September 21, 1998).

2.4. Treatment and toxicity assessments

In a previous oral single dose and 28-day repeated dose study doses up to 3000 mg/kg/day were well tolerated in both sexes of SD rats (Han et al., 2014). Therefore, doses of 0 (vehicle control), 300, 1000 and 3000 mg/kg/day were selected for this 90-day repeated dose study, and general and pathology observations were performed similar to the previous study (Han et al., 2014). The dosing volume, 10 mL/kg, was based on the most recent body weight and all measurements and examination records were calculated or collected using the Path/Tox system. Animal condition and behavior were checked once daily throughout the acclimation and recovery periods. Clinical observations were recorded twice daily, before and after dosing, during the treatment period and once during the recovery period and on the day of necropsy.

Animals were weighed on arrival, before the randomization, before dosing on the first day of dosing and once weekly thereafter. Final body weight was measured on the day of necropsy. Cage food consumption was recorded once during the acclimation period and once weekly during the treatment and recovery periods. Individual food consumption was calculated as g/rat/day. The amount of food consumed by each rat was determined by weighing each feeder at the beginning and end of the week and dividing by the number of animals in the cage. External eye examinations were performed on all animals before dosing began. External and fundus examination of animals in the vehicle control and highest dose (3000 mg/kg/day) groups were performed with an indirect ophthalmoscope (Vantage Plus Digital, Keeler Ltd., London, UK) in the last week of

treatment before necropsy. Eyes were examined after mydriasis induced with a mydriatic agent, mydrin-P (Santen Pharmaceutical Co., Osaka, Japan).

Urine samples were collected overnight for 16 h from animals housed in metabolism cages in the last week of treatment and before recovery necropsy. Each animal was housed in an individual metabolism cage; food was withdrawn overnight, but water was available. Urinalysis was performed using a urine automatic analyzer (COBAS U411 urine analyzer, Roche Diagnostics, Mannheim, Germany) and urine stick (Multistix 10 SG, Siemens, Forchheim, Germany) to evaluate the following parameters: volume, color, specific gravity, pH, and protein, ketone body, erythrocyte, glucose, bilirubin, nitrite and urobilinogen content. Microscopic examination of urine cast, epithelial cell, red blood cell and white blood cell content was also performed.

All animals were fasted overnight before the necropsy and blood collection. Blood samples were collected from all animals for hematology, coagulation and serum chemistry from the caudal vena cava under isoflurane anesthesia during necropsy. Blood samples were collected into tubes containing EDTA-2K for hematology analysis. White blood cell count, red blood cell count, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelet, differential leukocyte count (neutrophil, lymphocyte, monocyte, eosinophil, basophil and large unstained cells) and reticulocyte count were analyzed using the ADVIA 2120i hematology system (Siemens, Tarrytown, NY, USA). In addition, blood samples treated with 3.2% sodium citrate were analyzed for prothrombin time and activated partial thromboplastin time using the ACL 9000 (Instrumentation Laboratory, Milan, Italy).

Blood samples were collected for serum chemistry analysis in tubes lacking anticoagulant and placed at room temperature for at least 90 min before centrifugation at 1600× *g* for 10 min. Blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (CREA), glucose (GLU), total cholesterol (TCHO), albumin/globulin ratio (A/G), total protein (TP), albumin (ALB), creatine kinase (CK), triglycerides (TG), total bilirubin (TBIL), gamma glutamyl transpeptidase (GGT), phospholipids (PL), calcium (Ca), inorganic phosphorus (IP), sodium (Na), potassium (K), and chloride (Cl) blood levels were measured using an automatic TBA 200FR NEO analyzer (Toshiba., Tokyo, Japan).

Following the blood sampling, the animals were killed by exsanguination from the vena cava and aorta under isoflurane anesthesia. Complete necropsy examinations were performed on all animals. Absolute weight of the brain, pituitary gland, adrenal gland, liver, spleen, kidneys, heart, thymus, lungs, submandibular and sublingual salivary glands, thyroid gland, testes, epididymides, seminal vesicle, prostate, uterus (with cervix) and ovaries (with oviducts) was measured and the relative organ weight as a percentage of the terminal body weight was determined.

Following a detailed external and internal examination, all tissues were collected from each animal, including; adrenal glands, aorta, brain, cecum, colon, duodenum, epididymides, esophagus, eye with optic nerve, femur with marrow, Harderian gland, heart, ileum, jejunum, kidneys, liver, lung with bronchi, mammary gland in females, mandibular lymph node, mesenteric lymph node, ovaries with oviduct, pancreas, pituitary gland, prostate, rectum, submandibular and sublingual salivary glands, sciatic nerve, seminal vesicle, skeletal muscle, skin, thoracic spinal cord, spleen, sternum with marrow, stomach, testes, thymus, thyroids, tongue, trachea, urinary bladder, uterus with cervix, and vagina. All tissues were preserved in a 10% neutral buffered formalin solution except the eyes with optic nerve, testes and epididymides. The testes and epididymides were fixed in Bouin's fixative and the eye with optic nerve was fixed in Davidson's fixative for approximately 48 h before being transferred to 70% alcohol. The tissues were sectioned and stained with hematoxylin and eosin (H&E) before microscopic examination. All preserved tissues from animals in the vehicle control and the highest dose group were examined.

2.5. Identification of allergic reaction

According to the scheduled necropsy for dosing and recovery animals, approximately 0.6 mL of blood was collected into a tube lacking anticoagulant. The tube was stored at ambient room temperature for at least 30 min and centrifuged at 11,700× *g* for 3 min at room temperature and the serum was separated.

IgE and histamine ELISA kits (Abnova Corporation, Taipei City, Taiwan) were used to measure IgE and histamine concentrations. The results were measured using a SpectraMax M3 microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Data analyses and calculations were conducted with SoftMax Pro software (ver. 5.4.1, Molecular Devices) and Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA). Data were obtained from a four-parameter logistic curve fitted using calibrators. When the measured value was out of range, each corresponding sample was reanalyzed after adjusting the dilution factor.

2.6. Statistical analysis

The data, including body weight, food consumption, clinical pathology, IgE and histamine were analyzed for variance homogeneity using Bartlett's test for the main animals and F-test for the recovery animals. Statistical analysis was performed separately for male and female animals. Homogeneous data were analyzed using analysis of variance (ANOVA) for the main animals and the *t*-test for the recovery animals, while inter-group differences were analyzed using Dunnett's *t*-test. Heterogeneous data were analyzed using the Kruskal–Wallis test and the significance of inter-group differences between the control and treated groups were assessed using Dunn's rank sum test. The statistical analyses were performed by comparing the dose groups to the vehicle control group using the Path/Tox system and SAS/STAT software (version 9.2; SAS Institute Cary, NC, USA). The results of the comparisons are indicated only when p-values are less than 0.05 or 0.01.

3. Results and discussion

3.1. Formulation analysis

Dose formulations in the range 30–300 mg/mL have been shown to be stable for 24 h at room temperature, for 3 days when stored at 2–8 °C and for 28 days when stored in a deep freezer at approximately −70 °C. Furthermore, dose formulation was homogenous in the range of 30–300 mg/mL.

Dose formulations for Day 1 and Week 13 were considered homogeneous because CV results were within 10% of the mean of the top, middle and bottom results (0.23–9.53%).

Concentrations of all dose formulations for the dosing start date (Day 1) and Week 13 were acceptable as the mean concentration was ±20% of the nominal concentration. Results for dose formulations in each group were in the range of 93.49–102.78%.

3.2. General observations

No treatment-related mortality or clinical signs were observed throughout the study period. Changes in body weight during the treatment period are shown in Fig. 1. There were no treatment-related changes in body weight in either sex during the

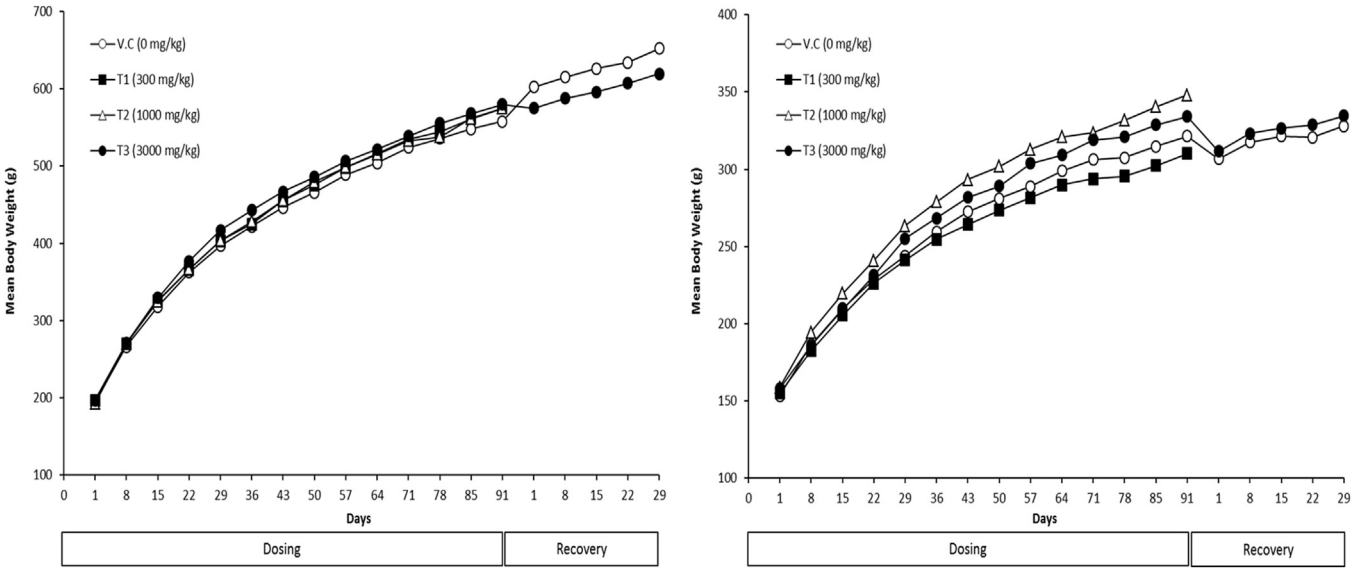

Fig. 1. Mean body weight of males (left) and females (right) treated with fdTML for 90-days.

treatment or recovery periods. In the ophthalmologic examination, no abnormal findings were observed.

3.3. Clinical pathology

There were no significant changes in the urinalysis parameters (data not shown), hematology (data from Day 92 in Table 2), coagulation (data from Day 92 in Table 2), or serum chemistry (data

Table 2
Hematological and coagulation values of rats treated orally with fdTML for 90-days.

Parameters	Vehicle control	300 mg/kg	1000 mg/kg	3000 mg/kg
Males				
WBC ($\times 10^3/\mu L$)	9.67 ± 2.018	11.74 ± 3.569	9.34 ± 1.036	10.07 ± 2.059
RBC ($\times 10^6/\mu L$)	9.19 ± 0.293	9.13 ± 0.572	9.02 ± 0.387	9.02 ± 0.306
HGB (g/dL)	16.0 ± 0.57	15.8 ± 0.53	15.7 ± 0.52	15.5 ± 0.46
HCT (%)	49.9 ± 1.80	49.4 ± 2.02	48.7 ± 2.00	48.9 ± 1.25
MCV (fL)	54.4 ± 1.38	54.2 ± 1.67	53.9 ± 1.19	54.3 ± 1.48
MCH (pg)	17.4 ± 0.53	17.4 ± 0.74	17.4 ± 0.39	17.2 ± 0.67
MCHC (g/dL)	32.0 ± 0.34	32.0 ± 0.70	32.2 ± 0.49	31.7 ± 0.50
PLT ($\times 10^3/\mu L$)	1038 ± 119.3	1058 ± 91.0	1059 ± 130.7	1067 ± 109.7
RET% (%)	2.20 ± 0.335	3.15 ± 2.184	2.39 ± 0.469	2.4 ± 0.440
NEU% (%)	17.0 ± 4.19	17.4 ± 4.58	17.4 ± 6.17	14.9 ± 3.63
LYM% (%)	78.3 ± 4.61	77.9 ± 5.06	77.6 ± 6.22	80.7 ± 4.11
EOS% (%)	1.1 ± 0.33	0.9 ± 0.33	1.0 ± 0.25	1.0 ± 0.20
MON% (%)	2.1 ± 0.42	2.3 ± 0.36	2.4 ± 0.56	2.0 ± 0.48
BAS% (%)	0.4 ± 0.22	0.5 ± 0.13	0.4 ± 0.17	0.3 ± 0.12
LUC% (%)	1.0 ± 0.51	1.0 ± 0.28	1.3 ± 0.70	1.1 ± 0.62
PT (sec)	14.3 ± 0.37	14.1 ± 0.48	14.3 ± 0.42	14.6 ± 0.48
APTT (sec)	16.0 ± 1.44	14.3 ± 16.1	16.1 ± 2.00	15.7 ± 1.86
Females				
WBC($\times 10^3/\mu L$)	6.72 ± 2.468	7.02 ± 1.980	7.41 ± 2.288	6.85 ± 2.661
RBC($\times 10^6/\mu L$)	8.17 ± 0.415	8.40 ± 0.290	8.39 ± 0.317	8.11 ± 0.755
HGB (g/dL)	15.3 ± 0.58	15.6 ± 0.33	15.6 ± 0.45	15.3 ± 0.49
HCT (%)	46.9 ± 1.81	47.9 ± 1.43	48.3 ± 1.67	47.6 ± 1.83
MCV (fL)	57.5 ± 2.31	57.1 ± 1.06	57.6 ± 2.11	59.2 ± 6.65
MCH (pg)	18.7 ± 0.83	18.6 ± 0.59	18.7 ± 0.82	19.0 ± 1.85
MCHC (g/dL)	32.5 ± 0.35	32.7 ± 0.77	32.4 ± 0.78	32.2 ± 0.81
PLT ($\times 10^3/\mu L$)	969 ± 65.4	974 ± 144.7	1171 ± 494.8	946 ± 117.2
RET% (%)	2.70 ± 0.876	2.27 ± 0.376	2.36 ± 0.299	4.18 ± 6.075
NEU% (%)	12.7 ± 6.68	11.6 ± 4.02	11.8 ± 5.14	11.9 ± 3.49
LYM% (%)	82.2 ± 6.99	82.9 ± 4.18	82.5 ± 5.61	82.5 ± 3.86
EOS% (%)	1.2 ± 0.53	1.2 ± 0.40	1.2 ± 0.60	1.2 ± 0.48
MON% (%)	2.2 ± 0.86	2.4 ± 0.88	2.5 ± 0.63	2.6 ± 0.78
BAS% (%)	0.4 ± 0.05	0.4 ± 0.16	0.4 ± 0.14	0.4 ± 0.16
LUC% (%)	1.5 ± 0.6	1.5 ± 0.54	1.6 ± 0.51	1.3 ± 0.30
PT (sec)	15.0 ± 0.61	14.8 ± 0.28	14.7 ± 0.36	15.1 ± 0.68
APTT (sec)	13.8 ± 1.36	15.3 ± 1.59	14.3 ± 1.77	13.8 ± 1.44

Each value represents the mean ± SD for 10 rats.

from Day 92 in Table 3) for the treatment groups during the treatment and recovery periods.

The mean AST level in females given 3000 mg/kg/day was significantly higher ($p = 0.026$) than the control group. Alanine aminotransferase is a hepatic enzyme associated with changes in liver weight, histopathology findings of abnormal hepatocytes and CYP450 activity (Ennulat et al., 2010). It has a wide normal range in similar aged rats from 47.0 to 172.2 IU/L (Giknis and Clifford, 2006; Petterino and Argentino-Storino, 2006). There were no increases in AST-related findings or changes in any other parameters and only AST from one animal was higher (278.1 IU/L) than the normal range. Therefore, AST was not considered to be associated with the treatment or toxicological findings.

3.4. Gross observations, organ weight and histopathology

Absolute organ weights are shown in Table 4. No treatment-related changes in absolute or relative organ weight (data not shown) were observed in the treated animals during the treatment and recovery periods. In addition, no treatment-related macroscopic or microscopic findings were observed in the treated animals.

Absolute spleen weight was significantly higher ($p = 0.014$) and non-significantly higher (1.31-fold) than control in two males given 300 mg/kg/day; this result was associated with enlarged spleens. Organ weight change is a useful indicator in toxicity studies. However, there are many factors that affect spleen weight including inter-animal variability, stress and physiological factors unrelated to treatment and spleen weight changes are often unrelated to histopathologic findings (Michael et al., 2007). In this study, the changes were unrelated to the treatment as no dose-dependent changes or related histopathologic findings were observed.

Mononuclear infiltration in organs including kidneys, liver, heart, epididymides, prostate, and others were observed in both sexes of the control and treatment groups throughout the study period. Mononuclear cells such as lymphocytes, monocytes and macrophages are distributed throughout the body via blood. Mononuclear infiltration in kidneys, liver, heart, epididymides, and prostate unrelated to the treatment in up to 100% of observed SD rats have been reported (Giknis and Clifford, 2012). Since mononuclear infiltration in organs is common in animals, it is not considered to indicate toxicity.

3.5. Allergic reaction

A food allergy is an adverse immunological reaction that can be caused by IgE- or non-IgE-mediated immune mechanisms. IgE-mediated food allergies (type I food allergy) account for the majority of food allergies (Nauta et al., 2008). Histamine is the most important inflammatory mediator of IgE-mediated reactions. Allergic reactions, including contact and respiratory forms, have been reported from various types of mealworms and Orthoptera (Linares et al., 2008). Furthermore, the Tenebrionid family potentially has significant allergens for workers exposed to grains or grain products (Schroeckenstein et al., 1990).

The IgE and histamine analysis results are shown in Table 5. There were no statistically significant changes in either IgE or histamine concentrations indicating allergic reaction throughout the study period. Moreover, there was no dose-dependent trend or recovery effect in IgE or histamine concentrations. The changes in IgE and histamine concentrations resulted predominantly in no more than twofold increases, but there was one group with a 4.2-

Table 3
Serum chemistry values of rats treated orally with fdTML for 90-days.

Parameters	Vehicle control	300 mg/kg	1000 mg/kg	3000 mg/kg
Males				
GLU (mg/dL)	118.7 ± 20.03	129.5 ± 32.51	132.0 ± 23.04	131.6 ± 22.69
BUN(mg/dL)	14.5 ± 1.48	14.6 ± 2.00	14.7 ± 1.43	15.1 ± 1.82
CREA (mg/dL)	0.54 ± 0.061	0.54 ± 0.086	0.57 ± 0.051	0.60 ± 0.050
TP (g/dL)	6.95 ± 0.297	7.06 ± 0.221	7.11 ± 0.323	6.95 ± 0.346
ALB (g/dL)	4.37 ± 0.100	4.35 ± 0.082	4.38 ± 0.125	4.34 ± 0.130
A/G (ratio)	1.7 ± 0.161	1.61 ± 0.073	1.61 ± 0.109	1.68 ± 0.128
AST (IU/L)	136.8 ± 12.80	151.2 ± 47.89	136.2 ± 26.66	131.5 ± 13.72
ALT (IU/L)	37.5 ± 4.81	44.6 ± 25.71	34.3 ± 5.34	30.7 ± 9.71
TBIL (mg/dL)	0.104 ± 0.0112	0.117 ± 0.0557	0.101 ± 0.0103	0.096 ± 0.0118
ALP (IU/L)	248.4 ± 47.83	215.8 ± 42.72	238.0 ± 47.58	249.0 ± 45.44
CK (IU/L)	790 ± 161.2	847 ± 234.8	839 ± 284.1	799 ± 156.2
TCHO (mg/dL)	62.5 ± 9.03	55.2 ± 8.74	66.3 ± 17.64	64.2 ± 19.56
TG (mg/dL)	47.2 ± 15.70	41.9 ± 10.23	53.7 ± 25.95	47.2 ± 11.48
PL (mg/dL)	96 ± 13.9	88 ± 12.0	102 ± 21.0	99 ± 22.3
GGT (IU/L)	0.00	0.00	0.00	0.01 ± 0.035
Females				
GLU (mg/dL)	127.2 ± 22.53	132.2 ± 136.9	136.9 ± 17.94	131.8 ± 31.73
BUN (mg/dL)	15.3 ± 2.13	15.4 ± 2.87	14.2 ± 2.85	15.4 ± 2.26
CREA (mg/dL)	0.64 ± 0.049	0.65 ± 0.068	0.63 ± 0.065	0.66 ± 0.091
TP (g/dL)	7.41 ± 0.503	7.56 ± 0.538	7.79 ± 0.527	7.61 ± 0.847
ALB (g/dL)	4.79 ± 0.263	4.93 ± 0.325	5.06 ± 0.265	4.93 ± 0.483
A/G (ratio)	1.85 ± 0.133	1.89 ± 0.151	1.86 ± 0.120	1.85 ± 0.160
AST (IU/L)	108.4 ± 18.67	135.2 ± 96.98	129.8 ± 41.81	144.4 ± 48.64*
ALT (IU/L)	28.5 ± 9.29	48.9 ± 55.11	44.7 ± 28.06	44.2 ± 26.94
TBIL (mg/dL)	0.155 ± 0.0663	0.145 ± 0.0242	0.165 ± 0.0273	0.199 ± 0.0887
ALP (IU/L)	137.3 ± 26.68	129.7 ± 36.96	114.8 ± 27.38	140.0 ± 42.95
CK (IU/L)	501 ± 189.3	531 ± 183.9	550 ± 164.7	587 ± 181.6
TCHO (mg/dL)	73.6 ± 20.21	90.2 ± 14.67	89.7 ± 11.94	93.1 ± 28.58
TG (mg/dL)	38.1 ± 11.12	38.5 ± 13.06	46.8 ± 23.80	40.3 ± 12.97
PL (mg/dL)	147 ± 30.8	173 ± 23.5	176 ± 24.3	175 ± 54.9
GGT (IU/L)	0.04 ± 0.126	0.59 ± 1.842	0.03 ± 0.057	0.37 ± 0.774

Each value represents the mean ± SD for 10 rats.
*: Significant differences from vehicle control group ($p < 0.05$).

Table 4
Absolute organ weights of rats treated orally with fdTML for 90-days.

Parameters	Vehicle control	300 mg/kg	1000 mg/kg	3000 mg/kg
Males				
Adrenal gland (g)	0.070 ± 0.0051	0.072 ± 0.0128	0.066 ± 0.0112	0.072 ± 0.0106
Brain (g)	2.115 ± 0.1020	2.170 ± 0.1041	2.141 ± 0.0934	2.121 ± 0.0557
Heart (g)	1.577 ± 0.1282	1.729 ± 0.1820	1.566 ± 0.2164	1.746 ± 0.1765
Kidneys (g)	3.759 ± 0.3863	3.901 ± 0.5509	3.724 ± 0.5974	3.810 ± 0.3923
Liver (g)	13.866 ± 1.3785	15.788 ± 1.9627	15.332 ± 3.2292	16.191 ± 2.2529
Pituitary gland (g)	0.012 ± 0.0016	0.013 ± 0.0013	0.013 ± 0.0020	0.013 ± 0.0019
Prostate (g)	0.685 ± 0.2068	0.810 ± 0.1176	0.692 ± 0.1181	0.643 ± 0.1552
Spleen (g)	0.790 ± 0.0987	1.136 ± 0.6454*	0.799 ± 0.1205	0.867 ± 0.1157
Testes (g)	3.534 ± 0.2279	3.560 ± 0.2016	3.481 ± 0.2758	3.525 ± 0.2089
Thymus (g)	0.341 ± 0.0776	0.388 ± 0.0598	0.391 ± 0.1015	0.368 ± 0.0512
Epididymides (g)	1.563 ± 0.1192	1.670 ± 1.1139	1.578 ± 0.2255	1.572 ± 0.0875
Lung (g)	1.717 ± 0.0997	1.936 ± 0.2887	1.692 ± 0.2084	1.802 ± 0.1272
Seminal vesicle (g)	2.186 ± 0.2175	2.280 ± 0.2550	2.002 ± 0.2874	2.105 ± 0.1968
Thyroid/parathyroid (g)	0.024 ± 0.0045	0.027 ± 0.0040	0.026 ± 0.0054	0.029 ± 0.0115
Salivary gland (g)	0.764 ± 0.0890	0.863 ± 0.1195	0.776 ± 0.1227	0.807 ± 0.0762
Females				
Adrenal gland (g)	0.083 ± 0.0104	0.077 ± 0.0071	0.079 ± 0.0128	0.080 ± 0.0081
Brain (g)	1.979 ± 0.0755	1.985 ± 0.0712	2.042 ± 0.0647	1.984 ± 0.0400
Heart (g)	0.998 ± 0.1334	0.961 ± 0.0757	1.079 ± 0.0622	1.009 ± 0.0616
Kidneys (g)	2.270 ± 0.2036	2.065 ± 0.1915	2.205 ± 0.1835	2.159 ± 0.1456
Liver (g)	8.478 ± 0.8480	8.233 ± 0.8664	8.894 ± 0.7510	8.679 ± 1.3834
Pituitary gland (g)	0.134 ± 0.0158	0.128 ± 0.0189	0.136 ± 0.0191	0.125 ± 0.0154
Prostate (g)	0.018 ± 0.0042	0.017 ± 0.0034	0.017 ± 0.0020	0.018 ± 0.0064
Spleen (g)	0.522 ± 0.0544	0.524 ± 0.1150	0.599 ± 0.1631	0.619 ± 0.1869
Testes (g)	0.307 ± 0.0648	0.346 ± 0.0827	0.335 ± 0.0668	0.382 ± 0.1052
Thymu s(g)	1.281 ± 0.0781	1.232 ± 0.0965	1.294 ± 0.0754	1.277 ± 0.1487
Epididymides (g)	0.022 ± 0.0036	0.020 ± 0.0049	0.020 ± 0.0055	0.020 ± 0.0028
Lung (g)	0.792 ± 0.2653	0.672 ± 0.2567	0.784 ± 0.3440	0.633 ± 0.1858
Seminal vesicle (g)	0.489 ± 0.0456	0.498 ± 0.0401	0.469 ± 0.0491	0.471 ± 0.0419

Each value represents the mean ± SD for 10 rats.
*: Significant differences from vehicle control group ($p < 0.05$).

Table 5
IgE and Histamine values of rats treated orally with fdTML for 90-days.

Parameters	Vehicle control	300 mg/kg	1000 mg/kg	3000 mg/kg
Males				
IgE (ng/mL)				
Day 92	15.1 ± 13.9	13.9 ± 16.9	13.7 ± 11.5	12.7 ± 9.6
Day 121[a]	14.7 ± 11.3	NA[b]	NA	27.7 ± 22.1
Histamine				
Day 92	53.2 ± 16.2	75.7 ± 28.4	63.3 ± 26.3	72.3 ± 20.3
Day 121	47.3 ± 10.7	NA	NA	55.0 ± 25.4
Females				
IgE (ng/mL)				
Day 92	13.3 ± 10.4	22.4 ± 22.9	55.8 ± 81.9	27.2 ± 32.9
Day 121	14.6 ± 12.5	NA	NA	18.0 ± 14.1
Histamine				
Day 92	49.3 ± 18.7	56.5 ± 19.3	59.2 ± 38.8	61.7 ± 18.0
Day 121	61.1 ± 22.6	NA	NA	57.5 ± 38.5

Each value represents the mean ± SD for 10 or 5 rats.
[a] Recovery Day 30.
[b] Not applicable.

fold increase at 1000 mg/kg/day for the female group on Day 92. Specifically, the changes in IgE concentrations only appeared in the fdTML-administrated female group, which resulted in 1.7-fold, 4.2-fold and 2.0-fold IgE increases at 300, 1000 and 3000 mg/kg/day, respectively, on Day 92, and a 1.9-fold increase at 3000 mg/kg/day on Day 121. Individual data (data not shown) for 2 out of 10 animals showed marked induction of 140 and 259 ng/mL IgE in the 1000 mg/kg/day group, and 1 out 10 animals showed an induction of 104 ng/mL IgE in the 3000 mg/kg/day group, all of which were much higher than the mean concentration in each corresponding group and were attributed to high degree of variation. These individual data might imply a prevalence rate of fdTML but it remains unclear in this study. To obtain more information on the relationship between allergenicity and fdTML, a large number of animals is needed to examine allergenic effects and prevalence rates of fdTML in future investigations.

The whole body of edible insects is usually consumed; therefore, we evaluated tentative hypersensitivity in rats after repeated administration of freeze-dried powder of whole insects. Some food insects were reported to contain cross-sensitizing allergens, such as tropomyosin of crustaceans or arginine kinase of arthropods, but specific insect allergens are largely unknown (Belluco et al., 2013). In addition, the total serum IgE provides a rough indication of food allergy as atopy (Metcalfe et al., 2013) and is a good allergy predictor in children (Satwani et al., 2009). In this study, tentative hypersensitivity was therefore evaluated using total IgE and histamine concentrations after repeated administration of freeze-dried powder of whole insects. Because this study simultaneously examined food toxicity and allergenicity, it is difficult to define the effect of fdTML on allergic responses based on specific histamine or total IgE concentrations measured in this study. For future investigations, time-dependent histamine release or fdTML-specific IgE measurements should be made to screen out fdTML-unrelated effects and clarify fdTML-induced allergic responses.

Since a high degree of variation has been observed in the correlation between increased IgE and histamine concentrations, and allergic reactions, ranging from several-fold to several-hundred-fold higher (Ji et al., 2013; Stone et al., 2010; Yamamoto et al., 2007), it was difficult to define allergic responses from IgE and histamine increases in this study. In rats, the basal concentration of total IgE was reported to be $\leq$ 30 ng/mL (Diaz-Sanchez and Kemeny, 1991). In humans, one report found no relationship between IgE and allergic reaction when the median value of total IgE was $\leq$100 ng/mL, suggesting an odds ratio of 1 (Salo et al., 2011). Therefore, more information such as toxicology data is needed to determine allergic responses.

Theoretically, any food can cause an allergy. Common foods, such as cow milk, eggs, and peanut,s can trigger food allergy and they affect approximately 4% of children and adults; however, these are not considered to be life-threatening food allergens to all people (Longo et al., 2013; Van Gramberg et al., 2013) and do not cause a severe clinical manifestation at all times. In contrast, it is reported that the high prevalence of sensitization to house dust mites is related to high prevalence of bronchial asthma or atopic dermatitis (Terreehorst et al., 2002). The pathogenesis of allergic diseases can be determined by abundant secretion of cytokines, such as IL-4 and IL-13, or proliferation and activation of mast cells and basophils (Amin, 2012). Although we conducted an allergic reaction test under limited conditions in this study, our results indicated that adverse immune responses were not shown based on hematological and pathological examinations, clinical signs, or molecular markers, implying that potential hypersensitivity from edible insect fdTML consumed as a food source is rare.

4. Conclusion

The administration of fdTML up to 3000 mg/kg/day to SD rats for 90-days did not result in any adverse effects or toxicity findings, including an allergy assessment. Therefore, the NOAEL (No Observed Adverse Effect level) was determined to be in excess of 3000 mg/kg/day for both sexes of SD rats given the experimental conditions of this study.

Conflicts of interest

There are no competing financial interests.

Acknowledgments

This research was supported by a grant (#PJ008969) from the Agenda program, Rural Development Administration, and a grant (#311006-3) from the Bio-industry Technology Development project, Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.yrtph.2016.03.006.

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