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Ninety-day oral toxicity study of rice-derived γ-oryzanol in Sprague-Dawley rats

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ABSTRACT

A 90-day oral toxicity study of γ-oryzanol, a rice-derived triterpenoid ferulate, was performed by oral gavage administration to male and female Sprague-Dawley rats at doses of 0, 1000, and 2000 mg/kg body weight/day. All rats administered γ-oryzanol survived throughout the study period. Both male and female rats showed no toxicologically significant changes of the general signs, examination findings, body weight, food consumption, functional observational battery results, ophthalmological findings, urinalysis, hematology tests, clinical chemistry tests, organ weights, and necropsy findings. Moreover, there were no histopathological changes related to administration of γ-oryzanol in males and females from the 2000 mg/kg body weight/day group. In conclusion, the no observed adverse effect level (NOAEL) of γ-oryzanol exceeded 2000 mg/kg body weight/day for both male and female rats under the conditions of this study.

1. Introduction

γ-Oryzanol is a rice-derived lipophilic compound that is used as a prescription drug (Hi-Z, Otsuka Pharmaceutical Co. Ltd., Japan), dietary supplement, or additive to foods and animal feed. It is made from a byproduct (soap stock) producing in purification processes of rice bran oil [1]. As a prescription drug, it has been employed to treat various conditions in Japan, including hyperlipidemia [2,3] and neurologic symptoms such as menopausal syndrome [4,5], autonomic ataxia [6,7], and irritable bowel syndrome [8,9]. It has also been reported that γ-oryzanol improves muscle strength [10] and performance of endurance exercise [11]. Based on such evidence, γ-oryzanol has been used as a dietary supplement in the USA [10]. Moreover, recent studies have shown that γ-oryzanol enhances adipocyte differentiation [12] and glucose-stimulated insulin secretion via activation of the c-AMP/PKA pathway [13], leading to an antidiabetic effect.

γ-Oryzanol is an ester of ferulic acid and several different triterpenoids, among which the main triterpenoids are 24-methylene cycloartanol, cycloartenol, campesterol, and β-sitosterol [14]. The composition of the triterpenoids differs depending on the type of rice from which γ-oryzanol is derived [14]. It has been reported that 24-methylenecycloartanol ferulate shows antitumor activity against breast cancer cell lines [15], while cycloaltenol ferulate suppresses mast cell degranulation [16] and β-sitosterol ferulate has strong antioxidant activity [17]. A recent study showed that campesterol ferulate is a mixture of stereo isomers [18]. It has been reported that ferulic acid is a metabolite of γ-oryzanol [19], but the other metabolites of triterpenoids have not been reported so far.

Subacute and chronic toxicity studies of γ-oryzanol have already been performed because it is used as a prescription drug [20]. Its teratogenicity and developmental toxicity have also been investigated [21]. The composition of triterpenyl ferulates differs depending on the rice source and the extraction and purification processes. Our HPLC analysis of medicinal γ-oryzanol and γ-oryzanol used in foods confirmed differences of the triterpenoids, especially 24-methylene cycloartanol and cycloartenol.

In spite of the increased use of γ-oryzanol in processed foods, especially in the USA, there has been no formal toxicological assessment of the effects of ongoing consumption of γ-oryzanol. Therefore, it is necessary to verify the safety of food-grade γ-oryzanol in rodents at doses relevant to human consumption of this substance as a food ingredient. Accordingly, we conducted a 90-day toxicity study in rats to assess the toxicity of γ-oryzanol manufactured by our company as a food additive.

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2. Materials and methods

2.1. Test sample and reagents

2.1.1. Analytical characterization of γ-oryzanol

γ-Oryzanol (CAS No. 11042-64-1) is a white or slightly yellow powder. The γ-oryzanol used in this study was manufactured by Oryza Oil & Fat Chemical Co. Ltd. (Lot. M-506). The composition of the triterpene ferulates in γ-oryzanol was determined by LC–MS/MS using a modification of the method of Fang et al [22]. Cycloartenol ferulate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was used as the reference standard. Then 10 μL of the filtered solution was injected into an LC–MS/MS system consisting of an HPLC apparatus (1100 series, Agilent Technology, Santa Clara, CA, USA) and a mass spectrometer (API-4000, Applied Biosystems, Foster city, CA, USA). HPLC was performed with a column (CAPCELL PAK C18 SG120, ϕ4.6 mm × 250 mm, particle size: 5 μm; Shiseido, Tokyo, Japan) at 30 °C. A mixture of methanol and water (99:1) was used as the mobile phase for HPLC and the flow rate was set at 1 mL/min. The conditions for MS were as follows: ionization method: APCI (positive, negative), corona current: 2 μA (positive, negative), nebulizer gas: nitrogen at 60 psi, and dry gas temperature: 300 °C. Individual chromatograms, MS spectra, and product ion spectra were obtained. MS/MS spectra was obtained by using a collision energy of 40 eV (positive) or −50 eV (negative) and the most abundant ions in the MS spectra as precursors.

HPLC chromatograms of γ-oryzanol and cycloartenol ferulate are shown in Fig. 1. Five peaks a), b), c), d), and e) were detected in the chromatogram of γ-oryzanol and the retention time of the cycloartenol ferulate peak was around 19.45 min (Fig. 1, lower). For peaks *a* to *e* and the peak of the standard, their most abundant ions and the corresponding product ions were obtained with positive or negative collision energy. The results were compared with the data reported by Fang et al. [22] and Endo et al. [23]. As a result, all peaks were identified as follows: a, cycloartenol ferulate; b, 24-methylenecycloartanol ferulate; c; campesterol ferulate; d, cycloblanol ferulate; and e, a mixture of sitosterol ferulate and cycloartanol ferulate (Fig. 2).

The triterpene ferulates in γ-oryzanol were cycloartenol ferulate (34%), 24-methylenecycloartanol ferulate (40%), campesterol ferulate (17%), cycloblanol ferulate (2%), and a mixture of sitosterol ferulate and cycloartanol ferulate (6%).

2.1.2. Reagents

Corn oil was obtained from Sigma-Aldrich Co., (St. Louis, MO, USA) and isopto atropine was obtained from Alcon (Fort Worth, TX, USA).

2.2. Regulatory guidelines and ethics

This study was conducted in accordance with the Good Laboratory Practice Regulations ("Standards and Regulations for Chemical Testing Laboratories") established by the Ministry of Environment, Republic of Korea (Jan. 1, 2015) as notification No. 2014-240. In addition, this study followed the "OECD Principles of Good Laboratory Practice" (Organization for Economic Co-operation and Development, ENV/MC/CHEM (98)17, revised in 1997) and the "OECD Guideline For The Testing Of Chemicals 408, Repeated-Dose 90-day Oral Toxicity Study in Rodents" (Organization for Economic Co-operation and Development, adopted 21st September 1998). The experimental plan was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) (Approval No.:150283). The experiments were conducted in accordance with the Animal Protection Act of Korea and the Guide for the Care and Use of Laboratory Animals.

2.3. Animals

Male and female Sprague-Dawley [Crl: CD (SD)] rats aged 5 weeks were obtained from Charles River Laboratories Japan, Inc. (Atsugi, Japan). Body weight ranged from 111.0 to 140.3 g for males (n = 46) and from 103.8 to 127.7 g for females (n = 46). The rats (n = 2 or 3) were housed in stainless steel wire mesh cages (26.0 cm wide × 35.0 cm long × 21.0 cm high) and were acclimatized for 12 days at 20.6–22.8 °C and 44–45% humidity with a 12 h light (150–300 lx)/dark cycle. Pellet rodent chow (Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C) obtained from Harlan Laboratories, Inc. (Indianapolis, IN, USA) was provided *ad libitum*. Tap water from the Cheongju-si public supply was filtered, irradiated with ultraviolet light, and provided *ad libitum*. After the quarantine-acclimation period, 40 males and 40 females were

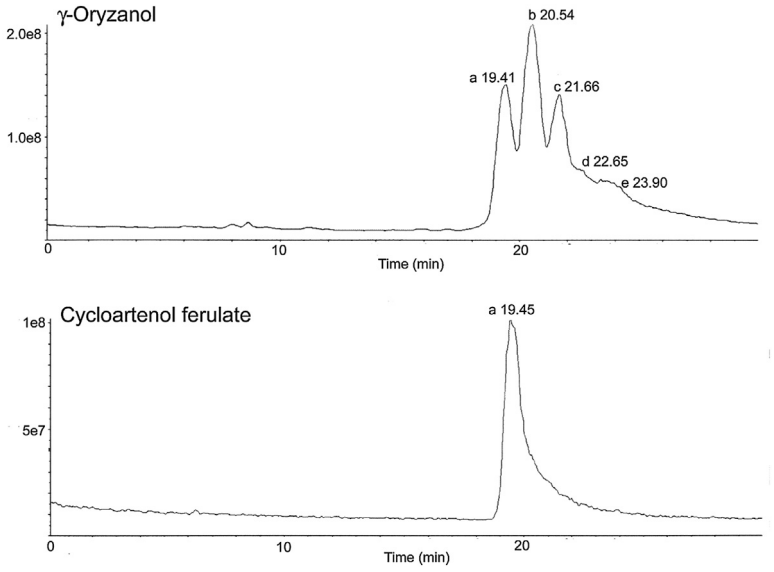

Fig. 1. HPLC chromatograms of γ-oryzanol and cycloartenol ferulate. HPLC conditions were as follows: column (CAPCELL PAK C18 SG120, ϕ4.6 mm × 250 mm), column temperature: 30 °C, mobile phase: methanol and water (99:1), and flow rate: 1 mL/min. Peaks *a* to *e* indicate the following ferulate esters of triterpenoids; *a*: cycloartenol, *b*: 24-methylenecycloartanol, *c*: campesterol, *d*: cycloblanol, *e*: a mixture of sitosterol and cycloartanol.

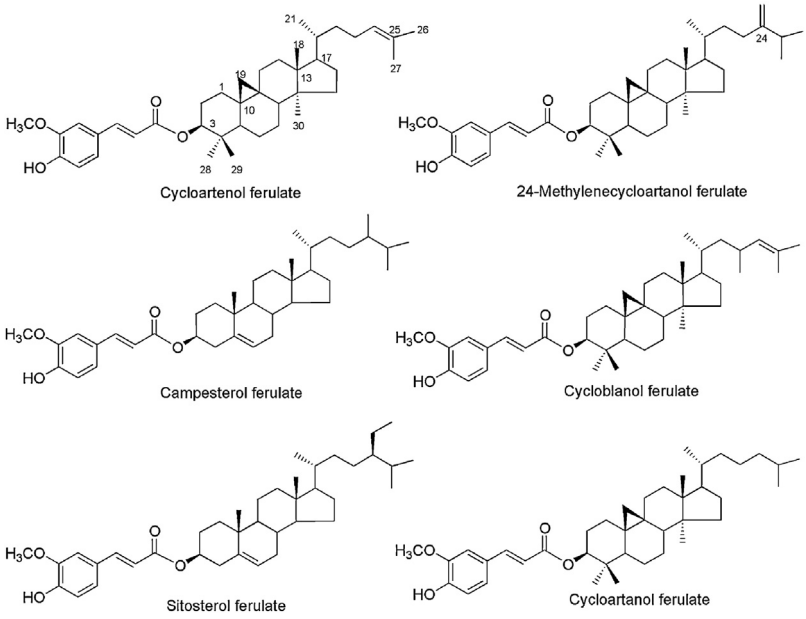

Fig. 2. Triterpene ferulates in γ-oryzanol.

selected and randomly assigned to 4 groups (10 animals of each sex per group) with equalization of the mean group body weight, which ranged from 224.2 to 259.8 g for males (n = 40) and from 167.1 to 205.1 g for females (n = 40). The rats were housed individually in stainless steel wire mesh cages during the study period.

2.4. Administration and observations

In the study reported by Hanesato et al. [20], the maximum dose of oryzanol was 1000 mg/kg body weight/day. We found that a dose of 3000 mg/kg body weight/day was technically difficult to administer in our preliminary study, so doses of 1000 and 2000 mg/kg body weight/day were chosen for the present study. As γ-Oryzanol is contained in approximately 0.5 to 1% rice bran oil [24], 1000 and 2000 mg of γ-Oryzanol are equivalent to 100–200 g of rice bran oil. γ-Oryzanol was suspended in corn oil (1000 or 2000 mg in a volume of 6 mL). A fresh suspension was prepared daily and was used within 4 h after preparation. Rats were administered the γ-oryzanol suspension (6 mL/kg body weight/day) once daily for 90 consecutive days by gavage with a gastric tube connected to a 3- or 5-mL disposable syringe. The suspension was thoroughly stirred just prior to administration to maintain homogeneity. Control animals received the vehicle (corn oil) at 6 mL/kg body weight/day. Body weight was measured before administration.

Throughout the study, all rats were observed once daily to assess clinical observations and twice daily for mortality and moribundity. Observation involved assessment of the skin, fur, eyes, mucous membranes, secretions, and excretion, as well as checking autonomic activity (lacrimation, piloerection, pupil size, and unusual respiration), stereotypic behavior (excessive grooming and repetitive circling), bizarre behavior (self-mutilation and walking backward), changes in gait, posture, and the response to handling, and the presence of chronic or tonic convulsions. Daily food consumption was calculated from total food consumption over 7 days.

2.5. Functional observation battery and motor activity

Functional observational battery testing was conducted on all surviving rats in the dosing groups at 90 days according to the method of Mattsson et al. [25] and MacDaniel et al. [26]. The visual response, proprioceptive stimuli, auditory stimuli, pain response, aerial righting reflex, and hind limb landing foot splay were each measured once. Grip strength was measured using a push-pull gauge (RX-2, Aikoh Engineering Co., Ltd., Osaka, Japan). Measurement was repeated 3 times each for the forelimbs and hind limbs, and the maximum value was used. Locomotor was measured using an activity monitor (Medd-Ofa-RS, Med Associate Inc., St. Albans, VT, USA) to monitor each animal every 10 min for 1 h.

2.6. Ophthalmological examination

Ophthalmological examination was conducted on both eyes of all rats prior to dosing and on both eyes of all rats in the control and γ-oryzanol (2000 mg/kg body weight/day) groups at 13-week administration period. A mydriatic agent (1% isopto atropine) was instilled into the eyes prior to examination and the anterior segment, transparent media, and ocular fundus were observed using an ophthalmoscope (All Pupil II, Keeler Ltd., Berkshire, UK).

2.7. Urinalysis

On day 90, fresh urine was collected for 3 h from 5 rats of each sex per group in the fasting state, and a 21-h urine sample was subsequently collected while allowing access to food. Rats in metabolic cages (Biotoxtech, Cheongju, Republic of Korea) were allowed free access to water during both urine collection periods. The urine samples were stored at4°C until analysis was performed within 2 h of sample collection. The Combur^{10}Test® M stick (Roche Diagnostics, Mannheim, Germany) was used to measure pH, protein, glucose, and occult blood, while urine chemistry was done with a Cobas u 411 urine analyzer (F. Hoffmann-La Roche, Basel, Swizerland). Urine color and turbidity were checked by visual inspection. The 24-h urine was used for measurement of the urine volume and specific gravity. The specific gravity was measured with a Gravimeter (VET 360, Reichert Technologies, New York, USA).

2.8. Hematology and blood chemistry tests

Rats were fasted overnight for approximately 18 h before blood collection. Then the rats were anesthetized with isoflurane on day

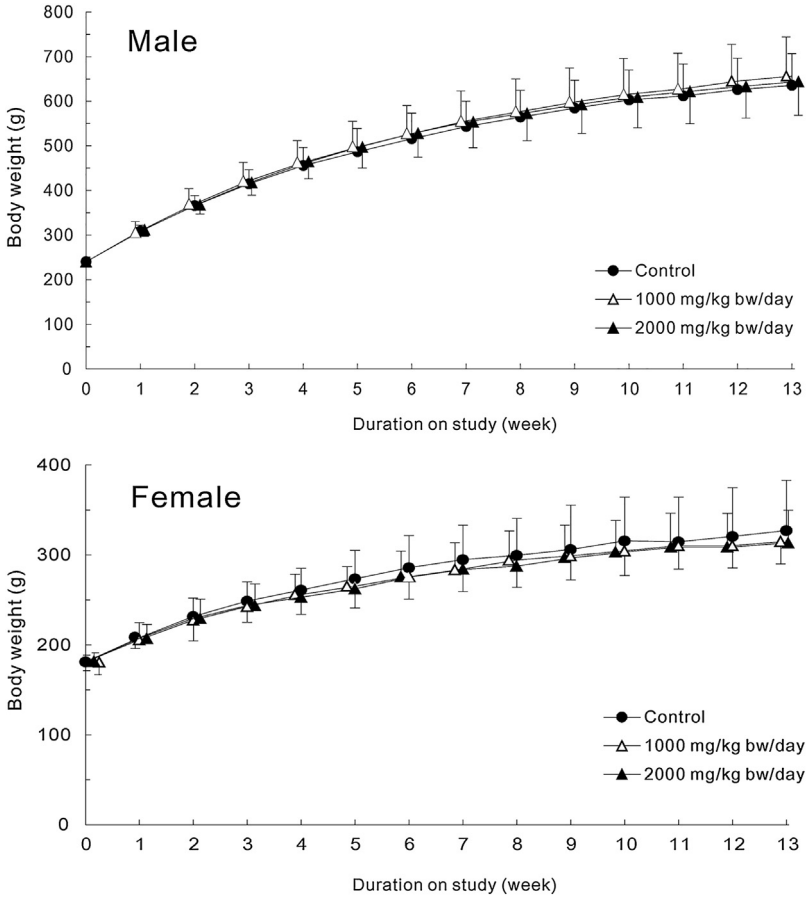

Fig. 3. Body weight of male and female rats. Values represent the mean ± SD (n = 10). No significant differences were observed.

91 and blood was collected from the abdominal aorta. For hematology test, approximately 3 mL of blood sample volume was collected and placed in a vacutainer containing 5.4 mg of K_2 EDTA. For evaluation of coagulating parameters, approximately 2 mL of blood was collected in a tube containing 3.2% sodium citrate. For clinical chemistry tests, approximately 5 mL of blood sample was collected.

The blood samples for hematology tests were stored at 4°C and analysis was carried out within 2 or 3 h after collection. A sample of approximately 1 mL was placed in a vacutainer containing EDTA and the following parameters were measured using an auto analyzer (ADVIA 2120i, Siemens, Berlin, Germany): total erythrocyte count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, total leukocyte count (WBC), and differential WBC counts (including neutrophils, lymphocytes, monocytes, eosinophils, basophils, and reticulocytes).

In addition, approximately 2 mL of blood mixed with 3.2% sodium citrate was centrifuged at 3000 rpm for 10 min to obtain plasma for evaluation of the prothrombin time and activated partial thromboplastin time using an automated coagulation meter (Coaprest 2000, Sekisui Medical Co. Ltd., Tokyo, Japan). For coagulation tests, blood samples were centrifuged within 1 h after collection. Furthermore, blood for clinical chemistry tests collected from the abdominal aorta into a vacutainer was centrifuged at 3000 rpm for 10 min to obtain serum within 1 h after collection and stored at −20°C until examination. The following parameters were analyzed using an auto analyzer (7180, Hitachi High-Technologies Co., Tokyo, Japan) and an electrolyte analyzer (EasyLyte, Medica Co., Bedford, MA, USA): alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), γ-glutamyl transpeptitase (GGT), blood urea nitrogen (BUN), creatinine, total protein, albumin, albumin/globulin (A/G) ratio, total cholesterol, triglycerides, glucose, phosphorus (P), calcium (Ca), chloride (Cl), sodium (Na), and potassium (K).

2.9. Necropsy

All rats were sacrificed by exsanguination from the abdominal aorta under isoflurane anesthesia on day 91. A complete macroscopic postmortem examination was performed on all rats, including the external surfaces of the carcass and the internal organs. All grossly visible abnormalities were recorded. Organs were weighed (paired organs were weighed together) and the organ-to-body weight (final body weight) ratios were calculated based on the fasting weight. The organs weighed were the brain, thymus, heart, liver, kidney, spleen, adrenal, testis, epididymis, ovary and uterus. At necropsy, the following organs and tissues were harvested and stored in 10% neutral buffered formalin (the testes and eyes including the optic nerve were harvested and fixed in Davidson's fixative before storage in 10% neutral buffered formalin): brain (cerebrum, cerebellum, and pons), pituitary, thyroid, parathyroid, thymus, lung including bronchi, trachea, heart, liver, spleen, kidney, adrenal, aorta, salivary glands (submandibular, sublingual and parotid glands), esophagus, stomach, duodenum, ileum, jejunum, colon, cecum, rectum, pancreas, epididymis, testis, seminal vesicle with coagulating gland, prostate, uterus including cervix, ovary, fallopian tube, vagina, urinary bladder, submandibular lymph node, mesenteric lymph node, mammary gland (inguinal), skin (inguinal), skeletal muscle (thigh), sciatic nerve, eye, optic nerve, harderian gland, nasal turbinates, sternum including bone

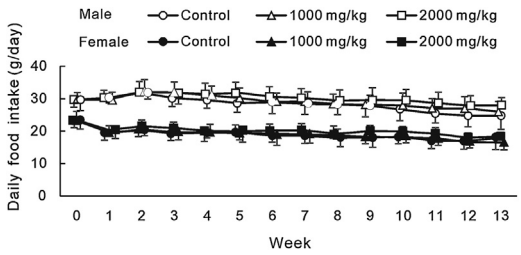

Fig. 4. Daily food intake. Each point represents the mean ± SD (n = 10). No significant differences were observed.

marrow, femur including bone marrow, spinal cord (cervical, midthoracic, and lumbar), and any gross lesions. For histopathological evaluation, specimens of the preserved tissues were trimmed, dehydrated, and embedded in paraffin by standard methods, after which the paraffin-embedded tissues were sectioned and stained with hematoxylin & eosin. All residual organs and tissues were preserved in 10% neutral buffered formalin.

Histopathological examination was performed on all tissues from the control group and the γ-oryzanol (2000 mg/kg body weight/day) group, as well as for macroscopic lesions of the uterus/cervix in the γ-oryzanol (2000 mg/kg body weight/day) group.

2.10. Statistical analysis

Statistical analysis was performed using SAS software (version 9.3, SAS Institute Inc., Cary, NC, USA). Data on the body weight, food consumption, urine volume, functional observational battery, hematology, clinical chemistry, and organ weight were analyzed by using Bartlett's test for homogeneity of variance (significance level: $P < 0.05$). One-way analysis of variance (ANOVA) was initially employed for homogeneous data. If a significant difference was found, Dunnett's test was used for multiple comparisons (significance levels: $P < 0.05$ and 0.01, two-tailed) as described earlier [27]. The Kruskal-Wallis test was performed on heterogeneous data. If a significant difference was identified, Steel's test was applied for multiple comparisons (significance levels: $P < 0.05$ and 0.01, two-tailed) as described earlier [27].

3. Results

3.1. Clinical observations and body weight

All rats survived until the end of the study in the 0 (control), 1000 and 2000 mg/kg body weight/day groups. No abnormalities of clinical observations or body weight changes (Fig. 3) were observed in the control group. Excretion of γ-oryzanol was sporadically observed in the stools of all males and females from day 57–58 to day 90 in the 1000 mg/kg body weight/day group and white substance originated from test substance was macroscopically observed in feces of all males and females from day 2–3 to day 90 in the 2000 mg/kg body weight/day group. In addition, an open toe wound with nail damage, hemorrhage, and/or crust formation was observed on the left hind limb of one male on days 50–57 and on the right forelimb of one female on days 44–49 in the 2000 mg/kg body weight/day group. These findings were considered to be incidental changes unrelated to γ-oryzanol, since they were not observed in any other rats throughout the study. No abnormalities were identified by detailed examination of males and females in the 1000 and 2000 mg/kg body weight/day groups. Compared with the control group, there were no statistically significant differences of the body weight (Fig. 3) and food consumption

Table 1
Mean functional observations; sensory motor function observations (1).

	Control	1000 mg/kg bw/day	2000 mg/kg bw/day
Male			
Visual response	3 ± 0	3 ± 0	3 ± 0
Touch response	3 ± 0	3 ± 0	3 ± 0
Click response	3 ± 0	3 ± 0	3 ± 0
Tail pinch response	3 ± 0	3 ± 0	3 ± 0
Aerial righting reflex	0 ± 0	0 ± 0	0 ± 0
Female			
Visual response	3 ± 0	3 ± 0	3 ± 0
Touch response	3 ± 0	3 ± 0	3 ± 0
Click response	3 ± 0	3 ± 0	3 ± 0
Tail pinch response	3 ± 0	3 ± 0	3 ± 0
Aerial righting reflex	0 ± 0	0 ± 0	0 ± 0

Values represent the mean ± SD (n = 10). Each parameter was scored as follow. Visual response; score 1: no response, score 2: The animal is aware of a stimulating bar and the response is trace, score 3: The animal approaches slowly and smells a stimulating bar. Touch response; score 1: no response, score 2: slight responses such as wiggling of ears, score 3: The animal turns around slowly, score 4: The animal stiffens, wards off a stimulating bar, or squeaks. Click response; score 1: no response, score 2: slight wiggling of ears, score 3: twitching of body, score 4: jumping, squeaking. Tail pinch response (pain response): score 1: no response, score 2: positive but slow response, score 3: Squeaking, turning back, score 4: stronger response than normal (getting away by loosing tail from a tweezer). Aerial righting response: score 0: normal (landing on four limbs).

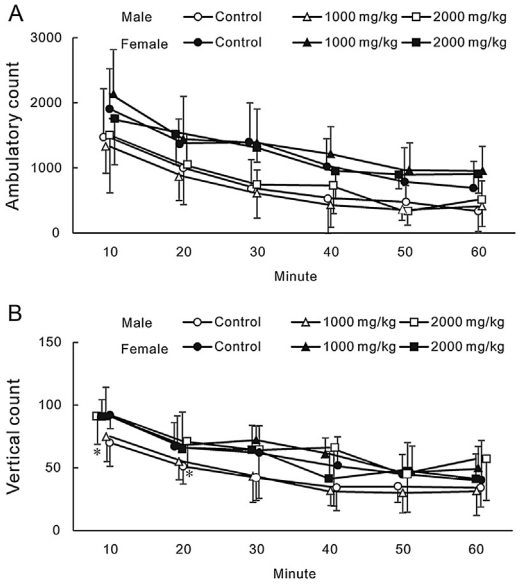

Fig. 5. Mean results for observation of spontaneous motor activity. Each point represents the mean ± SD (n = 10). Asterisk denotes a significant difference ($p < 0.05$) from the control for each sex.

of males and females (Fig. 4) in the 1000 and 2000 mg/kg body weight/day groups.

3.2. Functional observational battery and motor activity

Compared with the control group, there were no differences of the visual response, touch response, click response, tail pinch response, and aerial righting response in males and females from the 1000 and 2000 mg/kg body weight/day groups (Table 1). Similarly, there were no significant differences of), and ambulatory counts (Fig. 5) between the control group and males and females in the 1000 and 2000 mg/kg body weight/day groups. On the other hand, the vertical count was significantly increased from 0–10 to 10–20 min during the spontaneous motor activity test in male rats from the 2000 mg/kg body weight/day group compared with the control group (Fig. 5). However, there were no differences of the female vertical counts or the ambulatory counts in both sexes. In

Table 2
Mean functional observational battery findings; sensorimotor function (2).

	Hind limb landing foot splay (mm)	Forelimb grip strength (kgf)	Hind limb grip strength (kgf)
Males			
Control	84.4 ± 26.4	1.17 ± 0.26	0.62 ± 0.20
1000 mg/kg bw/day	75.6 ± 25.4	1.11 ± 0.21	0.59 ± 0.16
2000 mg/kg bw/day	85.3 ± 22.8	1.16 ± 0.34	0.59 ± 0.16
Females			
Control	55.4 ± 12.1	0.54 ± 0.17	0.60 ± 0.20
1000 mg/kg bw/day	53.2 ± 16.6	0.57 ± 0.16	0.56 ± 0.21
2000 mg/kg bw/day	57.4 ± 14.6	0.54 ± 0.18	0.59 ± 0.22

Values represent the mean ± SD (n = 10). No significant differences were observed.

addition, there were no ocular abnormalities in males and females treated with γ-oryzanol, even in the 2000 mg/kg body weight/day group (Table 3).

3.3. Urinalysis findings

There were no significant differences of urine volume between the control group and the γ-oryzanol groups in either males or females (Table 4). The urine color, transparency, pH, protein content, glucose content, and specific gravity were not obviously affected by γ-oryzanol (1000 or 2000 mg/kg body weight/day). Occult blood (>25 Ery/μL) was detected in the urine of one male (1000 mg/kg body weight/day) and one female (2000 mg/kg body weight/day).

3.4. Laboratory test results

There were no differences of hematological parameters in males and females from the 1000 and 2000 mg/kg body weight/day groups when compared with control values (Table 5). Blood urea nitrogen (BUN) and total cholesterol (T-Chol) were significantly increased in males from the 2000 mg/kg body weight/day group (Table 6). However, there were no significant differences of blood chemistry parameters in males and females from the 1000 mg/kg body weight/day groups and females from the 2000 mg/kg body weight/day group compared with the control values.

Table 3
Summary of ophthalmic examination.

	Control	2000 mg/kg bw/day
Male		
Right eye		
Pupil light reflex	10/10	10/10
Anterior segment	10/10	10/10
Transparent media	10/10	10/10
Fundus	10/10	10/10
Left eye		
Pupil light reflex	10/10	10/10
Anterior segment	10/10	10/10
Transparent media	10/10	10/10
Fundus	10/10	10/10
Female		
Right eye		
Pupil light reflex	10/10	10/10
Anterior segment	10/10	10/10
Transparent media	10/10	10/10
Fundus	10/10	10/10
Left eye		
Pupil light reflex	10/10	10/10
Anterior segment	10/10	10/10
Transparent media	10/10	10/10
Fundus	10/10	10/10

Number of not remarkable animals/number of examined animal.

Table 4
Summary of urinalysis results.

Sex		Males Control	1000 mg/kgbw/day	2000 mg/kgbw/day	Females Control	1000 mg/kgbw/day	2000 mg/kgbw/day
Volume (mL)		6.5 ± 1.9	6.6 ± 1.2	8.0 ± 3.1	9.6 ± 5.3	4.7 ± 3.6	4.7 ± 2.4
Color	Pale yellow	0	0	0	1	0	0
	Yellow	5	5	5	4	5	5
Transparency	Clear	3	3	3	5	4	4
	Mild turbidity	1	1	1	0	1	0
	Turbidity	1	1	1	0	0	1
pH	5.0	0	0	0	1	0	0
	6.0	2	1	2	2	3	2
	6.5	0	1	1	0	1	0
	7.0	3	1	0	1	1	1
	8.0	0	2	2	1	0	2
	9.0	0	0	0	0	0	0
Protein	–	0	0	0	2	0	0
	25 (mg/dL)	4	3	3	3	5	5
	75	1	2	1	0	0	0
	150	0	0	1	0	0	0
	500	0	0	0	0	0	0
Glucose	0	5	5	5	5	5	5
	50 (mg/dL)	0	0	0	0	0	0
Occult blood	–	3	3	4	5	5	4
	10 (Ery/mL)	2	1	1	0	0	0
	25	0	0	0	0	0	1
	50	0	0	0	0	0	0
	150	0	1	0	0	0	0
Specific	1.000–1.010	0	0	0	0	0	0
Gravity	1.011–1.020	0	0	0	1	0	0
	1.021–1.030	0	0	0	1	0	0
	1.031–1.040	0	0	0	1	1	0
	1.041–1.050	2	0	1	2	1	2
	1.051–1.060	0	2	2	0	2	0
	>1.060	3	3	2	0	1	3

Volumes are shown as the mean ± SD (n = 5). No significant differences of urine volume were observed. For evaluation of urine volume and specific gravity, the 24-h urine was used. Fresh urine was used for the other parameters.

Table 5
Hematological parameters.

Males	RBC ($\times 10^6$ cells/μL)	Hemoglobin (g/dL)	Hematocrit (%)	RBC Indices MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3$ cells/μL)	Reticulocytes (%)
Control	8.62 ± 0.28	14.9 ± 0.7	44.8 ± 1.8	52.0 ± 1.7	17.3 ± 0.6	33.3 ± 0.4	843 ± 50	1.75 ± 0.18
1000 mg/kg bw/day	8.74 ± 0.29	15.1 ± 0.5	45.2 ± 1.2	51.8 ± 1.4	17.3 ± 0.5	33.3 ± 0.3	818 ± 100	1.95 ± 0.22
2000 mg/kg bw/day	8.61 ± 0.24	15.2 ± 0.5	45.5 ± 1.4	52.9 ± 1.9	17.7 ± 0.7	33.4 ± 0.4	860 ± 95	1.95 ± 0.33
	WBC ($\times 10^3$ cells/μL)	Differential WBC counts (%) Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	PT (Sec)	APTT (Sec)
Control	8.39 ± 1.88	19.8 ± 4.7	75.7 ± 4.8	2.1 ± 0.5	1.6 ± 0.3	0.2 ± 0.1	17.5 ± 0.7	15.8 ± 1.2
1000 mg/kg bw/day	10.89 ± 2.02	18.1 ± 8.5	77.6 ± 8.5	1.9 ± 0.6	1.2 ± 0.8	0.3 ± 0.1	17.8 ± 0.5	16.3 ± 1.4
2000 mg/kg bw/day	10.13 ± 3.02	18.7 ± 3.9	76.8 ± 4.0	2.3 ± 0.4	1.3 ± 0.5	0.3 ± 0.1	17.8 ± 0.6	16.2 ± 1.0
Females	RBC ($\times$106 cells/μL)	Hemoglobin (g/dL)	Hematocrit (%)	RBC Indices MCV (fL)	MCH (pg)	MCHC (g/dL)	Platelets ($\times 10^3$ cells/μL)	Reticulocytes (%)
Control	7.84 ± 0.23	14.6 ± 0.5	43.1 ± 1.1	55.1 ± 1.4	18.6 ± 0.5	33.8 ± 0.5	849 ± 93	1.87 ± 0.36
1000 mg/kg bw/day	7.89 ± 0.57	14.7 ± 0.5	42.7 ± 2.2	54.2 ± 2.0	18.7 ± 1.4	34.4 ± 1.9	863 ± 45	1.89 ± 0.39
2000 mg/kg bw/day	8.10 ± 0.34	15.0 ± 0.8	44.0 ± 2.2	54.3 ± 1.6	18.5 ± 0.5	34.0 ± 0.2	894 ± 96	1.84 ± 0.35
	WBC ($\times 10^3$ cells/μL)	Differential WBC counts (%) Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	PT (Sec)	APTT (Sec)
Control	4.91 ± 1.50	18.2 ± 8.5	77.5 ± 9.0	2.2 ± 0.9	1.4 ± 0.4	0.2 ± 0.1	17.5 ± 0.9	14.4 ± 1.9
1000 mg/kg bw/day	5.86 ± 1.82	14.2 ± 5.8	81.0 ± 6.5	2.2 ± 0.7	1.3 ± 0.3	0.2 ± 0.1	17.8 ± 0.6	14.5 ± 1.3
2000 mg/kg bw/day	5.01 ± 1.22	17.1 ± 5.7	77.4 ± 6.3	2.5 ± 0.9	1.7 ± 0.5	0.3 ± 0.1	17.8 ± 0.7	14.3 ± 1.4

Abbreviations: RBC, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin, MCHC; mean corpuscular hemoglobin concentration (MCHC); WBC, white blood cells; PT, prothrombin time; APTT, activated partial thromboplastin time.
Values represent the mean ± SD (n = 10). No significant differences were observed.

Table 6
Blood chemistry parameters.

Males	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Glucose (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)
Control	36.9 ± 17.2	91.7 ± 23.9	302.4 ± 75.7	0.55 ± 0.10	131 ± 13	10.8 ± 1.1	0.44 ± 0.03	64 ± 9
1000 mg/kg bw/day	30.7 ± 10.9	84.4 ± 20.7	325.3 ± 101.5	0.59 ± 0.24	134 ± 12	11.8 ± 1.1	0.45 ± 0.05	74 ± 10
2000 mg/kg bw/day	47.9 ± 35.9	121.4 ± 59.3	299.3 ± 66.4	0.56 ± 0.11	123 ± 6	12.2 ± 1.4*	0.46 ± 0.03	90 ± 19**
	Triglyceride (mg/dL)	Phospholipid (g/dL)	A/G ratio	P (mg/dL)	Ca (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
Control	66 ± 25	5.8 ± 0.1	0.68 ± 0.04	5.93 ± 0.49	10.1 ± 0.2	140 ± 1	4.50 ± 0.32	104.7 ± 1.0
1000 mg/kg bw/day	69 ± 36	5.9 ± 0.4	0.65 ± 0.04	5.98 ± 0.48	10.1 ± 0.3	141 ± 1	4.45 ± 0.26	105.1 ± 0.6
2000 mg/kg bw/day	67 ± 19	6.0 ± 0.2	0.66 ± 0.08	5.90 ± 0.34	10.2 ± 0.2	141 ± 1	4.38 ± 0.19	105.6 ± 0.7
Females	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Glucose (mg/dL)	BUN (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)
Control	41.4 ± 31.9	93.3 ± 76.5	137.2 ± 39.7	0.21 ± 0.15	115 ± 11	14.2 ± 1.9	0.54 ± 0.03	87 ± 13
1000 mg/kg bw/day	33.6 ± 8.7	78.1 ± 20.7	176.8 ± 94.0	0.33 ± 0.20	116 ± 6	13.4 ± 0.7	0.53 ± 0.04	84 ± 12
2000 mg/kg bw/day	31.8 ± 11.6	78.6 ± 37.3	160.7 ± 45.1	0.31 ± 0.18	113 ± 8	13.9 ± 1.9	0.52 ± 0.03	96 ± 27
	Triglyceride (mg/dL)	Phospholipid (g/dL)	A/G ratio	P (mg/dL)	Ca (mg/dL)	Na (mmol/L)	K (mmol/L)	Cl (mmol/L)
Control	42 ± 37	6.8 ± 0.2	0.81 ± 0.05	4.22 ± 0.46	10.5 ± 0.3	141 ± 1	3.92 ± 0.18	106.9 ± 0.8
1000 mg/kg bw/day	34 ± 16	6.6 ± 0.2	0.81 ± 0.06	4.60 ± 0.60	10.5 ± 0.2	141 ± 1	3.93 ± 0.18	106.8 ± 1.5
2000 mg/kg bw/day	34 ± 24	6.7 ± 0.5	0.83 ± 0.05	4.54 ± 0.56	10.6 ± 0.5	141 ± 1	3.97 ± 0.22	106.6 ± 0.7

Values represent the mean ± SD (n = 10). Asterisks denote significant differences from the control group.
Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase, ALP, alkaline phosphatase; GGT, γ-glutamyl transpeptitase; BUN, blood urea nitrogen; A/G, albumin/globulin; P, phosphorus; Ca, calcium; Cl, chloride; Na, sodium; K, potassium.
* $p < 0.05$.
** $p < 0.01$.

3.5. Organ weights and necropsy findings

Compared with the control group, there were no significant differences of organ weights in males and females from the 1000 and 2000 mg/kg body weight/day groups (Table 7). At necropsy, macroscopic examination did not reveal any changes related to administration of γ-oryzanol and the macroscopic findings observed in this study were considered to be incidental (see Supplemental Tables 1 and 2 in the online version at DOI:http://dx.doi.org/10.1016/j.toxrep.2016.12.001). Microscopic examination also did not reveal any test substance-related changes (Tables 8 and 9). See Supplementary Fig. S1 in the online version at DOI:http://dx.doi.

Table 7
Absolute Organ weights (g).

Males	Final body weight	Brain	Thymus	Heart	Liver	Spleen	Kidneys	Adrenals	Testes	Epididymis
Control	635.5 ± 66.9	2.18 ± 0.13	0.37 ± 0.11	1.66 ± 0.18	16.8 ± 2.2	0.85 ± 0.17	3.47 ± 0.27	0.058 ± 0.006	3.74 ± 0.42	1.45 ± 0.17
1000 mg/kg bw/day	656.3 ± 87.8	2.21 ± 0.13	0.36 ± 0.07	1.62 ± 0.19	16.4 ± 2.2	0.93 ± 0.22	3.34 ± 0.45	0.059 ± 0.007	3.84 ± 0.34	1.58 ± 0.12
2000 mg/kg bw/day	643.1 ± 63.6	2.18 ± 0.09	0.33 ± 0.12	1.60 ± 0.18	16.5 ± 2.9	0.86 ± 0.15	3.47 ± 0.41	0.060 ± 0.009	3.74 ± 0.40	1.48 ± 0.16
Females		Brain	Thymus	Heart	Liver	Spleen	Kidneys	Adrenals	Ovaries	Uterus
Control	327.2 ± 55.8	1.99 ± 0.13	0.29 ± 0.06	0.96 ± 0.07	8.5 ± 0.9	0.51 ± 0.12	1.87 ± 0.12	0.066 ± 0.011	0.081 ± 0.016	0.80 ± 0.30
1000 mg/kg bw/day	315.0 ± 34.7	1.96 ± 0.08	0.30 ± 0.09	0.95 ± 0.10	7.9 ± 0.8	0.51 ± 0.21	1.90 ± 0.21	0.072 ± 0.010	0.078 ± 0.015	0.81 ± 0.29
2000 mg/kg bw/day	313.2 ± 23.1	1.97 ± 0.12	0.30 ± 0.09	0.95 ± 0.08	7.7 ± 0.8	0.55 ± 0.06	1.90 ± 0.119	0.066 ± 0.009	0.075 ± 0.015	0.94 ± 0.49

Values represent the mean ± SD (n = 10). No significant differences were observed.

Table 8
Summary of histopathological findings in males.

Organs	Observations	Control	1000 mg/kg bw/day	2000 mg/kg bw/day
Adrenal	No. of microscopic findings	10/10	N/A	10/10
	Vacuolation, zona fasciculata	Grade 1: 2	/	Grade 1: 2
		Grade 2: 0	/	Grade 2: 1
Eyes	No. of microscopic findings	10/10	N/A	10/10
	Focal retinal dysplasia	0/10	/	Grade 1: 1
Heart	No. of microscopic findings	10/10	N/A	10/10
	Focal infiltration of monocytes	Grade 1: 2	/	Grade 1: 1
Kidneys	No. of microscopic findings	10/10	N/A	10/10
	Focal hyaline cast	Grade 1: 1	/	0/10
	Pyelitis	0/10	/	Grade 2: 1
	Tubular basophilia	Grade 1: 5	/	Grade 1: 3
Liver	No. of microscopic findings	10/10	N/A	10/10
	Focal infiltration of monocytes	Grade 1: 2	/	Grade 1: 2
	Periportal vacuolation	Grade 1: 4	/	Grade 1: 2
		Grade 2: 3	/	Grade 2: 3
	Sporadic hepatocyte vacuolation	Grade 2: 1	/	Grade 2: 3
Lungs including bronchi	No. of microscopic findings	10/10	N/A	10/10
	Alveolar focal macrophage aggregation	0/10	/	Grade 1: 1
	Focal fibrosing alveolitis	0/10	/	Grade 1: 1
	Focal osseous metaplasia	Grade 1: 1	/	Grade 1: 1
Prostate	No. of microscopic findings	10/10	N/A	10/10
	Interstitial lymphocyte infiltration	Grade 1: 1	/	0/10
Salivary gland, parotid	No. of microscopic findings	10/10	N/A	10/10
	Focal infiltration of monocytes	Grade 1: 1	/	0/10
Spleen	No. of microscopic findings	10/10	N/A	10/10
	Deposition of brown pigment	Grade 1: 3	/	Grade 1: 1
		Grade 2: 0	/	Grade 2: 1
	Extramedullary hematopoiesis	Grade 1: 1	/	Grade 1: 2
Testes	No. of microscopic findings	10/10	N/A	10/10
	Unilateral dilation of rete testis	0/10	/	Grade 1: 1

Abbreviations: /, not examined; N/Anot applicable (only animals with gross lesions were examined). Severity was classified into 4 grades; grade 1: minimalgrade 2: slight, grade 3: moderate, grade 4: severe.

org/10.1016/j.toxrep.2016.12.001 indicates microscopic images of kidneys and uterus including cystic tubles and a cyst in females. All microscopic findings in various organs and tissues were considered to be incidental or related to administration of the vehicle (corn oil) and were of no toxicological significance.

4. Discussion and conclusion

While various biological activities of γ-oryzanol have been reported, there are few published toxicity studies. Only *in vitro* and *in vivo* carcinogenicity studies have been reported. In the Ames test, γ-oryzanol did not exhibit any mutagenicity for *Salmonella* and *Escherichia Coli* strains under conditions with and without S9 mix [28]. With regard to *in vivo* studies, Tamagawa et al. [29,30] reported that oral administration of γ-oryzanol (200–2000 mg/kg body weight/day) for 79 weeks did not promote tumorigenesis in female mice (B6C3F1). Similarly, they confirmed that oral administration of γ–oryzanol for 105 weeks had no negative effects in F344 male rats Tamagawa et al. [29,30]. The administration periods and doses exceeded those used in the current study for examination of subacute toxicity and these previous studies demonstrated that γ-oryzanol does not show carcinogenicity. Instead, γ-oryzanol has been reported to suppress tumors of Zymbal's gland in rodents [31]. In addition, Hirose et al. [32] reported a preventive effect of γ-oryzanol against carcinoma and adenoma of the lung, large intestine, urinary bladder, kidney, liver, and thyroid gland. They also reported a suppressive effect on tumors of the auditory canal and mammary gland [33,34]. Based on such evidence, the safety of γ-oryzanol with regard to carcinogenesis seems to have been confirmed. However, examination of the subacute toxicity of γ-oryzanol after clarification of the composition of each constituent triterpene ferulate has not been performed before.

Therefore, this study was conducted to assess cumulative toxicity when γ-oryzanol was administered orally to 6-week-old SD rats once daily for 90 days. All rats survived until the end of the study and there was no mortality related to γ-oryzanol. The test sample was observed to be mixed in the stools of males and females in the 1000 and 2000 mg/kg body weight/day groups, but this finding was not considered to be of toxicological significance and simply indicated that γ-oryzanol was excreted without being absorbed. From the reported value [35], 3% of a dose of γ-oryzanol was absorbed in male rats when it was administered at 50 mg/kg body weight. Although it was not clarified whether there was a gender difference in absorption, the absorption rate of γ-oryzanol seems to be

Table 9
Summary of histopathological findings in females.

Organs	Observations	Control	1000 mg/kg bw/day	2000 mg/kg bw/day
Harderian gland	No. of microscopic findings	10/10	N/A	10/10
	Focal infiltration of lymphocytes	Grade 1: 1	/	0/10
	Focal hyperplasia	0/10	/	Grade 1: 1
Kidneys	No. of microscopic findings			
	Focal hyaline cast	Grade 1: 1	/	0/10
	Focal infiltration of monocytes	0/10	/	Grade 1: 2
	Cystic tubules	3/10	/	0/10
	Pyelitis	Grade 1: 1	/	0/10
		Grade 2: 1	/	0/10
Liver	No. of microscopic findings	10/10	N/A	10/10
	Focal infiltration of monocytes	Grade 1: 1	/	Grade 1: 5
	Periportal vacuolation	Grade 1: 1	/	0/10
	Sporadic hepatocyte vacuolation	Grade 2: 1	/	Grade 2: 3
Lungs including bronchi	No. of microscopic findings	10/10	N/A	10/10
	Alveolar focal macrophage aggregation	0/10	/	Grade 1: 1
	Focal fibrosing alveolitis	0/10	/	Grade 1: 1
Mammary gland: inguinal	No. of microscopic findings	10/10	N/A	10/10
	Lobuloalveolar hypertrophy	0/10	/	Grade 1: 1
Ovaries	No. of microscopic findings	10/10	N/A	10/10
	Follicular cyst	0/10	/	1/10
Spleen	No. of microscopic findings	10/10	N/A	10/10
	Deposition of brown pigment	Grade 1: 6	/	Grade 1: 5
		Grade 2: 1	/	Grade 2: 3
	Extramedullary hepatopoiesis	0/10	/	Grade 1: 1
Thyroid	No. of microscopic findings	0/10	N/A	1/10
Uterus including cervix	No. of microscopic findings	10/10	1/10	10/10
	Cyst in cervix lined by stratum germinativum	0/10	1/10/	0/10
Vagina	No. of microscopic findings	10/10	N/A	10/10
	Mucification of epithelium	0/10	/	Grade 1: 1

Abbreviations: /, not examined; N/A, not applicable (only animals with gross lesions were examined). Severity was classified into 4 grades; grade 1: minimal, grade 2: slight, grade 3: moderate, grade 4: severe.

low and most of each dose was excreted in the feces in this study. Also, there were no changes of body weight related to γ-oryzanol during the dosing period and no macroscopic findings or lesions were noted in the gastrointestinal tract.

When spontaneous motor activity was assessed, the vertical count was significantly increased during 0–10 and 10–20 min in males from the 2000 mg/kg body weight/day group compared with the control group. However, this was considered to be an incidental finding without toxicological significance due to lack of consistency between the sexes, and there were also no significant changes of the total count. Although γ-oryzanol is used to treat neurologic symptoms, there were no abnormalities of motor activity related to neurological dysfunction in this study.

Apparent occult blood (150 Ery/mL) was observed in the urine of one male in 1000 mg/kg body weight/day group. Similar occult blood was detected in one female in the 2000 mg/kg body weight/day group. hind limb landing foot splay, grip strength (Table 2 These findings were not considered to be of toxicological significance because the changes were slight [36] and showed an incidental distribution. Blood urea nitrogen and cholesterol were increased in males from the 2000 mg/kg body weight/day group, but these findings were not considered to be related to γ-oryzanol since the changes were slight [36] and inconsistent between the sexes. Moreover, γ-oryzanol (at doses around 200 mg/kg body weight) has been reported to suppress cholesterol absorption [37] by binding to cholesterol [38]. In the present study, administration of γ-oryzanol at a 10-fold higher dose did not induce excessive hypocholesterolemia. Instead, total cholesterol increased slightly in males given 2000 mg/kg body weight/day. This increase of total cholesterol was not significant and remained within the normal range (male: 44–132 mg/dL, female: 44–146 mg/dL). The change was probably not caused by γ-oryzanol because there was no significant increase of total cholesterol in females. Similarly, a hypoglycemic effect of γ-oryzanol was not confirmed. Considering these results, the increase of cholesterol in male rats was not toxicologically significant.

There were no γ-oryzanol-related changes of the body weight, food consumption, examination findings, hematological data, organ weight, ophthalmological findings, and necropsy findings in animals of both sexes from the 1000 and 2000 mg/kg body weight/day groups, as well as no histopathological changes in males and females from the 2000 mg/kg body weight/day group. In conclusion, the no observed adverse effect level (NOAEL) of γ-oryzanol was higher than 2000 mg/kg body weight/day in male and female rats under the conditions of this study. This present investigation concluded that 90 day oral gavage administration of food grade γ-oryzanol (NOAEL 2000 mg/kg BW) does not cause serious damages in rats.

Conflict of interest declaration

Biotoxtech Co., Ltd. was contracted by Oryza Oil & Fat Chemical Co. Ltd. to develop the protocol and conduct, analyze, and interpret, and report the animal study described herein. Oryza Oil & Fat Chemical Co. Ltd. approved the study plan, and also supported LC–MS/MS analysis and preparation of the manuscript. The authors declare no additional conflicts of interest in regard to this research and the authorship and/or publication of this article.

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