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90-Day repeated-dose toxicity study of licorice flavonoid oil (LFO) in rats

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ABSTRACT

Licorice flavonoid oil (LFO) is a new functional food ingredient consisting of licorice hydrophobic polyphenols in medium-chain triglycerides (MCT).
As part of a safety evaluation, a 90-day oral toxicity study in rats was conducted using an LFO concentrate solution (2.90% glabridin). Male and female animals were assigned to one of 12 groups (10 males or females per group) and received corn oil (negative control), MCT (vehicle control), or 400, 600, 800 or 1600 mg/kg of the LFO concentrate solution.In conclusion, LFO concentrate solution induced an anticoagulation effect in both sexes, although there was a clear sex difference. Based on thezse findings, it is concluded that the no-observed-adverse-effect level (NOAEL) for the LFO concentrate solution is estimated to be 800 mg/kg/day for female rats, and approximately 400 mg/kg/day for male rats.

1. Introduction

Licorice flavonoid oil (LFO) derived from licorice, *Glycyrrhiza glabra*, is a new functional food ingredient consisting of licorice hydrophobic polyphenols in medium-chain triglycerides (MCT). It has been developed by Kaneka Corporation. Recently, we found that LFO is effective in reducing visceral fat accumulation and elevated blood glucose levels in obese diabetic KK-Ay mice (Nakagawa et al., 2004). It has been suggested that the mechanism of LFO's anti-obesity effect is mediated by lipid metabolism-related gene expression in the liver (Aoki et al., 2007a). Furthermore, clinical studies have shown that LFO is effective in suppressing body weight gain by reducing body fat mass in overweight volunteers (Tominaga et al., 2006). These studies indicate that LFO may help to prevent lifestyle-related diseases such as obesity associated with metabolic syndrome.

In terms of safety, information on the hydrophobic fraction of licorice *glabra* has been limited, although licorice has a long history of human consumption. Clinical studies have shown that LFO is safe because no clinically significant adverse events occurred when it was given daily to healthy or overweight subjects for up to 12 weeks (Aoki et al., 2007b; Tominaga et al., 2006). Furthermore, as part of a series of LFO preclinical safety assessments, the genotoxicity of LFO has been investigated using three different methods, including a bacterial reverse mutation assay, a chromosomal aberration assay and a micronucleus *in vivo* assay (Nakagawa et al., in press). The results from a bacterial reverse mutation assay and micronucleus *in vivo* assay were all negative. In a short-term S9 mix chromosomal aberration test, however, LFO induced clastogenic activity at higher concentrations. From these results, although LFO showed clastogenic effects *in vitro* at higher concentrations, no genotoxic potential was observed in the *in vivo* assays at higher doses compared with the proposed clinical dose. Therefore, LFO can be considered to be non-genotoxic *in vivo*. In this paper, we report for the first time the results of a 90-day subchronic toxicity study in rats that has been conducted prior to human clinical studies. The first objective of the present study was to evaluate the toxicological potential and determine the no-observed-adverse-effect level (NOAEL) of licorice flavonoid oil (LFO) concentrate solution in the rat. Secondly, it was intended that the study would provide helpful and valuable information for the subsequent human studies and on usage as a dietary supplement.

2. Materials and methods

2.1. Study design

The first study was performed at the DIMS Institute of Medical Science, Inc. (Ichinomiya, Japan), in accordance with the Good Laboratory Practice (GLP) Standards of the Japanese Ministry of Health and Welfare Ordinance No. 21 (March 26th, 1997) and in compliance with the "Guidelines for Designation of Food

Abbreviations: APTT, activated partial thromboplastin time; LFO, licorice flavonoid oil; MCT, medium-chain triglycerides; PT, prothrombin time.

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Additives and for Revision of Standards for Use of Food Additives" of the Japanese Ministry of Health and Welfare (Eika No. 29, March 22nd, 1996). The second study was conducted at Kaneka Corporation (Hyogo, Japan) under non-GLP conditions.

During both studies, the animals were treated in accordance with the Guidelines for Animal Experimentation, Japanese Association for Laboratory Animal Science (1987).

2.2. LFO test substance

To prepare the LFO test substance, an extract of licorice (*G. glabra*) root was obtained with ethanol. After filtration and concentration, the ethanolic layer was mixed with medium-chain triglycerides (MCT) having a fatty acid composition of C8:C10 = 99:1. The concentration of glabridin, which is the major component of the solution, was adjusted to 3.0 ± 0.5%. This solution was defined as "LFO concentrate solution," which has the brand name "KANEKA GLAVONOID™". LFO concentrate solution comprises 30% licorice ethanol extract and 70% MCT, with the latter functioning as a carrier/diluent. With respect to the former, polyphenols including licorice prenyl-flavonoids such as glabridin, which are active components of LFO, account for almost all of the licorice ethanol extract. As other constituents apart from MCT and polyphenols, LFO concentrate solution contains a very small quantity of phospholipids (0.03%), sterols (0.07%), and free fatty acids (0.27%). In the present studies, LFO concentrate solution containing 2.90% glabridin (Lot No. 50605001, Kaneka Corporation) was used as the LFO test substance. The LFO concentrate solution is our own product and has been commercially available as a functional food ingredient. The LFO test substance was prepared in an identical manner to the LFO concentrate solution, which means that for this study, we prepared the LFO test substance on a laboratorial scale, conforming to exactly the same specification and manufacturing process as those for the LFO concentrate solution.

2.3. Preparation of the test substance solution in study 1

The LFO test substance (LFO concentrate solution) was diluted with MCT (Lot No. C030401, Riken Vitamin Co. Ltd., Tokyo, Japan) to adjust to the intended concentration. The working solution was prepared once every two weeks and stored in the refrigerator (temperatures set at 2–10 °C). The stability, homogeneity and content of the working solution were analyzed and the preparations were confirmed to be appropriate.

2.4. Animals and treatments in study 1

Four-week-old male and female Crj:CD(SD) rats (SPF) were purchased from Charles River Japan Inc. (Shiga, Japan) and used in the study following a 2-week acclimation period. On the day before study initiation, rats were allocated to one of 12 groups using a computerized stratified body weight technique. There were 6 groups each of male and female rats (10 rats/group). The rats were housed in pairs in transparent plastic cages with chipped hardwood bedding and received an irradiated solid diet, CE-2 (Clea Japan, Inc., Tokyo, Japan) and water *ad libitum*. The animal room was maintained on a 12-h light/dark cycle; temperature and humidity were maintained within specified ranges of 20.0–24.5 °C and 49–65% relative humidity. LFO test substance was administered orally by gavage (2 ml/kg b.w.) to animals at doses of 400, 600, 800 and 1600 mg/kg/day for 90 days. One control groups of each sex received corn oil (control) and one control group of each sex received MCT (vehicle control). The dosage was determined at weekly intervals based on the actual body weight.

2.5. Observations and measurements in study 1

2.5.1. Clinical observations, body weight and ophthalmoscopy

All animals were checked for general condition twice daily. Body weights were measured at the initiation of the experiment, and then at weekly intervals. At the scheduled termination, final body weights were measured following an overnight fast (approximately 16 h).

An ophthalmoscopic examination of all the animals was conducted before allocation. The eyes of 6 rats in each of groups 1–11 and 3 rats in group 12 (all surviving animals) were examined at week 13. The examination was carried out using a model L-3740 instrument (Inami, Co. Ltd., Tokyo, Japan).

2.5.2. Feed and water consumption

Two-day feed and water consumption per cage were measured at weekly intervals throughout the study and calculated as daily mean per rat.

2.5.3. Urinalysis, hematology and clinical chemistry

At treatment week 13, 6 rats each in groups 1–11 and 3 rats in group 12 (all surviving animals) were placed in urine-collection cages without food and water, and 4-h urine outputs (9:00–13:00) were collected. Urine pH was measured with a pH meter in fresh urine specimens obtained by forced micturition. Urine volume was measured and color was observed macroscopically. The other estimations performed were as follows: occult blood, ketone body, glucose, proteins, urobilinogen and bilirubin were measured using Multi-stick (Bayer Medical Ltd., Tokyo, Japan). Urine sediment was examined using staining and microscopy techniques. Specific gravity was measured using an Atago Serum Protein Refractometer N (Atago Co., Ltd., Tokyo, Japan). Sodium (NA), potassium (K) and chlorine (CL) were measured using an automatic analyzer, model 7070 (Hitachi, Ltd., Tokyo, Japan).

At the end of the treatment period, blood samples for hematology and clinical chemistry were taken at necropsy from all surviving animals via the abdominal aorta under anesthesia following an overnight fast. The following hematological parameters were measured on samples collected using EDTA-2 K as an anticoagulant: red blood cell count (RBC), white blood cell count (WBC), hemoglobin (HGB), hematocrit (HCT), platelet count (PLT), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). All parameters were measured using an automatic blood cell counter, model F-820 (Sysmex Co., Kobe, Japan), with the exception of MCH, MCV and MCHC, which were calculated. Blood cell morphology, including differential white blood cell percentage (myelocytes (MY), neutrophil stab forms (ST), neutrophil segmented forms (SEG), eosinophils (EOS), basophils (BA), monocytes (MO), lymphocytes (LY)) and reticulocyte count, was assessed using staining microscopy techniques. Clotting measurements, namely prothrombin time (PT) and activated partial thromboplastin time (APTT), were made on samples of blood collected in tubes containing sodium citrate as an anticoagulant.

The following clinical chemistry measurements were made on sera obtained by centrifugation of the aforementioned blood samples: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyltranspeptidase (γ-GTP), total bilirubin (T-BIL), blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), total cholesterol (T-CHO), phospholipids (PL), triglycerides (TG), total protein (TP), albumin (ALB), albumin/globulin ratio (A/G), inorganic phosphates (IP), calcium (CA), magnesium (MG), sodium (NA), potassium (K) and chlorine (CL). All parameters were determined using an automatic analyzer, model 7070 (Hitachi Ltd., Tokyo, Japan).

Hematological examinations, including blood coagulation (5 male rats and 1 female rat) and clinical chemistry (4 male rats), were also performed on moribund animals in the 1600 mg/kg group.

2.5.4. Pathology

All surviving animals were terminated by exsanguination at the end of the treatment period. Gross observations were made at autopsy and recorded. The following organs from each rat were weighed, and organ to body weight ratios determined: abdominal fat (around the uterus, around the testes and around the kidneys), adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostate (ventral), salivary glands, seminal vesicles, spleen, testes, thymus, thyroid and uterus. Samples of these organs and of the aorta, bone and bone marrow, epididymides, esophagus, eyes, Harderian glands, large intestine, lymph nodes, mammary glands, musculature, nasal cavity, oviducts, pancreas, sciatic nerve, skin, small intestine, spinal cord, stomach, tongue, trachea, urinary bladder, vagina, Zymbal's glands, and any gross lesions were fixed in 10% buffered formalin. A full histopathological examination was performed on hematoxylin–eosin stained tissue sections of the organs and tissues listed above for the control, vehicle control and female 1600 mg/kg group rats and all male rat groups rats. Microscopic examination was also carried out for specimens of any gross lesions for the 400, 600 and 800 mg/kg female groups. All rats found dead and moribund animals terminated during the treatment period were also examined histopathologically.

2.6. Protocol of study 2

We conducted the second study to determine the mechanism of the anticoagulant effect that had been induced by LFO concentrate solution in the first study. Twenty-six 5-week-old male Crj:CD(SD) rats (SPF) were purchased from Charles River Japan (Shiga, Japan) and housed in an animal room maintained with a 12-h light/dark cycle, at a constant temperature of 22 ± 2 °C and a relative humidity of 55 ± 20%. The animals were observed daily and were used after a 1-week acclimation period for the experiments.

The experimental design is shown in Fig. 1. Rats were allocated to 4 groups (5 rats/group) and 2 satellite groups (3 rats/group). In groups 1 and 5 (control group), a basal diet (CE-2) was fed to the rats for 20 days. In groups 2, 3 and 6, LFO concentrate solution (Lot No. 50605001) as 5% of the diet was fed for 20 days. In group 4, LFO concentrate solution as 5% of the diet was fed for 13 days and the food was replaced with the basal diet on day 13 and fed to the rats for the remaining 7 days. The rats in group 3 were given two i.p. injections of Vitamin K (70 mg/kg/day b.w.) on days 13 and 14. Vitamin K (drug formulation of vitamin K1, 10 mg/ml) was purchased from Isei Co., Inc. (Yamagata, Japan). Blood samples were collected from a jugular vein from conscious animals on day 11 (satellite groups 5 and 6) and on day 15 (groups 1 to 4), and from the abdominal aorta under anesthesia on day 20 (groups 1 to 4). PT and APTT were measured from blood samples collected on days 15 and 20. These parameters were also measured on day 11 using blood samples from animals in the satellite groups (groups 5 and 6) to determine whether these were prolonged before injection of Vitamin K or the cessation of LFO administra-

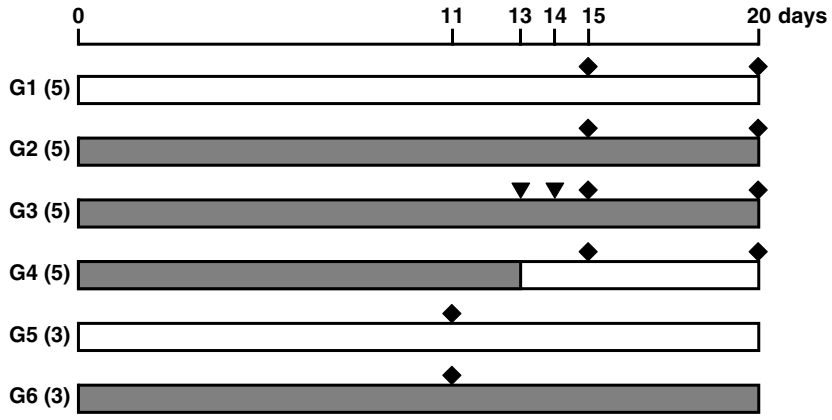

Fig. 1. Protocol for study 2. Values in parentheses are number of animals. (▼) vitamin K 70 mg/kg/day body weight i.p.; (◆) collection of blood samples; (□) basal diet; (■) LFO 5% in the basal diet. In groups 1 and 5 (control group), a basal diet (CE-2) was fed to the rats for 20 days. In groups 2, 3 and 6, LFO concentrate solution as 5% of the diet was fed for 20 days. In group 4, LFO concentrate solution as 5% of the diet was fed for 13 days and the food was replaced with the basal diet on day 13 and fed to the rats for the remaining 7 days.

tion. The following parameters were measured only in blood samples collected on day 20: the concentration of fibrinogen (factor I) and the activities of factors II, VII, IX and X. The concentration or activities of coagulant factors were measured using the light scattering method for fibrinogen, PT method for factor II, VII and X and the APTT method for factor IX (Adachi, 1996).

2.7. Statistical analysis

In study 1, the significance of differences in each parameter between the vehicle control (MCT) group and treated groups were analyzed. Body weights, quantitative urinalysis, hematology, clinical chemistry and organ weights were assessed using Bartlett's test. If homogeneous, the data were analyzed with Dunnett's multiple comparison test (one-sided); if not homogeneous, data were analyzed with Steel's test (one-sided). Differences in qualitative urinalysis, gross pathology and histopathology findings were analyzed using the Fisher's exact probability test (one-sided) and differences in the grade of lesions were considered using the Mann–Whitney *U*-test (two-sided) with Stat Light software (Yukms Co. Ltd., Tokyo, Japan). Statistical analysis was not performed for the male 1600 mg/kg group since there were only 2 surviving animals at the terminal sacrifice.

In study 2, statistical analysis was conducted with a multiple comparison test (parametric Dunnett type with or without log conversion, two-sided) after analysis of homogeneity of variance by Bartlett's test with SAS software (SAS institute, Cary, NC, USA).

The statistical significance of differences among groups was set at $P < 0.05$ in both studies.

Table 1
Incidence of death of animals in study 1

Week	Group[a]											
	Females						Males					
	1	2	3	4	5	6	7	8	9	10	11	12
1												
2			1[b]								1[b]	
3					1[b]							
4												1
5												
6												
7		1[b]				1[b]						3
8						1						
9												1
10												
11												
12												1
13												1
14											1[b]	1

[a] Dose levels for groups 1–6 or 7–12 were 0 (corn oil), 0 (MCT), 400, 600, 800 and 1600 mg/kg/day, respectively.
[b] Animals died due to error of administration.

3. Results

3.1. Results of study 1

3.1.1. Clinical observations and survival

A total of five females and ten males died or were terminated at unscheduled intervals during the study, including one female and eight males in the 1600 mg/kg group that died or were terminated due to adverse effects considered to be related to the test treatment (Table 1). Hematological analyses of blood samples from the moribund rats revealed an obvious decrease in red blood cell count, hemoglobin and hematocrit, as well as an obvious prolongation of PT and APTT. Furthermore, histopathological examination of these animals that died and the males that survived in this treatment group showed traces of hemorrhage in several organs. In the clinical observations for these animals, staining of the nose, bleeding in the oral cavity, swelling of the hindlimb, rough fur and/or prone position were noted at antemortem observations.

The death of one female rat in each of the MCT and LFO 400, 800 and 1600 mg/kg groups and two males in the 800 mg/kg group were probably caused by an accidental error in gavage administration because they died immediately after administration and an inflow of test substance into the lung was observed in almost all the animals that died.

Among the surviving animals, spontaneous formation of a skin crust occurred on the neck in one female given 400 mg/kg, and on the face in one male given 800 mg/kg.

3.1.2. Body weights and ophthalmoscopy

Significant changes in body weight were noted at weeks 7 and 11 in the female 400 mg/kg group and at weeks 3 and 10 in the male 600 mg/kg group. However, these changes were judged to be incidental changes because of the lack of dose dependency. No significant changes in body weight were observed in the female 600, 800 or 1600 mg/kg groups, or in the male 400 and 800 mg/kg groups. A tendency towards decreased body weight was noted in the surviving animals in the male 1600 mg/kg group from week 9 onwards (data not shown).

On ophthalmology at week 13, no abnormal findings in any of the six rats in each group (3 rats in the male 1600 mg/kg group) were observed (data not shown).

3.1.3. Feed and water consumption

No significant changes in feed consumption related to the test substance were observed (data not shown).

Although statistical analysis was not conducted, there appeared to be a trend toward increased water consumption in the female 800 mg/kg group from week 4 until the end of the treatment (excluding week 11), and in the female 1600 mg/kg group from week 2 until the end of the treatment (Table 2). A tendency for increased water consumption was observed in the male 600, 800 and 1600 mg/kg groups from week 1 or 2 until the end of the treatment. These changes were thought to be related to the test material because they were continuous throughout the treatment period.

3.1.4. Urinalysis

Selected urinalysis data are shown in Table 3. Urine volume was significantly elevated in the female 800 and 1600 mg/kg groups, and a tendency for increased urine volume was observed in the male 600, 800 and 1600 mg/kg groups. They were thought to reflect a tendency for increased water intake. Urine Na was significantly elevated in the female 800 mg/kg group and in the male 400 and 600 mg/kg groups, but without apparent dose dependence. No statistically significant changes were observed in any of other parameters examined.

Table 2
Water consumption values in study 1 (g/animal/day, mean)

Sex	Level (mg/kg)	Week												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Female	0 (Corn oil)	25.9	26.3	27.0	27.4	27.6	25.5	25.8	25.6	25.2	27.5	26.2	26.8	25.6
	0 (MCT)	27.2	26.9	27.5	26.2	25.3	28.6	27.3	24.4	26.5	28.3	29.0	26.3	25.6
	400	24.3	25.0	27.4	29.4	26.6	27.6	26.6	27.3	26.1	27.2	27.5	26.4	24.5
	600	25.0	26.3	26.7	27.8	28.0	28.1	28.1	26.6	29.6	28.2	28.0	28.9	26.8
	800	27.6	25.8	29.5	32.2	31.5	31.9	31.5	31.2	30.8	31.4	29.3	30.5	30.2
	1600	28.3	32.7	35.8	37.3	38.1	38.0	42.8	39.3	36.3	36.2	37.9	36.0	38.7
Male	0 (Corn oil)	33.6	36.2	36.4	36.5	34.4	36.1	35.5	34.8	35.4	36.0	34.5	34.3	33.9
	0 (MCT)	31.9	33.4	33.4	35.4	34.8	34.5	33.4	33.1	33.6	34.1	34.1	32.3	32.0
	400	32.0	34.5	34.5	37.3	35.9	34.8	34.2	32.8	38.4	34.0	32.7	31.9	33.1
	600	35.7	40.8	42.2	44.4	45.6	44.8	44.8	44.5	43.9	44.5	43.9	41.4	42.9
	800	33.5	38.0	39.3	40.1	42.7	42.5	41.1	40.8	41.2	41.7	41.8	40.1	38.9
	1600	37.2	44.9	48.0	53.7	53.4	52.3	61.7	55.7	55.4	54.3	49.6	53.8	54.3

The statistical analysis was not conducted because the raw data were given for each cage.

Table 3
Urinalysis values in study 1 (mean ± SD)

Sex	Level (mg/kg)	No. of samples	pH	Specific gravity	Urine volume (g)	Na (mEq/l)
Female	0 (Corn oil)	6	6.46 ± 0.35	1.05 ± 0.02	1.53 ± 0.90	101.2 ± 28.0
	0 (MCT)	6	7.00 ± 0.70	1.04 ± 0.02	1.30 ± 1.03	108.9 ± 37.8
	400	6	7.01 ± 0.69	1.03 ± 0.01	1.53 ± 0.84	104.7 ± 18.1
	600	6	6.71 ± 0.69	1.04 ± 0.01	1.57 ± 0.45	130.8 ± 30.6
	800	6	7.21 ± 0.38	1.03 ± 0.01	2.87 ± 0.90*	159.2 ± 38.0*
	1600	6	7.32 ± 0.39	1.03 ± 0.01	3.08 ± 1.76*	126.4 ± 48.2
Male	0 (Corn oil)	6	6.93 ± 0.29	1.05 ± 0.02	2.53 ± 1.37	101.3 ± 28.9
	0 (MCT)	6	7.13 ± 0.49	1.04 ± 0.02	2.35 ± 0.93	75.8 ± 32.9
	400	6	6.83 ± 0.58	1.04 ± 0.01	1.82 ± 0.86	122.1 ± 20.0*
	600	6	7.20 ± 0.52	1.03 ± 0.01	3.40 ± 0.84	120.5 ± 30.2*
	800	6	7.31 ± 0.30	1.03 ± 0.01	3.08 ± 1.30	106.4 ± 36.3
	1600	3	7.05 ± 0.47	1.03 ± 0.01	4.83 ± 2.40	89.9 ± 23.4

* Significantly different from vehicle control (MCT) group at $P < 0.05$.

Table 4
Hematology values in study 1 (mean ± SD)

Sex	Level (mg/kg)	No. of samples	WBC ($\times 10^2$/μj)	RBC ($\times 10^4$/μdl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT ($\times 10^4$/μl)	Reticulocyte (%)
Female	0 (Corn oil)	10	36.9 ± 9.6	750 ± 38	14.5 ± 0.6	42.9 ± 2.4	57.2 ± 2.0	19.3 ± 0.4	33.8 ± 1.0	103 ± 4	1.46 ± 0.28
	0 (MCT)	9	38.6 ± 15.2	723 ± 41	13.9 ± 0.6	41.4 ± 1.5	57.3 ± 2.0	19.3 ± 0.6	33.6 ± 1.0	104 ± 12	1.36 ± 0.35
	400	9	47.0 ± 13.8	735 ± 35	14.3 ± 0.4	42.8 ± 1.6	58.3 ± 1.4	19.4 ± 0.7	33.3 ± 0.7	10.7 ± 10	1.46 ± 0.33
	600	10	38.1 ± 13.5	716 ± 36	13.9 ± 0.5	41.4 ± 1.3	57.9 ± 2.4	19.4 ± 0.6	33.6 ± 0.8	101 ± 8	1.41 ± 0.32
	800	9	43.8 ± 4.8	728 ± 29	14.0 ± 0.5	41.4 ± 1.5	56.9 ± 1.6	19.3 ± 0.4	33.9 ± 0.8	115 ± 12	1.31 ± 0.31
	1600	8	47.9 ± 10.9	735 ± 35	14.2 ± 0.6	41.1 ± 2.2	55.9 ± 1.7	19.3 ± 0.5	34.5 ± 0.8	110 ± 11	1.32 ± 0.49
Male	0 (Corn oil)	10	75.1 ± 19.9	822 ± 32	15.2 ± 0.5	46.3 ± 1.7	56.3 ± 1.7	18.5 ± 0.7	32.8 ± 0.6	108 ± 8	1.36 ± 0.29
	0 (MCT)	10	78.4 ± 19.0	842 ± 38	15.4 ± 0.5	47.0 ± 1.1	55.9 ± 2.1	18.3 ± 0.7	32.7 ± 1.0	104 ± 9	1.45 ± 0.32
	400	10	91.4 ± 31.2	822 ± 28	15.1 ± 0.4	45.3 ± 1.2*	55.1 ± 1.9	18.4 ± 0.6	33.3 ± 0.5	108 ± 9	1.43 ± 0.36
	600	10	105.0 ± 25.7*	807 ± 42*	15.0 ± 0.5	44.7 ± 1.4**	55.4 ± 1.6	18.7 ± 0.6	33.7 ± 0.7**	112 ± 13	1.22 ± 0.29
	800	8	90.5 ± 33.7	787 ± 30**	15.0 ± 0.5	44.3 ± 1.8**	56.4 ± 2.6	19.0 ± 0.6*	33.8 ± 0.8**	105 ± 5	1.24 ± 0.27
	1600	2	108.5 ± 19.1	753 ± 4	14.5 ± 0.4	41.3 ± 0.4	54.8 ± 0.8	19.2 ± 0.4	35.1 ± 1.2	126 ± 13	2.94 ± 1.79

** Significantly different from vehicle control (MCT) group at $P < 0.01$.
* Significantly different from vehicle control (MCT) group at $P < 0.05$.

3.1.5. Hematology

Selected hematology data are shown in Table 4. RBC significantly decreased in the male 600 and 800 mg/kg groups. HCT significantly decreased in the male 400, 600 and 800 mg/kg groups. MCH was significantly elevated in the male 800 mg/kg group and MCHC was significantly elevated in the male 600 and 800 mg/kg groups. Similar tendencies were observed in the male 1600 mg/kg group.

WBC significantly increased in the male 600 mg/kg group but the increase was considered to be an incidental change because of the lack of dose dependence. No statistically significant changes were observed in any of the other parameters examined.

Data for blood coagulation tests are shown in Table 5. In these tests, PT was shown to be significantly prolonged in the female 1600 mg/kg group and the male 400 and 800 mg/kg groups. A non-significant tendency towards prolongation was also observed in the male 600 and 1600 mg/kg groups, with dose dependency. APTT was significantly prolonged in the female 1600 mg/kg group and the male 800 mg/kg group, and a similar tendency was observed in the male 600 and 1600 mg/kg groups. On hematological examination of moribund animals in the male 1600 mg/kg group, a tendency for decreases in RBC, HGB and HCT, and apparent prolongation of the PT and APTT, were observed. In five moribund male rats, PT values were 71.8, 74.6, 74.7 and more than 75.0 s in two rats, and APTT was more than 220.0 s in all five moribund rats. PT and APTT values for one moribund female rat were 75.0 and 193.8, respectively (data not included in table).

Table 5
Blood coagulation time in study 1 (mean ± SD)

Sex	Level (mg/kg)	No. of samples	PT (s)	APTT (s)
Female	0 (Corn oil)	10	10.71 ± 0.71	32.10 ± 10.71
	0 (MCT)	9	10.60 ± 0.63	29.31 ± 11.33
	400	9	10.30 ± 0.27	31.57 ± 9.12
	600	10	10.16 ± 0.63	29.75 ± 7.60
	800	9	10.03 ± 0.46	32.87 ± 9.07
	1600	8	12.99 ± 2.59*	52.70 ± 18.79**
Male	0 (Corn oil)	10	10.56 ± 0.79	28.48 ± 10.69
	0 (MCT)	9	11.09 ± 0.65	32.88 ± 9.88
	400	10	12.45 ± 1.28*	37.52 ± 9.15
	600	10	15.23 ± 5.40	48.91 ± 23.24
	800	8	21.89 ± 4.54**	81.83 ± 37.03**
	1600	2	24.10 ± 0.85	97.05 ± 1.20

* Significantly different from vehicle control (MCT) group at $P < 0.05$.
** Significantly different from vehicle control (MCT) group at $P < 0.01$.

3.1.6. Clinical chemistry

Clinical chemistry data are shown in Table 6. AST decreased significantly in the female 1600 mg/kg group and male 800 mg/kg group. ALT was significantly elevated in the female 600, 800 and 1600 mg/kg groups and in the male 400, 600 and 800 mg/kg groups, and a similar tendency was observed in the male 1600 mg/kg group. ALP was significantly elevated in the female 1600 mg/kg group. T-BIL was significantly elevated in the female 1600 mg/kg group, and a similar tendency was observed in the male 1600 mg/kg group. CRE was significantly elevated in the male 800 mg/kg group, and a similar tendency was observed in the male 1600 mg/kg group. GLU decreased significantly in the females 600 mg/kg and in the higher dosage groups. TP was significantly reduced in the male 600 mg/kg group and a similar tendency was observed in the male 800 and 1600 mg/kg groups. IP was significantly elevated in the female 1600 mg/kg group. MG was significantly reduced in the female 1600 mg/kg group. K was significantly lowered in the male 600 and 800 mg/kg groups, with a similar tendency observed in the male 1600 mg/kg group.

Changes in γ-GTP, T-CHO, PL and TG were judged to be incidental because of a lack of any dose dependence. No statistically significant changes were detected in any of the other parameters.

Table 6
Clinical chemistry values in study 1 (mean ± SD)

Sex	Level (mg/kg)	No. of samples	AST (U/l)	ALT (U/l)	ALP (I U/l)	γ-GTP (I U/l)	T-BIL (mg/dl)	BUN (mg/dl)	CRE (mg/dl)	GLU (mg/dl)	T-CHO (mg/dl)	PL (mg/dl)
Panel A												
Female	0 (Corn oil)	10	138 ± 34	24.7 ± 5.4	98 ± 19	0.76 ± 0.32	0.094 ± 0.020	19.5 ± 2.1	0.59 ± 0.03	127 ± 19	71.2 ± 9.6	168 ± 17
	0 (MCT)	9	134 ± 27	21.9 ± 2.7	89 ± 34	0.46 ± 0.30	0.109 ± 0.019	16.5 ± 2.8	0.57 ± 0.05	136 ± 16	81.9 ± 15.9	200 ± 41
	400	9	130 ± 25	23.9 ± 3.1	105 ± 22	0.78 ± 0.32	0.109 ± 0.020	17.2 ± 1.8	0.58 ± 0.04	123 ± 15	73.9 ± 12.3	173 ± 22
	600	10	141 ± 31	27.5 ± 6.1*	94 ± 21	0.66 ± 0.32	0.119 ± 0.031	19.3 ± 3.7	0.60 ± 0.07	120 ± 15*	72.9 ± 16.7	174 ± 29
	800	9	150 ± 14	29.3 ± 4.7**	95 ± 27	0.91 ± 0.41**	0.127 ± 0.027	19.0 ± 2.1	0.60 ± 0.05	115 ± 12**	73.4 ± 13.2	174 ± 26
	1600	8	103 ± 26*	42.1 ± 9.2**	120 ± 23*	0.44 ± 0.26	0.178 ± 0.056*	19.4 ± 3.1	0.60 ± 0.08	110 ± 11**	87.8 ± 12.5	204 ± 28
Male	0 (Corn oil)	10	170 ± 52	35.0 ± 6.1	179 ± 28	0.32 ± 0.20	0.091 ± 0.014	18.2 ± 2.0	0.55 ± 0.05	164 ± 24	89.0 ± 10.7	146 ± 16
	0 (MCT)	9	126 ± 26	25.4 ± 3.0	177 ± 36	0.37 ± 0.35	0.086 ± 0.015	17.5 ± 1.7	0.53 ± 0.05	150 ± 12	64.9 ± 12.0	126 ± 14
	400	10	135 ± 31	28.6 ± 2.2*	185 ± 35	0.25 ± 0.20	0.087 ± 0.011	16.9 ± 2.1	0.57 ± 0.05	147 ± 21	82.3 ± 15.1**	156 ± 22*
	600	10	116 ± 47	33.4 ± 5.1**	212 ± 33	0.42 ± 0.51	0.092 ± 0.009	15.9 ± 1.7	0.54 ± 0.05	150 ± 11	78.9 ± 12.6*	150 ± 22*
	800	8	74 ± 16**	36.1 ± 8.9*	215 ± 46	0.20 ± 0.19	0.100 ± 0.020	16.9 ± 2.3	0.59 ± 0.04*	161 ± 20	85.4 ± 8.7**	163 ± 31**
	1600	2	99 ± 13	72.0 ± 14.1	218 ± 28	1.20 ± 0.14	0.225 ± 0.064	19.7 ± 2.1	0.65 ± 0.07	146 ± 0	83.5 ± 6.4	155 ± 10
			TG (mg/dl)	TP (mg/dl)	ALB (mg/dl)	A/G	IP (mg/dl)	CA (mg/dl)	MG (mg/dl)	NA (mEq/l)	K (mEq/l)	CL (mEq/l)
Panel B												
Female	0 (Corn oil)	10	46.3 ± 14.5	6.70 ± 0.29	2.77 ± 0.14	0.706 ± 0.042	5.28 ± 1.05	9.91 ± 0.43	2.50 ± 0.26	143.1 ± 1.9	4.56 ± 0.52	105.1 ± 1.4
	0 (MCT)	9	96.1 ± 45.7	6.72 ± 0.39	2.84 ± 0.17	0.733 ± 0.032	5.19 ± 1.24	9.98 ± 0.31	2.41 ± 0.08	142.5 ± 0.9	4.30 ± 0.39	104.4 ± 1.4
	400	9	57.7 ± 30.0*	6.67 ± 0.34	2.78 ± 0.15	0.716 ± 0.032	6.12 ± 0.97	9.97 ± 0.25	2.41 ± 0.20	142.4 ± 1.1	4.48 ± 0.36	104.8 ± 0.7
	600	10	57.2 ± 25.9*	6.69 ± 0.42	2.79 ± 0.21	0.716 ± 0.033	5.56 ± 1.13	9.87 ± 0.51	2.34 ± 0.22	142.2 ± 2.1	4.26 ± 0.30	104.5 ± 2.0
	800	9	67.8 ± 47.2	6.53 ± 0.42	2.70 ± 0.19	0.703 ± 0.024	6.02 ± 1.05	9.99 ± 0.34	2.52 ± 0.13	142.8 ± 0.8	4.24 ± 0.23	104.8 ± 0.9
	1600	8	55.3 ± 23.2*	6.45 ± 0.44	2.71 ± 0.16	0.726 ± 0.039	6.38 ± 0.97*	9.85 ± 0.29	2.21 ± 0.14*	142.5 ± 2.0	3.98 ± 0.38	102.4 ± 2.5
Male	0 (Corn oil)	10	105.6 ± 24.5	6.33 ± 0.21	2.32 ± 0.06	0.580 ± 0.022	6.71 ± 0.54	10.16 ± 0.22	2.31 ± 0.14	141.9 ± 0.8	5.00 ± 0.21	102.0 ± 0.8
	0 (MCT)	9	132.9 ± 56.9	6.26 ± 0.13	2.36 ± 0.09	0.607 ± 0.038	6.60 ± 0.84	9.96 ± 0.35	2.27 ± 0.12	142.1 ± 0.8	4.78 ± 0.39	103.0 ± 1.3
	400	10	134.4 ± 22.8	6.24 ± 0.22	2.37 ± 0.08	0.615 ± 0.030	7.14 ± 0.72	10.04 ± 0.36	2.25 ± 0.18	142.1 ± 0.4	4.80 ± 0.19	102.3 ± 0.7
	600	10	124.3 ± 48.5	6.04 ± 0.13**	2.28 ± 0.06	0.608 ± 0.029	6.69 ± 0.59	9.88 ± 0.29	2.22 ± 0.20	142.6 ± 0.9	4.41 ± 0.29*	102.6 ± 1.1
	800	8	122.9 ± 105.1	5.96 ± 0.37	2.28 ± 0.14	0.619 ± 0.027	6.76 ± 0.94	9.93 ± 0.47	2.25 ± 0.21	143.1 ± 1.2	3.92 ± 0.41**	103.1 ± 1.3
	1600	2	58.5 ± 13.4	5.90 ± 0.14	2.25 ± 0.07	0.620 ± 0.057	6.35 ± 0.21	9.50 ± 0.28	2.25 ± 0.21	143.6 ± 1.4	3.93 ± 0.21	102.9 ± 0.1

* Significantly different from vehicle control (MCT) group at $P < 0.05$.
** Significantly different from vehicle control (MCT) group at $P < 0.01$.

3.1.7. Organ weight

Selected organ weight data are shown in Table 7. Absolute spleen weight was significantly lowered, and relative kidney weight significantly elevated in the female 1600 mg/kg group. The absolute and relative weights of abdominal fat around the uterus were significantly decreased in the female 1600 mg/kg group. The absolute and relative weights of abdominal fat around the kidneys were significantly decreased in the female 800 and 1600 mg/kg groups. Only the relative weight of abdominal fat around the kidneys was significantly reduced in the female 400 mg/kg group. A tendency for a decrease in absolute weight and relative weight of abdominal fat around the testes or kidneys was observed in the male 1600 mg/kg group.

Changes in other organs were judged to be incidental because of the lack of dose dependency.

3.1.8. Gross pathology

On the one female and eight males in the 1600 mg/kg whose deaths were due to treatment with the test material, traces of hemorrhage, such as dark red discoloration, were observed in several organs macroscopically. In the one surviving rat in the male 1600 mg/kg group, dark red discoloration of the musculature was found; the finding was also probably related to the test material.

3.1.9. Histopathology

In the histopathological examination, hemorrhage in several organs and tissues and hematopoiesis in the bone marrow were frequent in the eight rats that died and in the one rat that survived in the male 1600 mg/kg group and in one rat that died in the female 1600 mg/kg group. Signs of hemorrhage were observed in diverse organs and tissues in each rat and in uncharacteristic organs and tissues such as the lymph nodes, the thymus, nasal cavity, tongue, stomach, pancreas, testes, epididymides, prostate, skeletal musculature, brain, skin/subcutis, fat tissue and so on. Furthermore, several changes, such as inflammatory lesions, apoptosis, necrosis and atrophy were found in several organs in the rats that died due to the test material treatment. These were considered to be reactive changes accompanying the hemorrhage. In the salivary glands, minimal or slight hypertrophy of acinar cell was found in nine males in the 1600 mg/kg group.

Several changes, other than those already mentioned, were observed in the systemic organs and tissues in surviving animals, with such changes thought to be spontaneous in nature because no differences in the frequency or degree of these changes were found between the test material treatment groups and the MCT group.

3.2. Results of study 2

The administration of LFO concentrate solution induced obvious prolongation of PT and APTT in group 2 in comparison to the control group (Fig. 2). However, in group 3, PT and APTT values returned to normal levels within 2 days after vitamin K injection. Furthermore, in group 4, PT and APTT values returned to normal levels within 2 days after cessation of feeding the LFO concentrate solution. We also confirmed the prolongation of PT and APTT at day 11 in the rats given LFO concentrate solution using satellite animals (group 5 and 6).

At day 20, activity of the vitamin K-dependent coagulation factors II, VII, IX and X, was clearly decreased following administration of LFO concentrate solution in group 2, while this decrease completely resolved following injection of vitamin K in group 3 or by cessation of administration of LFO concentrate solution in group 4 (Fig. 3). The concentration of fibrinogen, a vitamin K-independent factor did not decrease, but increased significantly.

The PT and APTT in group 2 on day 20 remained prolonged (Fig. 2). In contrast, the effect of vitamin K was maintained for 7 days after injection even though LFO concentrate solution was fed until termination of the study.

4. Discussion

The changes judged to be related to administration of the test material will be discussed below. The survival rates related to the test material treatment were 90 and 20% in the female and male 1600 mg/kg groups, respectively. In the dead rats from these groups, evidence of hemorrhage in several tissues and organs was observed, and PT and APTT were clearly prolonged in the blood coagulation test performed to identify the cause of death in six moribund rats. Therefore, the hemorrhage in several tissues and organs was thought to be induced by abnormal blood coagulation.

The increased water consumption was presumed to reflect the bitter taste of the test material, since salivation was observed in the 800 and 1600 mg/kg dosage groups. This phenomenon was considered to be related to the administration of the test material, but was not considered to be an adverse effect because there were no related abnormalities in clinical chemistry, urinalysis and histopathological examinations.

In the hematology, changes in the RBC, HCT, MCH and MCHC were noted in the male 1600 mg/kg group, suggesting anemia. Similar changes in the male 800 mg/kg group were not as marked and no hemorrhage was observed histopathologically, although it was considered likely that these changes were due to the test material treatment. Changes or tendency towards changes in blood coagulation were judged to be due to the test substance because a dose-response relationship was observed.

In the clinical chemistry investigations, increases in ALT that were observed in the female 1600 mg/kg and male 800 and 1600 mg/kg groups were judged to be related to the test material treatment. Although the changes in ALT, ALP and T-BIL in the same groups were suggestive of hepatic damage, no changes in liver weight or in findings on histopathological examination were observed. Decrease or tendency towards decrease in K in male 600, 800 and 1600 mg/kg were judged to be related to treatment with the test substance. Among the other parameters, changes in GLU, IP, MG and CRE were considered possibly due to the effects of treatment with the test substance. These changes were less marked and no changes in the weight or in findings on histopathological examination of associated organs were detected. The decrease in AST was not considered to be of toxicological significance because the clinical implication of the observed decrease was unclear.

Changes in absolute spleen and relative kidney weights in the female 1600 mg/kg group were considered to be related to exposure to the test substance, but no remarkable changes were observed on histopathological examination. Changes in other organs were thought to be incidental because they did not indicate dose-dependent changes and no remarkable findings were observed on histopathological examination. The significant decrease or the towards decrease in abdominal fat around the uterus, testes or kidneys observed in the 800 and 1600 mg/kg groups was likely induced by the anti-obesity effects of the test material (Nakagawa et al., 2004; Aoki et al., 2007a). The macro- and micropathological examinations revealed hemorrhage in systemic organs and tissues in the eight rats that died and the one rat that survived in the male 1600 mg/kg group and in one rat in the female 1600 mg/kg group that died. These changes were probably induced by decreases in the function of the blood coagulation system due to the test material. In other findings, acinar cell hypertrophy of the salivary gland in the male 1600 mg/kg group was considered to be related to the

Table 7
Organ weights in study 1 (mean ± SD)

Sex	Level (mg/kg)	No. of samples	Liver (g)	Kidney (g)	Lung (g)	Spleen (g)	Heart (g)	Brain (g)	Pituitary (mg)	Thyroid (mg)	Salivary glands (g)	Fat (U/T)[a] (g)	Fat (K)[b]
Absolute organ weights													
Female	0 (Corn oil)	10	6.65 ± 0.88	1.65 ± 0.21	0.99 ± 0.10	0.455 ± 0.059	0.79 ± 0.08	2.00 ± 0.11	16.5 ± 2.1	17.0 ± 4.7	0.402 ± 0.041	4.20 ± 1.55	6.73 ± 3.26
	0 (MCT)	9	7.72 ± 0.09	1.85 ± 0.18	1.00 ± 0.11	0.491 ± 0.041	0.87 ± 0.07	2.00 ± 0.09	17.6 ± 4.8	16.8 ± 3.0	0.409 ± 0.052	6.19 ± 3.04	7.45 ± 2.68
	400	9	7.15 ± 1.07	1.72 ± 0.16	0.99 ± 0.07	0.477 ± 0.071	0.79 ± 0.10*	1.99 ± 0.06	16.4 ± 4.3	18.3 ± 2.7	0.418 ± 0.036	3.95 ± 2.01	4.81 ± 2.29
	600	10	7.37 ± 0.76	1.74 ± 0.21	0.99 ± 0.05	0.448 ± 0.045	0.82 ± 0.07	2.01 ± 0.09	17.4 ± 4.8	16.7 ± 3.7	0.424 ± 0.034	4.61 ± 0.86	5.75 ± 1.46
	800	9	7.43 ± 0.08	1.71 ± 0.19	0.99 ± 0.08	0.439 ± 0.077	0.82 ± 0.05	2.00 ± 0.11	15.7 ± 2.7	18.2 ± 4.1	0.414 ± 0.058	3.50 ± 1.79	4.27 ± 2.81*
	1600	8	7.88 ± 0.75	1.91 ± 0.12	1.02 ± 0.04	0.413 ± 0.059*	0.82 ± 0.03	2.03 ± 0.07	14.9 ± 2.1	17.1 ± 3.4	0.411 ± 0.033	3.08 ± 1.01**	3.07 ± 0.96**
Male	0 (Corn oil)	10	14.96 ± 2.11	3.17 ± 0.36	1.43 ± 0.17	0.720 ± 0.115	1.43 ± 0.22	2.18 ± 0.11	12.6 ± 1.6	24.7 ± 4.7	0.671 ± 0.075	9.64 ± 2.27	14.12 ± 3.05
	0 (MCT)	10	12.73 ± 1.14	2.94 ± 0.09	1.35 ± 0.08	0.679 ± 0.041	1.30 ± 0.12	2.13 ± 0.06	11.1 ± 1.9	21.6 ± 3.2	0.634 ± 0.054	8.21 ± 1.52	11.49 ± 1.83
	400	10	13.71 ± 2.07	3.02 ± 0.35	1.36 ± 0.12	0.716 ± 0.095	1.36 ± 0.12	2.13 ± 0.09	11.5 ± 1.5	22.2 ± 1.6	0.657 ± 0.061	9.30 ± 1.78	13.43 ± 3.03
	600	10	14.27 ± 1.71	3.18 ± 0.24**	1.40 ± 0.09	0.666 ± 0.153	1.47 ± 0.07**	2.26 ± 0.06**	14.1 ± 1.2**	25.3 ± 3.5*	0.689 ± 0.046*	8.22 ± 1.54	11.68 ± 2.22
	800	8	13.52 ± 2.07	2.84 ± 0.30	1.33 ± 0.16	0.707 ± 0.096	1.30 ± 0.09	2.15 ± 0.04	13.4 ± 1.9**	22.5 ± 6.0	0.630 ± 0.053	7.81 ± 2.57	10.16 ± 3.88
	1600	2	13.85 ± 1.55	2.83 ± 0.39	1.39 ± 0.23	0.689 ± 0.075	1.27 ± 0.28	2.18 ± 0.17	14.0 ± 2.8	23.0 ± 4.2	0.680 ± 0.125	3.90 ± 1.14	6.31 ± 1.83
			Liver (%)	Kidney (%)	Lung (%)	Spleen (%)	Heart (%)	Brain (%)	Pituitary (%)	Thyroid (%)	Salivary glands (%)	Fat (U/T)[a] (%)	Fat (K)[b] (%)
Organ to body weight ratio													
Female	0 (Corn oil)	10	2.63 ± 0.17	0.653 ± 0.032	0.392 ± 0.025	0.181 ± 0.027	0.312 ± 0.018	0.797 ± 0.060	0.0066 ± 0.0010	0.0066 ± 0.0014	0.159 ± 0.014	1.63 ± 0.52	2.58 ± 1.05
	0 (MCT)	9	2.84 ± 0.23	0.684 ± 0.056	0.371 ± 0.038	0.181 ± 0.014	0.322 ± 0.016	0.740 ± 0.055	0.0064 ± 0.0019	0.0061 ± 0.0013	0.151 ± 0.016	2.28 ± 1.11	2.75 ± 0.96
	400	9	2.86 ± 0.30	0.687 ± 0.027	0.400 ± 0.031*	0.192 ± 0.032	0.317 ± 0.028	0.802 ± 0.076	0.0067 ± 0.0016	0.0073 ± 0.0009	0.168 ± 0.019	1.54 ± 0.69	1.88 ± 0.77*
	600	10	2.86 ± 0.24	0.674 ± 0.046	0.387 ± 0.019	0.174 ± 0.013	0.318 ± 0.023	0.785 ± 0.078	0.0068 ± 0.0021	0.0064 ± 0.0013	0.165 ± 0.015	1.78 ± 0.29	2.22 ± 0.51
	800	9	2.89 ± 0.25	0.664 ± 0.053	0.385 ± 0.025	0.171 ± 0.029	0.322 ± 0.023	0.781 ± 0.067	0.0061 ± 0.0011	0.0071 ± 0.0014	0.162 ± 0.023	1.33 ± 0.06	1.60 ± 0.92**
	1600	8	3.10 ± 0.29	0.749 ± 0.027**	0.401 ± 0.014*	0.162 ± 0.021	0.321 ± 0.016	0.800 ± 0.047	0.0059 ± 0.0010	0.0066 ± 0.0013	0.162 ± 0.012	1.20 ± 0.35*	1.21 ± 0.36**
Male	0 (Corn oil)	10	2.86 ± 0.23	0.609 ± 0.032	0.274 ± 0.020	0.138 ± 0.015	0.273 ± 0.026	0.421 ± 0.028	0.0023 ± 0.0005	0.0047 ± 0.0008	0.129 ± 0.007	1.84 ± 0.31	2.69 ± 0.45
	0 (MCT)	10	2.61 ± 0.20	0.606 ± 0.036	0.278 ± 0.020	0.140 ± 0.012	0.268 ± 0.036	0.438 ± 0.031	0.0023 ± 0.0005	0.0044 ± 0.0005	0.130 ± 0.015	1.68 ± 0.25	2.35 ± 0.32
	400	10	2.72 ± 0.20	0.602 ± 0.055	0.271 ±0.021	0.143 ± 0.018	0.271 ± 0.018	0.426 ± 0.032	0.0023 ± 0.0005	0.0043 ± 0.0005	0.131 ± 0.010	1.85 ± 0.31	2.67 ± 0.53
	600	10	2.77 ± 0.23	0.619 ± 0.043	0.271 ± 0.012	0.129 ± 0.030	0.286 ± 0.020	0.440 ± 0.024	0.0029 ± 0.0003**	0.0051 ± 0.0007*	0.135 ± 0.014	1.60 ± 0.27	2.27 ± 0.39
	800	8	2.80 ± 0.27	0.591 ± 0.064	0.276 ± 0.021	0.147 ± 0.018	0.271 ± 0.017	0.448 ± 0.031	0.0026 ± 0.0005	0.0048 ± 0.0012	0.131 ± 0.007	1.62 ± 0.50	2.09 ± 0.69
	1600	2	2.88 ± 0.09	0.588 ± 0.033	0.289 ± 0.023	0.143 ± 0.004	0.263 ± 0.038	0.455 ± 0.002	0.0030 ± 0.0000	0.0050 ± 0.0014	0.141 ± 0.015	0.80 ± 0.17	1.33 ± 0.49

[a] Abdominal fat around the uterus or testes.
[b] Abdominal fat around the kidney. (g)
* Significantly different from vehicle control (MCT) group at $P < 0.05$.
** Significantly different from vehicle control (MCT) group at $P < 0.01$.

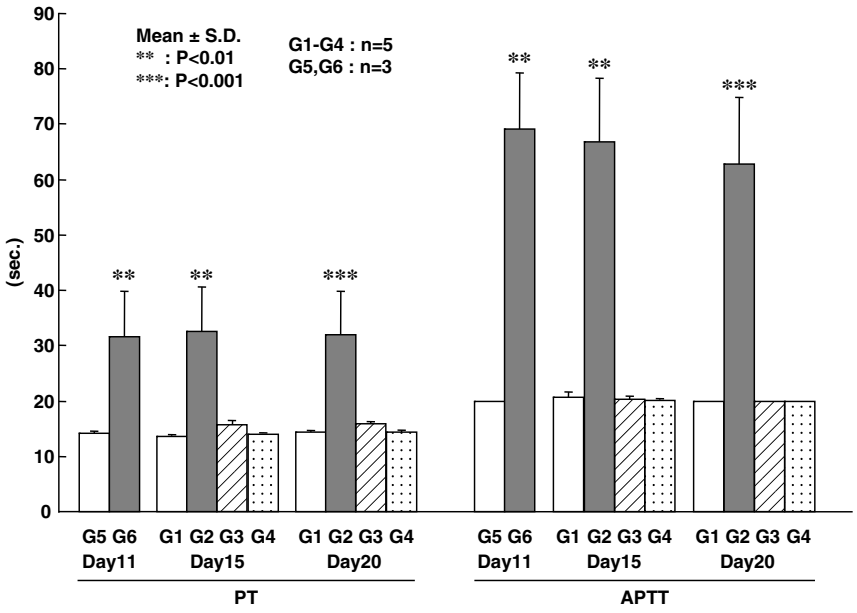

Fig. 2. PT and APTT at day 11, 15 and 20 in study 2. (□:G1, G5) basal diet; (■:G2, G6) LFO; (▨:G3) LFO + vitamin K; (▫:G4) cessation of LFO treatment at day 13. Each bar shows the mean ± SD of 3 or 5 animals.

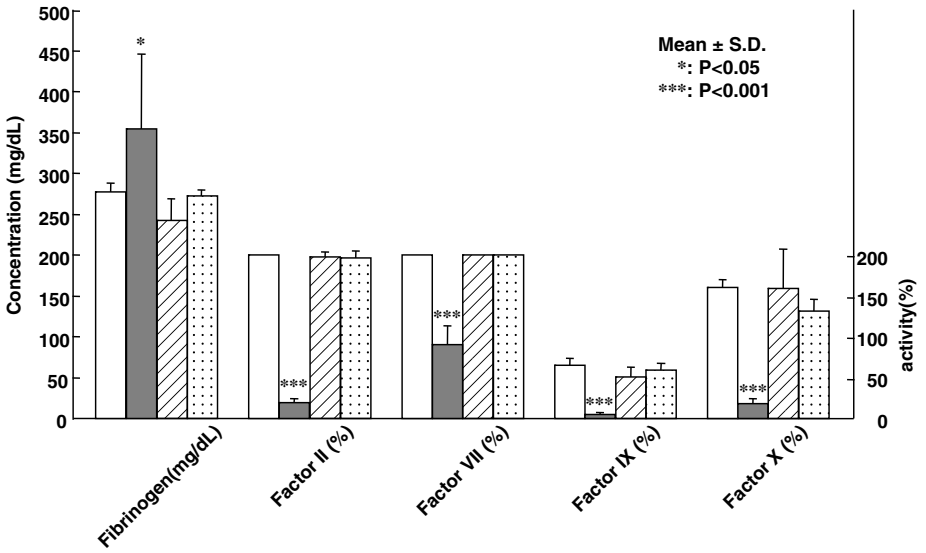

Fig. 3. Concentration of fibrinogen and activities of coagulation factor II, VII, IX and X at day 20 in study 2. (□:G1) basal diet; (■:G2) LFO; (▨:G3) LFO + vitamin K; (▫:G4) cessation of LFO treatment at day 13. Each bar shows the mean ± SD of 5 animals.

increase in water consumption and urine volume, but not considered to be an adverse effect because no damage or organ dysfunction was observed.

Although several changes were seen following administration of MCT in comparison to treatment with corn oil, the changes in body weight, clinical chemistry values and organ weight were not noted in earlier toxicity studies of MCT or tricaprylin (Traul et al., 2000; Webb et al., 1993; Ohta et al., 1970). Thus, we cannot conclude that the changes were due to the toxicity of MCT based on the results of this experiment.

In the second study, it was confirmed that the blood anticoagulant effect was caused by inhibiting the synthesis of vitamin K-dependent coagulation factors II, VII, IX and X. In contrast, the concentration of fibrinogen (factor I), which is independent of vitamin K, increased. The increase in fibrinogen was thought to be induced indirectly by the inhibition of transformation from fibrinogen to fibrin due to the inhibition of synthesis of vitamin K-dependent coagulation factors. Warfarin, which is used as a rodenticide or antithrombotic drug, is known to inhibit vitamin K_1-2,3 epoxide reductase and thus the synthesis of vitamin K, and subsequently the synthesis of coagulation factors II, VII, IX and X (Zivelin et al., 1993; Watt et al., 2005). Therefore, LFO is considered to have the same mechanism of action as warfarin in terms of inhibition of the synthesis of vitamin K-dependent coagulation factors. In study 1, a sex difference in the effects of LFO on blood coagulation time was observed. Values of PT and APTT at all dose levels in males were greater than those in females ($P < 0.05$ or trends), resulting in a higher incidence of hemorrhage in systemic organs and tissues in males than in females, and in a lower survival rate for males of 20%, compared with 90% in females at 1600 mg/kg. Hara et al.

(1994) reported that male rats are more sensitive to the effects of warfarin than female rats in terms of decreases in prothrombin (coagulation factor II) levels in plasma and prolongation of blood coagulation time. Therefore, LFO is also similar to warfarin from the perspective of sex differences in terms of the effects on blood coagulation.

Glycyrrhizin, a glycoside isolated from licorice (*G. glabra*), is known to inhibit platelet aggregation (Okimasu et al., 1983;Francischetti et al., 1997). However, LFO concentrate solution does not include glycyrrhizin (not more than 0.005%) from the aqueous extraction because the major component of LFO is ethanol extracts from licorice. The major components of LFO, aside from MCT, are flavonoids derived from licorice. In general, it is well known that some flavonoids have anticoagulant activity. In particular, coumarin and its derivatives are well-known as oral anticoagulants and their effects have been correlated with the level of vitamin K in the liver (Winn et al., 1987). Compounds in LFO that are known to have structures similar to coumarin include glabridin, glabrol, glabrene and 4′-*O*-methylglabridin. Therefore, the anticoagulant activity associated with LFO might be caused by these components.

In human studies of LFO, including a 4-week study at daily doses of 300, 600 and 1200 mg/man/day LFO, corresponding to 100, 200 and 400 mg/man/day of LFO concentrate solution as defined in the materials and methods of this paper, a 12-week study at a dose of 300 mg/man/day and a 4-week study at a dose of 1800 mg/man/day corresponding to 100 to 600 mg/man/day of LFO concentrate solution, no prolongation of blood coagulation time was observed (Aoki et al., 2007b; Tominaga et al., 2006). Therefore, the anticoagulant effect is not induced practically under conditions of typical exposure or usage in humans.

In conclusion, when rats were given LFO concentrate solution by gavage daily for 90 days, death attributable to the test material was observed in the female and male 1600 mg/kg groups. In male rats, the tendency towards anemia and the changes in clinical chemistry values suggested abnormalities of liver and kidney function in the 800 and 1600 mg/kg groups, although no histopathological abnormalities were found in these organs. Moreover, dose-dependent prolongation of PT was observed in male rats in all test material groups. However, the prolongation of PT in the male 400 mg/kg group was very minimal and within 2 s of control values in the MCT group. The values of blood coagulation parameters such as PT or APTT in the rat are similar to those in humans (Smith et al., 1987). In humans, it is well known that the normal ranges of PT and APTT are within 2 and 5 s of control values, respectively. Toxicological change is defined here as a change that induces functional decline and irreversible change in organs or cells. In the male 400 mg/kg group, there were no changes that suggested functional decline in clinical chemistry or on histopathological examination associated with the prolongation of PT. This was also true for findings of these examinations in the male 600 mg/kg group. Furthermore, the prolongation of PT and APTT resolved, with levels rapidly returning to normal following cessation of test material administration or the injection of vitamin K in study 2. These results indicated that this change was reversible and the liver function itself did not decline. Therefore, the changes in PT observed in the male 400 mg/kg group are considered to be of very little toxicological significance. As a result, the only clear toxicity of LFO concentrate solution observed in this study is an anticoagulation effect, with a clear sex difference, and the no-observed-adverse-effect level (NOAEL) was estimated to be 800 mg/kg/day for females. In males, the NOAEL of LFO concentrate solution was approximately 400 mg/kg/day, although the no-observed-effect level (NOEL) was less than 400 mg/kg/day.

Conflict of interest statement

The manuscript is unpublished and not considered to be published in other journal at the same time. All authors have contributed significantly, and they agree with the content of manuscript. The authors declare that there are no conflicts of interest.

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